SOUTH AFRICAN *PHOTORHABDUS* SPP.: GENETIC AND ANTIBIOTIC DIVERSITY

By

MATTHEW GEORGE DENNIS VAN WYNGAARD

Submitted in fulfilment of the academic requirements for the degree of

Master of Science in Microbiology

Discipline of Microbiology

School of Life Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Pietermaritzburg

South Africa

March 2016

ABSTRACT

As antibiotic producing symbionts of entomopathogenic nematodes, members of the genus *Photorhabdus* are candidates for biological control of insects and phytopathogenic microorganisms. The aim of this project was to establish the levels of genetic and antibiotic diversity amongst twenty *Photorhabdus* sp. isolates acquired from the South African Small Grain Institute, Agricultural Research Council (ARC) culture collection. These isolates represent a subset of *Photorhabdus* sp. isolates obtained from the Freestate and Western Cape provinces of South Africa.

Bacterial strain diversity was investigated using several techniques; which included phenotypic testing, genomic and proteomic fingerprinting approaches. Phenotypic characteristics were assayed using API 20E biochemical test strips in conjunction with several supplementary tests. Genomic fingerprinting involved 16S rRNA gene PCR-RFLP and RAPD-PCR. Phylogenetic relationships between the isolates were determined using partial 16S rRNA gene sequence analysis. MALDI-TOF-MS was used to generate proteomic profiles for isolate identification and mass spectra comparison.

Species-level differentiation between isolates and reference strains was achieved using 16S rRNA gene PCR-RFLP and partial 16S rRNA gene sequence analyses. Higher levels of strain differentiation between isolates were detected by RAPD-PCR and with MALDI-TOF-MS. In comparison to the high resolution of isolate diversity achieved by DNA-based analysis methods, the phenotypic characteristics proved to be of limited value. All methods evaluated suggest that the isolates are closely-related strains of *P. luminescens*. Two isolates were found to be non-*Photorhabdus* and identified as strains of *Xenorhabdus* sp. and *Pseudomonas* sp..

Antimicrobial activity amongst the isolates was screened for using disc-diffusion bioassays against *Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis*, *Rhizoctonia solani* and *Botrytis cinerea*. All isolates showed bioactivity against the Gram positive test organisms with weaker and variable antagonism against both fungal species. Three representative isolates were taken forward for further analysis of bioactive compounds produced. Antibiotic extraction and purification was attempted using several techniques including liquid-liquid extraction of broth supernatant using ethyl acetate, methanol extraction of cell pellet material and methanol extraction of lyophilised broth. Analysis of crude and partially purified antibiotic extracts was performed using C18 Sep-Pak clean-up, TLC, UPLC-ESI-TOF-MS and GC-MS.

All antimicrobial extraction methods yielded bioactive fractions. UPLC-ESI-TOF-MS proved the most valuable analytical method with data obtained suggesting that each isolate produced identical compounds. The dominant UPLC peak for all isolates and extraction procedures displayed an ESI-TOF-MS mass spectrum consistent with the antibiotic 3,5-dihydroxy-4-isopropyistilbene.

These results show that very limited genetic and phenotypic diversity existed between isolates. All isolates were identified as members of *P. luminescens* but did not cluster closely with any previously described subspecies on the basis of 16S rRNA partial gene sequence analysis. Likewise antimicrobial compound diversity between the three isolates assessed was found to be low, with the major bioactive compound identified as one previously described from *Photorhabdus* spp..

DECLARATION

I, Matthew George Dennis van Wyngaard, declare that

(i) The research reported in this thesis, except where otherwise indicated, is my original work.

(ii) This thesis has not been submitted for any degree or examination at any other university.

(iii) This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

(iv) This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

  (a) their words have been re-written but the general information attributed to them has been referenced;

  (b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced.

(v) Where I have reproduced a publication of which I am an author, co-author or editor, I have indicated in detail which part of the publication was actually written by myself alone and have fully referenced such publications.

(vi) This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References section.

Signed: …….

M. G. D. van Wyngaard (Candidate)

Signed: …….

C. H. Hunter (Supervisor)

ACKNOWLEDGEMENTS

I would like to acknowledge the National Research Foundation (NRF) for their generous funding of this research project, and the Agricultural Research Council (ARC) for supplying the *Photorhabdus* isolates used in this study.

My sincerest thanks to Charles Hunter for his support and guidance over the course of this research, and throughout the writing process.

I also owe thanks to the academic, technical and support staff, along with my postgraduate colleagues, who made my progress through the research and writing processes possible.

My sincerest gratitude to my family for their encouragement, with special thanks to my mother and father for their unwavering support throughout this research and always. A final thank you to Heather, whose continuous support and input were invaluable.

TABLE OF CONTENTS

LIST OF PLATES

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ARC	=	Agricultural Research Council
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxyribnucleotide
DSMZ	=	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	=	Ethylenediaminetetraacetic acid
GC-MS	=	Gas chromatography mass spectrometry
HPLC	=	High-performance liquid chromatography
MALDI-TOF-MS	=	Matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry
MIC	=	Minimum inhibitory concentration
MSP	=	Mass spectra profile
NA	=	Nutrient agar
NB	=	Nutrient broth
NBTA	=	Nutrient bromothymol blue agar
PCR	=	Polymerase chain reaction
PDA	=	Potato dextrose agar
RAPD	=	Randomly amplified polymorphic DNA
R_f	=	Retention factor
RFLP	=	Restriction fragment length polymorphism
RNA	=	Ribonucleic acid
rRNA	=	Ribosomal ribonucleic acid
ST	=	3,5-dihydroxy-4-isopropyistilbene
TCs	=	Toxin complexes
TLC	=	Thin-layer chromatography
TSA	=	Tryptic soy agar
TSB	=	Tryptic soy broth
TTC	=	Triphenyltetrazolium chloride
UPGMA	=	Unweighted pair group method with arithmetic mean
UPLC-ESI-TOF-MS	=	Ultra-performance liquid chromatography electrospray ionisation time-of-flight mass spectrometry
UV	=	Ultraviolet

INTRODUCTION

Members of the genus *Photorhabdus* are bacterial symbionts of *Heterorhabditis* nematodes; the two genera function together in an entomopathogenic relationship (Boemare and Akhurst, 2005). In this relationship, *Photorhabdus* spp. have been shown to produce a wide range of antimicrobial and insecticidal compounds which kill and preserve the insect from competing microorganisms in the soil environment while converting the cadaver into nutrients for the nematode offspring (Hu *et al*., 2006; Fischer-Le Saux *et al.,* 1999). The efficiency of the insecticidal complex has focussed research towards their application as biological control agents of microbial and insect pests (Hu *et al.,* 1999; Hu *et al.,* 1998). Insecticidal toxins expressed by *Photorhabdus* spp. have shown potential as target molecules for developing transgenic crops; they also have potential to be included in insecticidal sprays targetting crop-damaging insects that feed on plant tissue (ffrench-Constant *et al.,* 2007; Gerritsen *et al.,* 2005; Bowen *et al.,* 1998). In addition, the range of bioactive compounds produced by *Photorhabdus* spp., as well as their ability to preserve insect cadavers from competing microorganisms, have also attracted research interest (Bode, 2009; Piel, 2004).

Four species of *Photorhabdus* are currently recognised: *P. luminescens*, *P. temperata*, *P. asymbiotica* and *P. heterorhabditis* along with several subspecies (Ferreira *et al.,* 2014; Boemare and Akhurst, 2005). Although diversity within the genus *Photorhabdus* has been explored in various geographic locations such as Australia, North America, Europe and the Caribbean, little is known of the diversity and ecology of *Photorhabdus* spp. in the South African environment (Hazir *et al.,* 2004; Akhurst *et al.,* 2004; Fischer-Le Saux *et al.,* 1999; Fischer-Le Saux *et al.,* 1998). Furthermore, to the best of our knowledge no research has been undertaken to characterise and assess the diversity of antimicrobial compounds produced by a range of South African *Photorhabdus* isolates.

The aim of this study was to establish the levels of diversity amongst a collection of *Photorhabdus* spp. collected from various geographical locations within the Freestate and Western Cape provinces of South Africa. This subset of twenty *Photorhabdus* spp. isolates was obtained from the Agricultural Research Council (ARC). The initial research objective was to evaluate levels of species and strain diversity amongst these isolates using several approaches. These included: differentiation on the basis of a range of phenotypic traits; genomic assessments such as 16S rRNA partial gene

sequencing, 16S PCR restriction fragment length polymorphism (16S-PCR-RFLP) and randomly amplified polymorphic DNA (RAPD-PCR); and applying matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) as a means of characterisation on a proteomic basis. A subsequent objective of this research was to extract and characterise the antimicrobial compounds produced from representative isolates. This research investigated whether antimicrobial compounds produced demonstrate differences from previously characterised *Photorhabdus* compounds. After initial antimicrobial screening, three isolates representative of genetic groupings were selected for further antimicrobial compound assessment. Techniques applied here included investigation of several extraction and purification methods, and analysis using techniques such a thin-layer chromatography (TLC), Ultra-performance liquid chromatography electrospray ionisation time of flight mass spectrometry (UPLC-ESI-TOF-MS) and Gas chromatography mass spectrometry (GC-MS).

CHAPTER ONE

Literature review

1.1. Introduction

Photorhabdus is a fascinating bacterial genus owing to its unique life cycle and the ability to produce antibiotics which limit bacterial competition in insect cadavers (Hu *et al*., 2006). The genus *Photorhabdus* comprises a group of bioluminescent γ-proteobacteria that fall within the family Enterobacteriaceae (Boemare and Akhurst, 2005). Members of the genus form a symbiotic relationship exclusively with nematodes of the entomopathogenic genus *Heterorhabditis* (Boemare and Akhurst, 2006). Originally classified as *Xenorhabdus*, *Photorhabdus* was assigned its own genus due to genetic, phenotypic and fatty acid composition differences (Boemare and Akhurst, 2006; Boemare *et al.,* 1993). Differences between *Xenorhabdus* and *Photorhabdus* include several phenotypic characteristics, the genus of nematode host and the location of the bacterium in the nematode host gut. *Photorhabdus* spp*.* are distributed throughout the gut of *Heterorhabditis* spp*.* nematodes, whereas *Xenorhabdus* spp. are located in a gut vesicle of *Steinernematidae* spp. nematodes (Liu *et al.,* 2001). Nevertheless, there is a close relationship between the two bacterial genera in terms of their genetic makeup and the entomopathogenic symbiont niche they both occupy (Boemare and Akhurst, 2006; Rainey *et al.,* 1995).

All *Photorhabdus* spp. are highly pathogenic to insects, a dose of less than five cells via injection will kill within two to three days (Goodrich-Blair and Clarke, 2007). Due to this degree of pathogenicity and the wide range of antibiotics produced, *Photorhabdus* and its nematode symbiont have been investigated as a source of pharmaceutically active antimicrobial compounds (ffrench-Constant *et al.,* 2007a; Piel, 2004). Further, there is potential for *Photorhabdus* biocontrol use, both as an insecticidal nematode complex, as well as the use of separate insecticidal and antimicrobial metabolites, applied alone or via transgenic expression (ffrench-Constant *et al.,* 2007a; Bowen *et al.,* 1998).

The purpose of this literature review is to explore the diversity of the genus *Photorhabdus*, its metabolites and antimicrobial compounds, as well as methods applied in the study of the diversity of these bacteria.

1.2. *Photorhabdus* spp. phenotypic characteristics and taxonomy

Photorhabdus spp. are Gram negative rod-shaped bacteria. They exhibit a high degree of cell size variation, ranging from 0.5–2 µm by 1–10 µm, with some filamentous cells reaching up to 30 µm in length. These bacteria are facultative anaerobes and grow at a temperature range of 15–35°C, with some strains able to grow at 38°C. Protein inclusion bodies are found in a high percentage (5–80%) of cells in the stationary phase. Cells are motile by peritrichous flagella and have a tendency to swarm on agar surfaces (Boemare and Akhurst, 2005; Gerrard *et al.,* 2004). A range of antibiotics and pigments are typically synthesised and most strains are bioluminescent, emitting light at ~490 nm (Boemare and Akhurst, 2005; Forst and Nealson, 1996). All *Photorhabdus* spp. are highly virulent to insects, with little difference in their respective LT_{50}, which is the time required for 50% of infected insects to die after receiving a standard dose (Gerrard *et al.,* 2004; Forst and Nealson, 1996).

Four species of *Photorhabdus* are currently recognised: *P. luminescens* (type species), *P. temperata*, *P. heterorhabditis* and *P. asymbiotica* (Ferreira *et al.,* 2014; Boemare and Akhurst, 2005). Several subspecies within these four species have been described, including: *P. luminescens* subspecies *luminescens, laumondii, thracensis, akhurtsi, kayai, kleinii, sonorensis, caribbeanensi, noenieputensis* and *hainanensis* (Saenz-Aponte *et al.,* 2014; Ferreira *et al.,* 2013; Tailliez *et al.,* 2010; Boemare and Akhurst, 2006; Boemare and Akhurst, 2005; Hazir *et al.,* 2004); *P. temperata* subspecies *temperata, cinerea, khanii*, *tasmaniensis, stackebrandtii,* and *thracensis* (Saenz-Aponte *et al.,* 2014; Tailliez *et al.,* 2010; Tóth and Lakatos, 2008; Boemare and Akhurst, 2006; Boemare and Akhurst, 2005); and *P. asymbiotica* subspecies *australis* and *asymbiotica* (Tailliez *et al.,* 2010; Boemare and Akhurst, 2006; Boemare and Akhurst, 2005).

All species of *Heterorhabditis* nematodes have their respective *Photorhabdus* symbionts and cannot grow in their absence (Goodrich-Blair and Clarke 2007; Krasomil-Osterfeld, 1995). No *Photorhabdus*

spp. has been isolated freely from either soil or aquatic environments (Forst and Nealson 1996; Peel *et al.,* 1999). *Photorhabdus luminescens* is associated with *H. bacteriophora* and *H. indica* nematodes whereas *P. temperata* and is associated with *H. megidis*, *H. zealandica* and a subgroup of *H. bacteriophora* (Boemare and Akhurst, 2005). *Photorhabdus heterorhabditis* has been isolated from a *H. zealandica* nematode in South Africa (Ferreira *et al.,* 2014). *Photorhabdus asymbiotica* was named for its apparent lack of a nematode host due to its isolation solely from human clinical specimens (Boemare and Akhurst, 2005). However, Gerrard *et al.* (2006) found a *Photorhabdus* isolate obtained from a *Heterorhabditis* nematode that was genetically identical to a human clinical *P. asymbiotica* isolate.

1.2.1. Phase variation

Both *Photorhabdus* spp. and *Xenorhabdus* spp. undergo a phase shift when cultured in the laboratory (Boemare and Akhurst, 2005). This occurs when the culture characteristics of the wild-type isolate switches spontaneously to a variety with different characteristics (Boemare and Akhurst, 2005). The primary phase of *Photorhabdus* spp. is designated Phase one and is associated with the wild-type characteristics of strains isolated from the nematode, whereas the secondary phase appears after long term stationary phase growth in the laboratory and is termed Phase two (Boemare and Akhurst, 2005; Gerritsen *et al.,* 1995). Growth of *Heterorhabditis* nematodes depends on the presence of *Photorhabdus* cells, particularly of the primary phase culture. Nematode growth on secondary phase culture is poor (Goodrich-Blair and Clarke, 2007; Krasomil-Osterfeld, 1995). Reversion from Phase two to Phase one culture characteristics has not been demonstrated (Gerritsen *et al.,* 1992; Boemare and Akhurst, 2005).

Phase one colonies are typically mucoid, adhere to the inoculating loop and absorb dye. In addition, cells in the primary phase have proteinaceous inclusion bodies, exhibit bioluminescence, and produce antibiotics, pigments, lipases and proteases. Phase two variants generally produce smooth colonies with no adherence to the inoculating loop, do not absorb dye, are weakly bioluminescent, do not produce antibiotics, and have reduced lipase and protease activity levels (Boemare and Akhurst, 2005; Forst and Nealson, 1996; Gerritsen *et al.,* 1995; Gerritsen *et al.,* 1992). Phase two colonies can also exhibit pigmentation different to the primary form, are antigenically distinct and

differ in respiratory activity (Boemare and Akhurst, 2005; Gerritsen *et al.,* 1995; Smigielski *et al.,* 1994).

In general, the Phase two variants lack the characteristics of the primary phase, or exhibit them at greatly decreased levels (Gerritsen *et al.,* 1995). However, the classification of *Photorhabdus* strains into these two categories is not binary because a variety of phase variants have been distinguished that exhibit some properties of both phases (Forst and Nealson 1996; Gerritsen *et al.,* 1995). For example, Hu and Webster (1998) describe a variant producing small colonies on agar and exhibiting some Phase two culture traits. The small colony variant also produced primary phase colonies in addition to small colony variants when subcultured.

1.2.2. Culture handling

Photorhabdus spp. can be cultured on standard laboratory media such as tryptic soy agar (TSA) or nutrient agar (NA) at 28°C (Boemare and Akhurst, 2005). To combat phase shift, it is recommended to subculture colonies with Phase one traits weekly (Boemare and Akhurst, 2005). To maintain and select for primary phase variants, subculturing can be performed on a differential medium such as nutrient bromothymol triphenyltetrazolium agar (NBTA) medium which contains triphenyltetrazolium chloride (TTC) 0.004% (w/v) and bromothymol blue 0.0025% (w/v) (Fischer-Le Saux *et al.,* 1999). The bromothymol blue and TTC are only taken up by Phase one colonies (Boemare and Akhurst, 2005). Alternatively, other typical colony characteristics can be used to maintain a primary phase subculture such as choosing typically-pigmented, mucoid colonies (Boemare and Akhurst, 2005).

Storage of *Photorhabdus* for extended periods of time is complicated by the phase change phenomenon, therefore long-term culture storage at -80°C is recommended (Boemare and Akhurst, 2005). Refrigeration at 4°C is not recommended as it has been found to be lethal for many strains of *P. luminescens* (Peel *et al.,* 1999).

1.3. The *Photorhabdus*–nematode life cycle

Photorhabdus is located throughout the intestine of infective juvenile *Heterorhabditis* nematodes, known as stage 3 or dauer larva (Liu *et al.,* 2001; Fischer-Le Saux *et al.,* 1999). At this stage of its life cycle the nematode is non-feeding and the *Photorhabdus* cells do not divide (Boemare and Akhurst, 2005). When a juvenile nematode encounters an insect it penetrates via natural body openings, or directly through the cuticle, and releases 50–250 *Photorhabdus* cells into the insect haemolymph (ffrench-Constant *et al*., 2007; Hu *et al.,* 2006; Fischer-Le Saux *et al.,* 1999; Abu Hatab *et al.,* 1998). The bacteria then begin to divide, producing toxins, antibiotics, lipases and proteases which disrupt the insect's immune response, eventually killing it and protecting the cadaver from other opportunistic microorganisms (Eleftherianos *et al.,* 2007; ffrench-Constant *et al.,* 2007; Goodrich-Blair and Clarke, 2007; Sharad and Naved, 2005; Abu Hatab *et al.,* 1998).

The fertile hermaphrodite nematode begins to reproduce inside the insect cadaver after releasing *Photorhabdus* cells (Goodrich-Blair and Clarke, 2007). The rectal glands of the mother nematode develop high concentrations of *Photorhabdus* cells, which are the source of the *Photorhabdus* inoculum for progeny nematodes (Goodrich-Blair and Clarke, 2007). The juvenile nematodes are inoculated with *Photorhabdus* cells by a process known as *endotokia matricida* (Goodrich-Blair and Clarke, 2007). In this process juvenile nematodes grow within the mother nematode, consuming her. Nematodes feed on *Photorhabdus* cells and degraded insect tissue, and after the insect's resources are depleted, the progeny nematodes emerge from the insect cadaver as infective juveniles to repeat the infective cycle (Goodrich-Blair and Clarke, 2007; Abu Hatab *et al.,* 1998).

1.4. Active compounds

A range of antimicrobial and insecticidal compounds are produced by *Photorhabdus* to preserve the insect cadaver from opportunistic microorganisms in the soil environment (Hu *et al*., 2006; Piel, 2004; Fischer-Le Saux *et al.,* 1999; Krasomil-Osterfeld, 1995). The major classes of antimicrobial compounds produced by *Photorhabdus* spp. are the stilbene and anthraquinone pigments (Piel,

2004). In addition a number of insecticidal compounds are synthesised by *Photorhabdus* in the initial infection of the insect (ffrench-Constant *et al.,* 2007a).

1.4.1. Stilbenes

Stilbenes are a lipophilic class of antibiotics produced by all studied *Photorhabdus* spp. (Bode, 2009; Hu and Webster, 2000; Hu *et al.,* 1998). These compounds are typically produced by plants, and *Photorhabdus* is the only non-plant genus described with stilbene synthetic ability (Bode, 2009). The synthetic pathway for these compounds is based on condensation of β-ketoacyl intermediates as opposed to elongation of cinnamoyl-CoA by malonyl-CoA as occurs in plants (Bode, 2009).

Two hydroxystilbene antibiotics have been described in *Photorhabdus*: 3,5-dihydroxy-4-isopropyistilbene (ST) (Figure 1.1) and 3,5-dihydroxy-4-ethyistilbene (Figure 1.2) (Boemare and Akhurst, 2006; Forst and Nealson, 1996). Both stilbene compounds have been obtained from extractions of infected insect cadavers, though only ST has been described *in vitro* in tryptic soy broth (TSB) (Boemare and Akhurst, 2006; Hu *et al.,* 1998; Forst and Nealson, 1996). The mode of action of hydroxystilbene antibiotics is via the inhibition of RNA synthesis (Forst and Nealson, 1996). Activity against bacteria and fungi has been described for both compounds, while ST is also nematicidal and acts as a nematode repellent, with the exception of some *Heterorhabditis* species (Boemare and Akhurst, 2006; Hu *et al*., 1997; Forst and Nealson, 1996; Li *et al.,* 1995). Eleftherianos *et al.* (2007) reported ST to disrupt the phenoloxidase enzyme of the insect immune system and play an important role in the virulence of *Photorhabdus*.

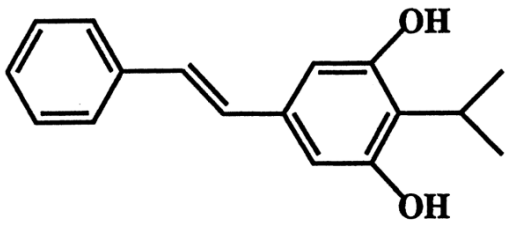


Figure 1.1. Structure of 3,5-dihydroxy-4-isopropyistilbene (Hu *et al.,* 1997).

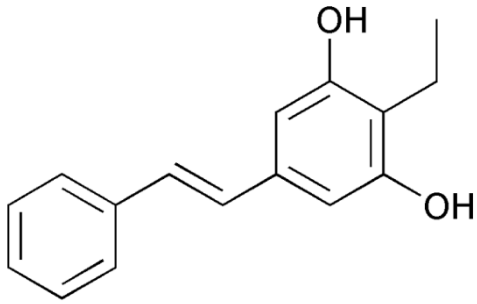


Figure 1.2. Structure of a 3,5-dihydroxy-4-ethyistilbene (adapted from Piel, 2004).

The ST compound is produced by the bacteria within 24 hours of the infection process of the insect and in broth culture assays, with detectable amounts present as early as 6 hours after inoculation (Eleftherianos *et al.* 2007; Hu *et al.,* 1999; Li *et al.,* 1995). In insect cadaver studies, Hu and Webster (2000) reported ST production within 24 hours of infection, with production increasing over the following 5 days and only slowly decreasing in concentration over the next 28 days. Hu *et al.* (1999) reported that ST levels remained relatively constant for a period of days after infection. In *in vitro* studies the concentration of ST was found to decrease over a one-week period after inoculation (Hu *et al.,* 1999; Li *et al.,* 1995).

A variant of the ST antibiotic, 2-isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3-diol (Figure 1.3) has been identified by Hu *et al.* (2006) from insects infected with *P. luminescens* and its nematode symbiont. This unstable compound was active at concentrations of 6.25–12.5 µg/ml against *Bacillus subtilis, Escherichia coli, Streptococcus pyogenes* and *Staphylococcus aureus* (Hu *et al.,* 2006). This

compound was also detected *in vitro,* but at a lower concentration than in the insect cadaver (Hu *et al.,* 2006). Interestingly, 2-isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3-diol was also active against mouse cancer cell lines to a greater extent than normal cell lines (Hu *et al.,* 2006).

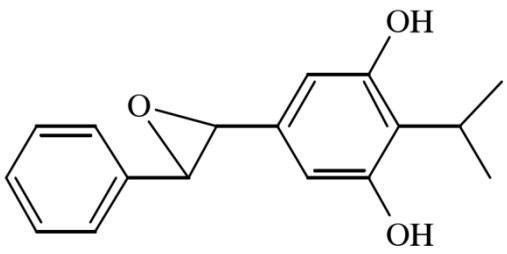


Figure 1.3. Diagram of 2-isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3-diol (adapted from Hu *et al.,* 2006).

1.4.1.1. Stilbene extraction

Extraction of *Photorhabdus* stilbene antibiotics is commonly achieved by ethyl acetate extraction of broth culture or infected insect cadavers (Eleftherianos *et al.,* 2007; Hu *et al*., 1997; Li *et al.,* 1995; Richardson *et al.,* 1988). Extraction of ST with acetone is reported to be more efficient (~95%) than recovery using methanol, diethyl ether, or ethyl acetate (Hu *et al*., 2006; Hu *et al*., 1997). Ethyl acetate fractions are commonly dried using $MgSO_4$ before evaporation using a rotary evaporator under partial vacuum at 30–60 °C (Eleftherianos *et al*., 2007; Williams *et al.,* 2005; Hu *et al.,* 1999; Li *et al*., 1995).

Purification of the resultant crude extracts has been achieved using a variety of methods. Hu *et al.* (1999) used flash silica gel chromatography with ethyl acetate and hexane (1 : 9) as the eluting solvent where ST was eluted early from the column. Similarly, Li *et al*. (1995) used increasing concentrations of ether in hexane as the eluting solvents where 60% ether eluted out ST. Hu *et al*. (1998) used 40% ether in hexane to separate ST from acetone extracts of infected insects and ethyl acetate extractions of broth culture supernatant. Further separation of ST was achieved with dichloromethane as the eluting solvent using silica flash chromatography of the ST-containing

fraction. Richardson *et al.* (1988) separated ST from an ethyl acetate extraction using increasing concentrations of dichloromethane in hexane in a silica column, where this antibiotic was eluted at 70% dichloromethane in hexane.

Chromatographic methods such as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) have been successful in the study of ST-containing fractions. Separation of ST using TLC was achieved by Hu *et al.* (1997) using a 98.5 : 1.5 chloroform : methanol mobile phase, where ST produced a band of R_f 0.59. The ST band was visualised under ultraviolet (UV) illumination at 254 nm, which concurs with Hathway (1962) who described an intense blue fluorescence of hydroxystilbenes when exposed to UV light. TLC was further found to be suitable for the quantification of ST from extracts using UV absorbance values (Hu *et al.,* 1997). HPLC separation of ST from other extracted compounds was also successful with acetonitrile and water as the mobile phase (Hu *et al.,* 1997).

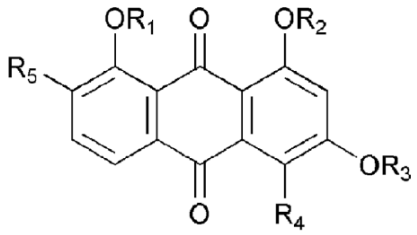
1.4.2. Anthraquinone pigments

Photorhabdus spp. are known for the production of a range of anthraquinone pigments, some of which function as antibiotics (Boemare and Akhurst, 2005). Anthraquinones are commonly isolated from fungi, lichen, plants and Actinomycetes (Betina, 1994). Biological activities of anthraquinones from various sources include:

- 2-methoxy-1,3,6-trihydroxyanthraquinone is an indirect cancer preventative antioxidant found in broccoli (Pawlus *et al.,* 2005).
- Antibiotic and purgative activities of anthraquinones in rhubarb extracts in traditional Chinese medicine (Ma *et al.,* 1989).
- An anthraquinone isolated from *Aspergillus glaucus* has shown antibacterial properties against Gram positive bacteria (Anke *et al.,* 1980).
- Three anthraquinone compounds isolated from *Cassia tora* (emodin, rhein and physcion) showed antifungal activity comparable to synthetic fungicides against phytopathogenic fungi (*Botrytis cinerea*, *Erysiphe graminis*, *Phytophthora infestans* and *Rhizoctonia solani*) (Kim *et al.,* 2004). Emodin (4,5,7-trihydroxy-2-methylanthraquinone) is also associated with

antibacterial activity against *S. aureus* and *B. subtilis*, though not against any of the Gram negative bacteria tested (Chukwujekwu *et al.,* 2006).

The synthesis of anthraquinones by Gram negative microorganisms is rare, with *Photorhabdus* being only the second genus described to do so (Bode, 2009). A variety of anthraquinone pigments produced by *Photorhabdus* spp. have been described by Piel (2004) (Figure 1.4). These compounds are produced by a type II polyketide synthesis pathway, a process similar to fatty acid formation via condensation of acetate and malonate units (Bode, 2009).



R^1 = Me, R^2 = H, R^3 = Me, R^4 = H, R^5 = H
R^1 = H, R^2 = Me, R^3 = Me, R^4 = H, R^5 = H
R^1 = H, R^2 = Me, R^3 = H, R^4 = H, R^5 = H
R^1 = H, R^2 = H, R^3 = Me, R^3 = H, R^4 = H
R^1 = H, R^2 = H, R^3 = H, R^4 = H, R^5 = H
R^1 = H, R^2 = Me, R^3 = Me, R^4 = H, R^5 = OMe
R^1 = Me, R^2 = H, R^3 = Me, R^4 = OH, R^5 = H

Figure 1.4. Structural variants of seven *Photorhabdus* anthraquinone pigments (Piel, 2004).

A range of anthraquinone antibiotics with activity against bacteria have been isolated from *Photorhabdus*-infected insect cadavers and a lesser number from broth culture (Boemare and Akhurst, 2006). Hu *et al.* (1998) describe three pigments produced by *P. luminescens* isolated from infected insects: 1,8-dihydroxy-3-methoxy-9,10-anthraquinone, 1-hydroxy-2,6,8-trimethoxy-9,10-anthraquinone and 1,4-dihydroxy-2,5-dimethoxy-9,10-anthraquinone. Li *et al.* (1995) isolated two yellow-coloured pigments from broth culture of *P. luminescens*, namely 3,8-dimethoxy-1-hydroxy-9,10-anthraquinone and 1,3-dimethoxy-8-hydroxy-9,10-anthraquinone.

Photorhabdus pigments differ in colour between strains. Composition of growth media appears to have an impact on colony colouration, as dictated by the pigments being produced (Boemare and Akhurst, 2005; Forst and Nealson, 1996). A pigment studied by Richardson *et al.* (1988) was subsequently identified as 1,6-dihydroxy-4-methoxy-9,10-anthraquinone, which is yellow below pH 9 and red at more alkaline pH.

1.4.2.1. Anthraquinone extraction

As anthraquinones are more commonly isolated from plants, extraction methodology has been geared to overcome a series of obstacles not relevant in the case of *Photorhabdus*. A typical plant extraction will involve heavy maceration of the target plant tissue followed by soaking in solvents such as methanol, ethanol, acetone, trichloromethane, and solvent mixtures for extended periods of time (Chukwujekwu *et al.,* 2006, Pawlus *et al.,* 2005, Kim *et al.,* 2004, Arrebola *et al.,* 1999, Ma *et al.,* 1989). Solubility of anthraquinones is not necessarily high in these solvents, as the solubility of the anthraquinone 2-methoxy-1,3,6-trihydroxyanthraquinone from *Morinda citrifolia* (Noni) was found to be low in methanol, acetone, and trichloromethane. However, this low solubility did not prevent extraction and identification of this particular compound (Pawlus *et al.,* 2005). Techniques to improve extraction yield include ultrasonic vibration and increased temperature via microwave radiation (Zhu *et al.,* 2008; Hemwimon *et al.,* 2007; Ma *et al.,* 1989). Extraction of *Photorhabdus* anthraquinones from broth culture has been achieved using similar methods to the extraction of the stilbene antibiotics. For example ethyl acetate extraction and elution from a silica column with 100% ether (Li *et al.,* 1995), or ethyl acetate extraction followed by elution from a silica column with 80% ethyl acetate or hexane (Richardson *et al.,* 1988).

1.4.3. Insecticidal toxins

A number of insecticidal toxins have been described from *Photorhabdus* spp. These include the toxin complexes (TCs), makes caterpillars floppy (Mcf) toxins, *Photorhabdus* virulence cassettes (PVCs), *Photorhabdus* insect-related (Pir) toxins and Txp40 (Wafa *et al.,* 2013; ffrench-Constant *et al.,* 2007a; Brown *et al*., 2006; Waterfield *et al.,* 2005).

The TCs are multi-subunit high molecular weight proteins (ffrench-Constant *et al.,* 2007a). The presence of TCs is not unique to *Photorhabdus* as they have been described in other members of the Enterobacteriaceae (Daborn *et al.,* 2001). The first description of TCs from *Photorhabdus* was by Bowen *et al.* (1998) as several peptides ranging in size from 25–207 kDa. Oral toxicity to insects has been described for subunits Tca and Tcd independently, although these have a synergistic effect when combined (ffrench-Constant and Bowen, 2000). Insecticidal activity of TCs has been demonstrated against thrips via direct feeding, and by feeding on coated leaf surfaces (Gerritsen *et al.,* 2005). It is suggested that TCs from different *Photorhabdus* isolates may have varying insect activity ranges (Gerritsen *et al.,* 2005). For example, certain described TCs are not orally active against thrips but show activity against caterpillars, whereas TCs of North American *P. temperata* strains demonstrate oral activity against thrips but not caterpillars (Gerritsen *et al.,* 2005). Oral toxicity is important for the use of these toxins as bio-insecticides, either by direct application or incorporation into transgenic plants (Yang and Waterfield, 2013). Transgenic expression of TcdA by *Arabidopsis* plants active against *Manduca sexta* caterpillars has been demonstrated (ffrench-Constant *et al.,* 2007a; Bowen *et al.,* 1998).

First described by Daborn *et al.* (2002), Mcf is a polypeptide, multi-domain toxin of 2,929 amino acids encoded by an 8.8 Kb gene. Mcf is believed to induce cell apoptosis and damage to the insect midgut upon injection, resulting in the characteristic loss of caterpillar body turgidity (Wafa *et al.,* 2013; ffrench-Constant *et al.,* 2007a). Domains of the toxin show similarity to Toxin B (*Clostridium difficile*) and to a cytolytic toxin produced by *Actinobacillus pleuropneumoniae* (ffrench-Constant *et al.,* 2007a).

PVCs act by inducing antifeeding and destroying insect haemocytes (Wafa *et al.,* 2013; ffrench-Constant *et al.,* 2007a). Putative effector proteins in the PVCs appear similar to certain portions of multi-domain toxins such as Mcf (*P. luminescens*), Toxin A (*C. difficile*), YopT (*Yersinia enterocolitica*) and CNF1 (*Escherichia coli*) (ffrench-Constant *et al.,* 2007a). Despite structural similarity to antibacterial R-type pyocins, the PVCs studied in *Photorhabdus* do not exhibit antibacterial activity (ffrench-Constant *et al.,* 2007a).

PirAB is a binary toxin with oral and injectable activity against mosquitoes and caterpillars (Wafa *et al.,* 2013; ffrench-Constant *et al.,* 2007a). PirAB appears to act via pore formation, with protein similarity and a putative activity mechanism similar to *Bt* Cry toxins (*Bacillus thuringiensis*) and leptinotarsin (*Leptinotarsa haldemani*) (ffrench-Constant *et al.,* 2007a).

Txp40 was initially isolated from *X. nematophila* by Brown *et al*. (2006), though is produced by species of both *Photorhabdus* and *Xenorhabdus*. Txp40 is a proteinaceous insecticidal toxin of 42 kDa that appears to target the midgut of the insect and to a lesser extent the fat bodies (Brown *et al*., 2006).

1.4.4. Other products of metabolism

Photorhabdus spp. produce a variety of metabolites which function in the processing of insect cadavers, including lipases, phospholipases, proteases and DNAses. Many of these metabolites are postulated to aid in bacterial pathogenicity (Sharad and Naved, 2005; Abu Hatab *et al.,* 1998; Forst and Nealson, 1996). Along with the stilbene and anthraquinone antibiotic compounds, *Photorhabdus* spp. also produce defective phage particles, bacteriocins and a siderophore photobactin (Figure 1.5) which have been shown to possess antibacterial activity (Piel, 2004, Forst and Nealson, 1996; Boemare *et al.,* 1992; Baghdiguian *et al.,* 1993).

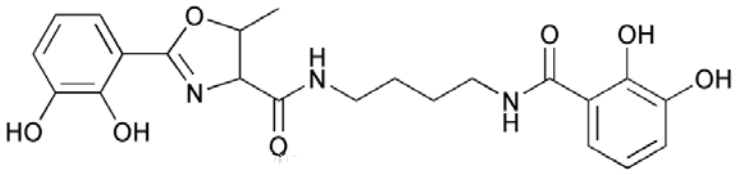


Figure 1.5. Diagram of photobactin (Piel, 2004).

1.5. Techniques for studying diversity of *Photorhabdus* spp.

1.5.1. Phenotyping of *Photorhabdus* spp.

Metabolic characteristics are the pre-genetic era standard for identification and classification of microbial species. Despite methodological limitations phenotypic characteristics are still widely used in modern microbiology and microbial taxonomy (Bochner, 2009). Complicated methods and issues with reproducibility have been improved upon by the availability of standardised commercially-available metabolic characteristic test kits (e.g. API (bioMérieux) and BIOLOG) and advances in phenotypic microarrays (Bochner, 2009).

Phenotypic characteristics have been used in differentiating *Xenorhabdus* and *Photorhabdus* strains (Boemare and Akhurst, 2005; Fischer-Le Saux *et al*., 1999). A number of metabolic characteristics are variable between *Photorhabdus* species and subspecies, these characteristics include: maximum growth temperature, indole production, DNase production, urease (Christensen's), esculin hydrolysis, tryptophan deaminase, Simmons' citrate, annular haemolysis (sheep or horse blood agar), acid production from mannitol and trehalose, and utilisation of L-fucose, DL-glycerate, L(-) histidine, myo-inositol, DL-lactate and D-mannitol (Boemare and Akhurst, 2006; Fischer-Le Saux *et al*., 1999). However the choice of media for phenotypic tests is an important consideration as these can have an impact on the test outcome for *Photorhabdus* strains. For example, Simmons citrate and Christensen's citrate media provide differing results and variable descriptions of DNase, urease and maltose fermentation activity were reported from the same *Photorhabdus* isolates (Akhurst *et al.,* 2004). These variations have been ascribed to differences in biochemical test protocols and incubating conditions (Akhurst *et al*., 2004). Additionally phase variation complicates the phenotyping of *Photorhabdus* and *Xenorhabdus*, as this affects the expression of metabolic characteristics (Boemare and Akhurst, 2006).

1.5.2. Genotyping of *Photorhabdus* spp.

In the last decade the phenotyping of bacteria has largely been superceded by molecular techniques (Li *et al.,* 2009). Substrates and reagents for phenotypic testing can be expensive and the processing

of large amounts of isolates is labour-intensive (Olive and Bean, 1999; Van Belkum, 1994). Genotyping methods offer a higher resolution of inter- and intra-species variation in bacteria while providing faster turnaround time, higher resolution and superior reliability (Li *et al.,* 2009, Rademaker *et al.,* 2005; Van Belkum, 1994). Bacterial DNA fingerprinting involves techniques to determine polymorphisms in genomes or gene loci. Commonly used genotyping techniques are PCR amplification, restriction enzyme digestion and fragment length analysis techniques (Rademaker *et al.,* 2005).

Methods of whole genome fingerprinting without the use of PCR include restriction fragment length polymorphism (RFLP) and pulse field gel electrophoresis (PFGE) (Li *et al.,* 2009). Briefly, RFLP of genomic DNA involves whole genome digestion using specific restriction endonucleases, fragment separation using gel electrophoresis and thereafter southern blotting using specific probes (Li *et al.,* 2009; Olive and Bean, 1999). The use of rRNA gene specific probes is termed ribotyping (Li *et al.,* 2009). While PFGE also uses restriction digestion of the whole genome, the target endonuclease cutting sites are rarer and resulting genome fragments are larger. These can then be separated using pulse field electrophoresis and DNA banding patterns compared (Li *et al.,* 2009). Widely used PCR-based fingerprinting methods include randomly-amplified polymorphic DNA (RAPD) and PCR-restriction fragment length polymorphism (PCR-RFLP) or amplified ribosomal DNA restriction enzyme analysis (ARDRA). PCR-based fingerprinting techniques exploit variations between product profiles visualised using gel electrophoresis for comparison between isolates based on similar and dissimilar bands (Li *et al.,* 2009). A number of such PCR-based methods have been used to elucidate the diversity and relationships between *Photorhabdus* isolates. These include 16S rRNA gene PCR-RFLP or ARDRA, DNA-DNA hybridisation, and 16S rRNA gene sequencing (Liu *et al.,* 2001; Fischer-Le Saux *et al*., 1998; Brunel, *et al*., 1997; Boemare *et al.,* 1993).

1.5.2.1. 16S rRNA gene PCR-RFLP fingerprinting

Many genes can and have been used for locus-specific RFLP; for example 16S rRNA, 23S, rRNA and 16S–23S intergenic spacer regions (Olive and Bean, 1999). 16S rRNA gene PCR-RFLP involves the amplification of the 16S rRNA gene followed by digestion by a site-specific restriction endonuclease.

The resulting restriction fragments can then be visualised by agarose gel electrophoresis for comparative purposes between strains and to analyse relatedness (Li et al., 2009).

PCR-RFLP analysis has been widely used for the study of diversity between bacterial species and strains (Rademaker *et al.,* 2005). A rapid fingerprinting method based on RFLP has been used to differentiate *Photorhabdus* and *Xenorhabdus* strains based on patterns of restriction digests of the amplified 16S rRNA gene (Brunel *et al*., 1997). Amplified 16S rRNA genes from *Photorhabdus* and *Xenorhabdus* have been successfully digested individually by a range of restriction endonuclease enzymes (e.g. CFOI, HINFI, DDEI, ALUI, HAEIII, and MSPI) (Brunel *et al*., 1997). Identification of *Xenorhabdus* species and separation of all *Photorhabdus* genotypes have been achieved using three restriction endonuclease enzymes, namely CFOI, ALUI AND HAEIII (Fischer-Le Saux *et al*., 1998; Brunel *et al*., 1997). However, these studies were performed before the description of the *P. temperata*, *P. asymbiotica* and *P. heterorhabditis* species.

Advantages of 16S rRNA gene PCR-RFLP fingerprinting are that it is cheaper and faster than 16S rRNA gene sequencing or DNA-DNA hybridisation (Brunel *et al*., 1997). For *Photorhabdus* specifically 16S rRNA gene PCR-RFLP has been found to be as effective in grouping isolates as DNA-DNA hybridisation (Fischer-Le Saux *et al*., 1998). Furthermore, phase variation between isolates is not considered to impact experimental outcomes (Brunel *et al*., 1997). A disadvantage of 16S rRNA gene PCR-RFLP is the effects of polymorphic gene copies within an isolate, which may result in complex banding patterns (Brunel *et al*., 1997). This occurrence can be recognised and accounted for by summing up fragment sizes, which should be less than or equal to the pre-digested 16S rRNA gene fragment size (Brunel *et al*., 1997).

1.5.2.2. RAPD-PCR fingerprinting

RAPD-PCR involves the use of a single short primer of 9–10 bp which can detect polymorphisms throughout genomic DNA when used at low, nonspecific annealing temperatures (Van Belkum, 1994). The amplimers are the result of the distance between the 3' ends of annealed primers and are therefore variable in length (Li *et al.,* 2009; Olive and Bean, 1999). When separated by gel

electrophoresis these amplimers result in a fingerprint for that particular genome (Li *et al.,* 2009). This fingerprinting technique has been successfully applied to differentiate closely-related bacterial species and strains (Olive and Bean, 1999). Unfortunately, there are a number of disadvantages associated with RAPD, namely inter-laboratory reproducibility issues and overcomplicated patterns which may be difficult to analyse (Olive and Bean, 1999). Furthermore, RAPD is sensitive to PCR run conditions and reagent concentrations, with variable banding profiles produced as a result (Li *et al.,* 2009; Rademaker *et al.,* 2005). However, strict standardisation of run conditions and reagent sources has improved the reliability of RAPD as a fingerprinting technique, as has the availability of commercial RAPD-PCR kits (Li *et al.,* 2009).

Research published using RAPD-PCR analysis on entomopathogenic nematode symbionts is limited, with only one study published to our knowledge (Kumar *et al.,* 2011). There is, however, potential for RAPD-PCR as a high-throughput screening technique for processing large volumes of isolates in diversity studies. Furthermore, *Photorhabdus* has limited 16S rRNA gene sequence diversity, therefore a genome-wide technique like RAPD-PCR may be better able to resolve differences between closely-related strains and isolates.

1.5.2.3. Gene sequencing

The 16S rRNA gene is important in bacterial identification due to its presence in all bacterial lineages (Li *et al.,* 2009). Hence, sequencing of the 16S rRNA gene has historically been the benchmark sequence used for bacterial species identification (Li *et al*., 2009; Mellmann *et al*., 2008; Janda and Abbott, 2002). Insufficient polymorphisms in this gene sequence complicates differentiation between closely-related bacterial strains, hence modern microbiology has moved towards other less conserved gene sequences for the purposes of diversity studies (Li *et al.,* 2009). In *Photorhabdus*, several alternative gene sequences to 16S rRNA genes have been used for relatedness studies. These include: *gyrB* (DNA gyrase subunit B), *recA* (recombinase), *dnaN* (DNA polymerase III beta subunit), and *gltX* (glutamyl-tRNA synthetase) (Orozco *et al.,* 2013).

A polyphasic or combined approach incorporating multiple phenotypic and genetic characterisations has been suggested as a more comprehensive assessment of strain diversity (Janda and Abbott, 2002). The use of a single gene has the potential for an incomplete assessment of strain diversity; therefore multiple gene sequences have been used. In the case of *Photorhabdus*, a polyphasic approach has been successfully applied using combined data gleaned from phenotypic testing, 16S rRNA gene sequences and DNA-DNA hybridisation (Boemare and Akhurst, 2006; Fischer-Le Saux *et al*., 1999).

1.5.3. Isolate characterisation using MALDI-TOF-MS

Matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) has been applied as a technique for the identification and classification of microorganisms (Welker and Moore, 2011). MALDI-TOF-MS offers differentiation of bacteria based on fractionation of proteins from disrupted bacterial cells with soft ionisation which allows mass spectra of large, unfragmented molecules (Welker and Moore, 2011; Lay, 2000). This technique offers fast turnaround time, high sensitivity, reproducibility, low consumable costs and analysis over a large range of *m/z* values (Krásný *et al.,* 2013; Freiwald and Sauer, 2009).

A common technique for MALDI-TOF-MS involves analysis of the whole bacterial cells from solid culture media (Dare, 2006; Fenselau and Demirev, 2001). This begins with lysed cells followed by co-crystallisation with a matrix compound on a target plate (Welker and Moore, 2011). Cells are lysed using techniques involving water, solvents and strong acids to expose both internal and external cell proteins for analysis. Examples of extraction protocols include ethanol-formic acid and trifluoroacetic (TFA) extractions, although it is possible for untreated cells to be analysed directly (Freiwald and Sauer, 2009; Fenselau and Demirev, 2001). The matrix compound is involved in transferring energy to cell components during ionisation as well as separating biopolymers on the target spot (Welker and Moore, 2011). Common matrix compounds for proteomic studies include 3,5-dimethoxy-4-hydroxycinnamic acid and α-cyano-4-hydroxycinnamic acid (HCCA) (Carbonnelle *et al.,* 2011).

Ribosomes are the predominant source of proteins in bacterial samples (Welker and Moore, 2011). Other protein sources include outer membrane proteins, DNA binding proteins, ribosome modulation factors, RNA chaperones, carbon storage regulators and cold shock proteins (Krásný *et al.,* 2013; Welker and Moore, 2011). There are two approaches to bacterial identification using mass spectra. One is the use of biomarker peaks, which are specific diagnostic peaks indicative of a species or strain (Wang *et al.,* 1998). Biomarker proteins are theoretically ideal for identification as they are always expressed during cell growth and are affected less by processing variables (Dare, 2006). An alternate approach to spectral analysis is the examination of mass fingerprints, which involves the comparison of the whole spectra patterns (Carbonnelle *et al.,* 2011). These mass fingerprints can then be compared between the samples in a set of isolates or with established reference spectra (Welker and Moore, 2011). The sample spectral fingerprint is compared to the reference spectra in a library and a similarity score is assigned mathematically (Krásný *et al.,* 2013). In addition to numerical score identification outputs, taxonomic dendrograms can also be generated, and open source programs such as SPECLUST offer cluster analysis of mass fingerprints (Welker and Moore, 2011; Alm *et al.,* 2006).

Identification databases have been incorporated into several MALDI-TOF-MS systems; examples include the Bruker Biotyper, SARAMIS by bioMérieux and Andromas by Andromas SAS (Krásný *et al.,* 2013). Commercial databases at present represent a small subset of known bacteria, of which the primary focus is clinical isolates (Krásný *et al.,* 2013; Welker and Moore, 2011). With environmental isolates, identification with these databases is often limited by under-representation; however custom spectral libraries offer flexibility with customisable reference libraries (Welker and Moore, 2011).

Spectral reproducibility is the key factor with inter-spectra analyses. This is affected by a number of factors: culture media, choice of matrix, cell lysation protocol, culture age, laser choice and run parameters (Šedo *et al,* 2011; Lay, 2000). However it is possible to control these factors with appropriate standardisation (Carbonnelle *et al.,* 2011). Certain biomarker peaks amenable for the purposes of bacteria identification are typically conserved even with growth and processing variables (Wang *et al*., 1998). The use of reference spectra with a limited number of high intensity biomarker peaks can also offer lower susceptibility to culture growth condition variations (Carbonnelle *et al*., 2011). The ability of the system to measure small-scale differences between

culture conditions and sample processing, illustrates the sensitivity of this method in bacterial differentiation (Lay, 2001).

1.6. Conclusion

Photorhabdus spp. occupy an interesting ecological niche, and the genus has gained attention as a source of a variety of antimicrobial compounds. In addition, there is potential for use of the entomopathogenic nematode complex or extracted bacterial metabolites for biocontrol purposes. Relatively little research has been conducted as to the diversity of *Photorhabdus* spp. extant in South Africa, and the compounds these may produce. This research aims to examine a selection of South African *Photorhabdus* isolates, in terms of species and compound diversity. This research work was approached in two stages. Characterisation of a selection of metabolic traits was performed to determine phenotypic diversity amongst the isolates. This was followed by genetic diversity assessment applying DNA fingerprinting techniques, sequence analysis of partial 16S rRNA gene sequences, and applying MALDI-TOF-MS for proteomic differentiation. Thereafter antimicrobial compound diversity assessments were performed. All isolates were examined in disc-diffusion bioassay against a range of fungal and bacterial species. Isolates were then selected on apparent diversity and carried forward for examination of compounds being synthesised using two different antibiotic extraction approaches and ESI- and GC- MS analysis. To our knowledge this is the first study into the diversity of amongst a range of South African *Photorhabdus* isolates and the first data on identification of an antimicrobial compound from these isolates.

CHAPTER TWO

Materials and methods

2.1. Bacterial isolates

Twenty isolates presumptively identified as strains of *Photorhabdus* spp. were evaluated in this study. These were sourced from the South African Small Grain Institute, Agricultural Research Council, Welkom, RSA from a collection of bacterial isolates obtained from nematodes identified as *Heterorhabditis bacteriophora* (Hatting *et al.,* 2009) (Table 2.1). Isolates originated from a total of eleven distinct sampling locations and a range of crop species, primarily in the Freestate at Bethlehem and Fouriesburg; with one isolate obtained from Clanwilliam in the Western Cape. Two reference strains *P. luminescens* DSM 3368 and *P. temperata* DSM 14550 were included in the study for comparative purposes (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)).

Table 2.1. Geographical and crop species origin of *Photorhabdus* isolates (Hatting *et al.,* 2009).

Isolate	Location	Habitat
22	Bethlehem - Freestate	Broccoli
32	Fouriesburg - Freestate	Blueberry
36	Fouriesburg - Freestate	Cherry
40	Fouriesburg - Freestate	Cherry
50	Fouriesburg - Freestate	Blueberry
66	Unspecified	Unspecified
91	Fouriesburg - Freestate	Apple (Sundowner)
96	Fouriesburg - Freestate	Apple (Breyburn)
151	Clanwilliam - Western Cape	Pine forest
161	Bethlehem - Freestate	Apple/*Otiorhynchus sulcatus*
164	Bethlehem - Freestate	Apple (Braeburn)
165	Bethlehem - Freestate	Apple (Braeburn)
170	Fouriesburg - Freestate	Apple
172	Fouriesburg - Freestate	Apple
173	Fouriesburg - Freestate	Apple
174	Fouriesburg - Freestate	Apple
175	Fouriesburg - Freestate	Apple/*Otiorhynchus sulcatus*
178	Bethlehem - Freestate	Raspberries
179	Bethlehem - Freestate	Raspberries
180	Bethlehem - Freestate	Raspberries

2.1.1. Culture preservation and maintenance of *Photorhabdus* isolates

Type strains *P. luminescens* DSM 3368 and *P. temperata* DSM 14550 used for reference purposes were obtained from the Leibniz-Institut DSMZ culture collection in the form of lyophilised broth cultures. These were revived by resuspending in sterile tryptic soy broth (TSB) (Biolab, Merck, Darmstadt, Germany), after which inoculum was streaked onto tryptic soy agar (TSA) plates (Biolab) and incubated at 28°C before preparation for long-term storage. The ARC isolates were provided as freshly-inoculated cultures in plastic Petri dishes on an agarised nutrient medium.

Isolates and reference strains were routinely cultured on nutrient bromothymol triphenyltetrazolium agar (NBTA) (Boemare and Akhurst, 2005). This medium comprised Nutrient agar (NA) (31 g/L) (Biolab) supplemented with 0.0025% (w/v) bromothymol blue (Sigma Aldrich, St. Louis, Missouri, USA) and filter-sterilised (0.22 µ) 0.004% (w/v) triphenyltetrazolium chloride (TTC) (Sigma Aldrich) which were added aseptically after autoclaving the basal medium at 121°C for 15 minutes (103.4 kPa). Bacterial cultures inoculated on NBTA were incubated inverted at 28°C for 48 h.

In order to avoid phase variation and to maintain wild-type characteristics, master cultures of the bacterial isolates were prepared for long-term storage. Isolates were subcultured by three-way streak onto NBTA and incubated at 28°C for 48 h. Primary phase colonies (Phase one) were identified and selected for on the basis of dye absorption by the colony, and a mucoid colony appearance (Boemare and Akhurst, 2005). A single Phase one colony pick-off was inoculated into a 100 mL flat-bottomed conical flask containing 20 mL of sterile TSB (Biolab). After incubation in a rotary shaking incubator at 28°C and 150 rpm for 24 h, glycerol stocks (20% v/v sterile glycerol) were made up in sterile 1 mL microfuge tubes, before storing at -80°C until use. *Photorhabdus* reference strains and isolates were revived from glycerol stocks by inoculation onto NBTA (28°C, 24–48 h). Each inoculum was subcultured onto NBTA twice before use.

2.2. Determination of phenotypic characteristics of *Photorhabdus* isolates

A selection of phenotypic tests were used to differentiate the *Photorhabdus* isolates. These were selected from a range of possible characteristics which have been previously applied to successfully differentiate *Photorhabdus* species (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005).

2.2.1. Phenotypic characterisation using API 20E test kit

Phenotypic characteristics of reference strains and isolates were assessed using API 20E test strips, which were prepared and inoculated according to the standard protocol detailed by the manufacturer (bioMérieux, Marcy-l'Étoile, France). Each inoculum was made up by resuspending a 24 h *Photorhabdus* sp. colony from nutrient agar (NA) into 5 mL sterile 0.85% (w/v) NaCl in distilled water. The inoculated API kits were incubated at 28°C and the results were recorded after 48 h according to the manufacturer's guidelines.

2.2.2. Phenotypic characteristics assessed using supplementary biochemical tests

Supplementary biochemical tests used to differentiate isolates included determination of amylase and catalase production, casein and esculin hydrolysis, fermentation of mannitol and trehalose, and citrate and urea utilisation.

2.2.2.1. Amylase production

Amylase production was assessed using TSA amended with 0.2% soluble starch (2.0 g/L) and autoclaved at 121°C for 15 minutes (103.4 kPa). Medium pH before autoclaving was pH 7.1 (Holding and Collee, 1971). Bacterial inoculum was aseptically applied as a single line streaked along the diameter of each plate in duplicate. After incubation for 48 h at 28°C, plates were flooded with 5% (w/v) Lugol's iodine solution to determine the presence or absence of starch. A zone of clearing

visible around a colony after addition of the iodine solution was interpreted as an amylase positive result; the absence of any zone of clearing was interpreted as an amylase negative response. An uninoculated plate was used as a negative control and incubated under the same conditions as experimental cultures.

2.2.2.2. Catalase production

The production of catalase by the *Photorhabdus* isolates was determined by inoculating a single line of inoculum onto a NA plate in duplicate. After 24 h of incubation at 28°C the plates were flooded with a 2% (v/v) H_2O_2 solution (Holding and Collee, 1971). Oxygen evolution as evidenced by the formation of bubbles was seen as a positive result for catalase production; whereas a lack of bubbles was considered a negative result. An uninoculated plate was included as a negative control and incubated under the same conditions as experimental cultures.

2.2.2.3. Casein hydrolysis

Protease production was assessed using skim milk agar medium made up by combining two solutions. Solution one consisted of: tryptone 17.0 g, soytone 3.0 g, sodium chloride 5.0 g, and bacteriological agar 15.0 g made up to 500 mL with distilled water and autoclaved at 121°C for 15 minutes (103.4 kPa). Solution two contained 100.0 g skim milk powder dissolved in 500 mL of water which was autoclaved separately at 121°C for 12 minutes (103.4 kPa) before being added to the first solution, with gentle mixing to avoid bubbles. Duplicate plates were inoculated with a single streak of inoculum across the diameter of the plate. Plates were incubated for 72 h at 28°C. A zone of clearing around the colony indicated a positive result; whereas an absence of clearing indicated a negative result. An uninoculated casein agar plate was used as a negative control and incubated under the same conditions as experimental cultures.

2.2.2.4. Esculin hydrolysis

The ability of an isolate to hydrolyse esculin to esculetin was determined using an agar medium consisting of: bacteriological agar 15.0 g/L, esculin 1.0 g/L and ferric ammonium citrate 0.5 g/L (Atlas, 2010). The medium pH was measured at pH 7.1 prior to autoclaving at 121°C for 15 minutes (103.4 kPa). Inoculum was applied as a single streak across the agar plate diameter, performed in duplicate, and monitored periodically over one week of incubation at 28°C. Production of a dark zone around the colony was seen as a positive result for esculin hydrolysis; an absence of a dark ring constituted a negative result. An uninoculated plate was used as a negative control and incubated under the same conditions as experimental cultures.

2.2.2.5. Carbohydrate fermentation

The fermentation of mannitol and trehalose was assessed using a basal agar medium adapted from Singer (2001) which comprised: $(NH_4)_2HPO_4$ 0.05 g/L, KCl 0.1 g/L, $MgSO_4.7H_2O$ 0.1 g/L, yeast extract 0.1 g/L, bacteriological agar 7.5 g/L and bromothymol blue 0.004 g/L. The medium was autoclaved at 121°C for 15 minutes (103.4 kPa) before being supplemented with each respective carbon source. Mannitol and trehalose were made up as individual solutions each containing 2.5 g carbon source in 50 mL distilled water; these solutions were filter sterilised (0.22 µ) and then added aseptically to the basal media for a final concentration of 10 g/L, before the agar plates were poured. Medium pH before autoclaving or carbohydrate amendment was measured at pH 7.1.

Fifty microlitres of inoculum was taken from a 24 h nutrient broth (NB) (Biolab) culture (28°C at 150 rpm) and applied, in duplicate, onto sterile filter paper discs (9 mm, Macherey-Nagal, Düren, Germany) placed aseptically onto the agar surface. Plates were incubated at 28°C for 7 d. The production of acid during fermentation of the respective carbohydrates was indicated by a colour change of the medium from blue to yellow in the region adjacent to bacterial growth. Negative controls were included in the form of basal media without any carbohydrate amendments, and uninoculated amended plates, both of which were incubated under the same conditions as experimental cultures.

2.2.2.6. Citrate utilisation

A Simmons citrate medium was used to determine citrate utilisation (Atlas, 2010). The medium contained: sodium citrate 2.0 g/L, $MgSO_4.7H_2O$ 0.20 g/L, $(NH_4)H_2PO_4$ 1.0 g/L, K_2HPO_4 1.0 g/L, NaCl 5.0 g/L, bromothymol blue 0.08 g/L and bacteriological agar 15.0 g/L made up to 1 L with distilled water, and autoclaved at 121°C for 15 minutes (103.4 kPa). The pH of the medium before autoclaving was measured at pH 6.9. Bacteria were inoculated in duplicate as a single streak across the agar plate diameter which was then incubated at 28°C for 7 d. A colour change of the agar medium from green to blue indicated citrate utilisation by the bacteria; no colour change was interpreted as a negative result. An uninoculated plate served as a negative control and was incubated under the same conditions as experimental cultures.

2.2.2.7. Urea utilisation

The utilisation of urea as a nitrogen source was assayed using a urea-enriched agar medium (Atlas, 2010). The basal medium contained: bacteriological agar 15.0 g/L, NaCl 5.0 g/L, KH_2PO_4 2.0 g/L, peptone 1.0 g/L, glucose 1.0 g/L and phenol red 0.012 g/L made up to 900 mL with distilled water. The medium was autoclaved at 121°C for 15 minutes (103.4 kPa). A filter-sterilised (0.22 µ) solution containing 20.0 g of urea in 100 mL distilled water was added when the molten basal agar had cooled to ~49°C after autoclaving. The plates were inoculated in duplicate with 50 µL of 24 h NB culture (28°C at 150 rpm) onto sterile filter paper discs (9 mm, Macherey-Nagal) placed aseptically onto the agar surface. Plates were incubated at 28°C for 7 d. A positive result was indicated by a medium colour change to pink or red; a lack of colour change constituted a negative result. Experimental controls were comprised of the basal medium lacking in urea and made up to 1.0 L volume. The pH of the medium after autoclaving is specified as pH 6.8 ± 0.2.

2.3. Determination of genetic diversity of *Photorhabdus* isolates

Genetic diversity amongst the *Photorhabdus* isolates was assessed using DNA fingerprinting approaches in the form of RAPD-PCR and 16S rRNA gene PCR-RFLP. Phylogenetic relationships of

isolates were determined by sequence analysis of 16S rRNA gene fragments. Phlyoproteomic relationships between isolates were also evaluated using MALDI-TOF-MS.

2.3.1. DNA extraction from *Photorhabdus* isolates

Cultures of each isolate were grown up in 10 mL of Luria-Bertani (LB) broth. This medium contained: tryptone 10.0 g/L, yeast extract 5.0 g/L, NaCl 10.0 g/L in distilled water, and adjusted to pH 7.5 before autoclaving at 121°C for 15 minutes (103.4 kPa). A single bacterial colony was inoculated into sterile LB broth and incubated overnight (18 h) at 28°C at 150 rpm in a rotary shaker-incubator. Thereafter, 1 mL of culture was taken and centrifuged in microcentrifuge tubes at 8000 x *g* for 5 minutes, and the culture pellet processed with a Nucleospin DNA Extraction Kit (Macherey-Nagal) using the standard protocol for Gram negative bacteria. Extracted DNA was stored in sterile 1.5 mL microfuge tubes at -20°C until use. The primers used in all PCR protocols are listed in Table 2.2 and were synthesised by Inqaba Biotech (Hatfield, Pretoria, RSA).

Table 2.2. Primers used to determine the genetic diversity amongst *Photorhabdus* isolates.

PCR Primer	Sequence (5–3´)	Reference
16S rRNA		Fischer-Le Saux *et al.* (1999)
Forward	GAAGAGTTTGATCATGGCTC	
Reverse	AAGGAGGTGATCCAGCCGCA	
RAPD-PCR		Daffonchio *et al*. (1998)
OPG 5	CTGAGACGGA	
OPG 8	TCACGTCCAC	
OPG 11	TGCCCGTCGT	
OPG 16	AGCGTCCTCC	

2.3.2. PCR product visualisation

All PCR products were visualised using agarose gel electrophoresis. 16S rRNA gene PCR and RAPD-PCR products electrophoresis was carried out using 1.5% (w/v) agarose gel (Laboratorois Conda, Madrid, Spain), whereas a 2% (w/v) agarose gel was used for 16S rRNA gene PCR-RFLP products. In each instance a 1x Tris/Borate/EDTA running buffer (TBE) (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8) was used.

The gels were pre-stained with 1x SYBR Safe (Invitrogen, California, USA). A volume of 4 µL DNA product was mixed with 1 µL 6x blue-orange loading dye (Promega, Madison, USA) before loading onto the gel. 1.5 kb and/or 100 bp molecular weight ladders (Promega) were included in each gel run to allow for estimation of amplicon fragment size. Gels were run for 60–120 minutes at 90 V in 1x TBE buffer, and then visualised under ultraviolet (UV) illumination. Images of each gel was captured on Syngene G:Box using GeneSnap software (Version 7.0.9) (Syngene, Cambridge, England).

2.3.3. 16S rRNA gene fragment PCR

The PCR primers used for 16S rRNA gene fragment amplification are listed in Table 2.2. Each PCR reaction (50 µL) comprised 0.4 µM primer, (1x) GoTaq Flexi buffer (Promega), 200 µM per dNTP, 0.5 µL of template DNA, 1.25 U/50 µL GoTaq polymerase (Promega) and $MgCl_2$ optimised to 1.5mM. Each PCR reaction was made up to a final volume 50 µL with nuclease-free H_2O (Promega). The PCR reactions were run in a Bioer XP Thermo Cycler (Model TC-XP-G, Bioer Technology Co. Ltd., Binjiang district, China). Reaction conditions were as follows: 2 minutes at 95°C; 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 2 minutes at 72°C; followed by 72°C for 5 minutes. Reactions were cooled to 4°C after the run before storage at -20°C until needed. The positive control of this PCR and for the subsequent 16S gene PCR-RFLP was a *P. luminescens* Isolate p22, which had been successfully amplified using this primer in previous studies.

2.3.3.1. PCR-RFLP analysis of 16S rRNA gene fragment PCR product

PCR-RFLP fingerprinting of 16S rRNA gene fragments from *Photorhabdus* isolates was carried out using HAEIII, HHAI, ALUI restriction endonucleases (Fermentas Fastdigest, Thermo Fisher Scientific, Lithuania) according to the method of Brunel *et al*. (1997). Prior to digestion with endonucleases, 50 µL of 16S rRNA gene PCR product from each isolate was purified using a DNA purification kit (Zymo DNA clean and concentrator kit, Zymo Research Corp., Irvine, USA) following the manufacturer's guidelines. Thereafter 10 µL of product was collected from each reaction and stored in sterile 1.5 mL microfuge tubes at -20°C until use. The final concentration of DNA after the clean up protocol was determined at 280 nm using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, USA).

Endonuclease digests of purified DNA were performed following the manufacturers guidelines by combining 2–4 µL of DNA (final amount 0.5–1 ng DNA per reaction), 2 µL reaction buffer and 1 µL of the respective endonuclease before making up to a final volume of 20 µL using DNAse-free water in sterile 100 µL PCR tubes. The endonucleases were incubated in a Bioer XP Cycler, with HAEIII and HHAI endonucleases at 37°C for 5 minutes; while ALUI was run at 37°C for 15 minutes followed by inactivation by heating at 65°C for 5 minutes.

Fingerprint banding patterns were then visualised using agarose gel electrophoresis and images captured using a Syngene G:Box with Syngene Genesnap software (Version 7.09) and analysed using Syngene GeneTools software (Version 4.01) (Syngene). Dendrograms depicting the relationships between isolates were generated using unweighted pair group method with arithmetic mean (UPGMA) based on band matching, Jaccard similarity coefficient and alignment by retention factor (R_f) values with a tolerance between 2–3%.

2.3.3.2. Phylogenetic analysis of 16S rRNA gene fragments

Sequencing of 16S rRNA gene fragments was carried out by Inqaba Biotech using an ABI 3500XL Genetic Analyzer (Applied Biosystems, California, USA), using both the forward and reverse primers

(Table 2.2). Seven isolates representative of the genetic diversity distinguished through PCR fingerprinting methods were chosen for sequencing along with the reference strains *P. luminescens* DSM 3368 and *P. temperata* DSM 14550. Chromas Lite (Version 2.0.1) was used to edit any sequence ambiguities. Consensus sequences were created using BioEdit (Version 7.1.3.0) (Hall, 1999). Sequences were aligned using MAFFT online (http://mafft.cbrc.jp/alignment/server) and adjusted to equivalent lengths (~1440 bp). A sequence identity matrix of all nine sequences was generated using BioEdit. The 16S rRNA gene sequence fragments were then submitted to GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparison with strains within the database using the MegaBLAST search algorithm with default search parameters.

Consensus sequences were aligned using MAFFT and a phylogenetic tree generated using MEGA5 (Version 5.0.5) (Tamura *et al.*, 2011). Phylogenetic analysis of 16S rRNA gene sequences were performed using the Neighbour-Joining method (Tamura *et al.*, 2004; Saitou and Nei, 1987; Felsenstein, 1985) and reliability of resultant tree topology was determined with boot strap analysis of 1000 replicates. Genetic distances were calculated using the Jukes-Cantor model (Jukes and Cantor, 1969). Reference strain sequences sourced from The Ribosomal Database Project database (http://rdp.cme.msu.edu) were included for comparison purposes.

2.3.4. DNA fingerprinting using RAPD-PCR

Randomly amplified polymorphic DNA PCR (RAPD-PCR) fingerprinting of isolates was assessed using four separate primers OPG 5, OPG 8, OPG 11 and OPG 16 (Table 2.2). In each instance the same PCR reaction conditions were used. Each PCR reaction comprised 0.4 µM primer, (1x) GoTaq Flexi reaction buffer (Promega), 200 µM per dNTP, 0.5 µL of template DNA, 1.25 U/50 µL GoTaq polymerase (Promega) and $MgCl_2$ optimised to 1.5 mM. Each PCR reaction was made up to a final volume of 25 µL with nuclease-free water (Promega). The PCR reactions were run in a Bioer XP Thermo Cycler with the following run parameters: 4 minutes at 94°C; 40 cycles of 94°C for 1 minute, 36°C for 1 minute and 72°C for 30 seconds; followed by 72°C for 5 minutes. PCR products were cooled to 4°C before storage at -20°C until use. The positive control of these protocols was *P. luminescens* isolate p22.

2.3.5. Whole-cell MALDI-TOF-MS analysis of *Photorhabdus* isolates

MALDI-TOF-MS analysis for bacterial identification was performed on a bench-top Bruker Microflex L20 MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an N_2 laser at 337 nm. FlexControl software (Version 2.4) was used for spectrum generation and processing, in conjunction with Biotyper (Version 3.0) software. The calibrant applied was *Escherichia coli* DH5α bacterial test standard (BTS) (Bruker Daltonics).

Cultures were grown on NA at 28°C for 24 h before being processed for MALDI-TOF-MS analysis following an ethanol-formic acid extraction protocol outlined in the Bruker Daltonics user manual (2012). One microlitre of supernatant from extract was spotted onto a stainless steel target plate and allowed to air-dry before overlaying with an equal volume of α-cyanohydroxycinnamic acid matrix (10 mg/mL dissolved in 50% (v/v) acetonitrile and 2.5% (v/v) trifluoroacetic acid). The target plate was then air-dried before analysis. Each sample spot was analysed with 40 laser shots from six different locations in the mass range of 1.9–20.1 kDa.

Identification of the *Photorhabdus* isolates was carried out using Biotyper software in conjunction with Biotyper Reference Library 1.0 (Version 3.1.2) which at the time of analysis (2012) consisted of 3995 entries for different bacterial species. The Biotyper software compared the spectra generated with those in the BDAL library and assigned a hierarchy of 10 possible identities, ranked according to score and confidence values.

Proteomic relatedness between isolates was determined using dendrograms generated from MALDI-TOF-MS data. Mass spectra profiles (MSPs) were generated from the *Photorhabdus* isolates and reference strains according to the recommended protocols in the Bruker Daltonics instruction manual (2012). Each isolate was analysed using 10 spots per isolate, with a single BTS calibrant spot per isolate set. Spectra were generated from 40 laser shots in 6 different positions per spot (240 total). Spectral processing and analysis was carried out with FlexControl (Version 2.4), followed by creation of an MSP using MALDI Biotyper and FlexAnalysis software. Of the 30 replicate spectra generated, a minimum of 20 were selected for the creation of an MSP entry, chosen according to

mass ranges of individual peaks falling within mass range specified in the Bruker Daltonics user manual (2012).

2.3.6. Extraction and analysis of antimicrobial compounds produced by *Photorhabdus* isolates

Analysis of antimicrobial compounds produced by the *Photorhabdus* isolates employed an initial screening step using disc-diffusion bioassays and was followed by evaluations of the effects of culture age on extract bioactivity. Several extraction methods were evaluated in an attempt to recover and characterise bioactive fractions contained in cell-free culture broth and cell pellets. Extracts were analysed using thin-layer chromatography (TLC), ultra-performance liquid chromatography electrospray ionisation time-of-flight mass spectrometry (UPLC-ESI-TOF-MS) and gas chromatography mass spectrometry (GC-MS) to ascertain the active compound(s) responsible for bioactivity. It should be noted that only colonies exhibiting Phase one characteristics were used for subculture purposes.

2.3.6.1. Screening bioassays for detecting antimicrobial activity

Photorhabdus isolates including type strains *P. luminescens* DSM 3368 and *P. temperata* DSM 14550 were assayed for antimicrobial activity using disc-diffusion bioassays. Starter cultures of each isolate were established by picking off single colonies (48 h on NBTA) and inoculating them into 20 mL sterile TSB. Broth cultures were incubated overnight at 28°C in a rotary shaker incubator at 120 rpm. One millilitre from each starter culture was aseptically transferred into 19 mL sterile TSB in 100 mL flat-bottomed conical flasks and incubated further at 28°C and 120 rpm. After 24 h, 48 h and 168 h of incubation respectively, 1.5 mL samples were aseptically removed from each culture and centrifuged at 8000 x *g* for 5 minutes in a benchtop centrifuge. Supernatants were then transferred into sterile 1.5 mL microfuge tubes and stored at -20°C until assayed.

Methanol extraction was performed on cell pellets remaining from centrifuged samples taken after 24 h and 168 h. Cell pellets were washed in 1 mL sterile quarter-strength Ringer's solution and

suspended by vortexing. The cell suspensions were then centrifuged at 8000 x *g* for 5 minutes and the supernatant removed and discarded. The pellets were resuspended in 1 mL methanol by vortexing; followed by centrifugation at 8000 x *g* for 5 minutes, after which the methanol supernatant was removed. These methanol extracts were stored in sterile 1.5 mL microfuge tubes at -20°C until use.

Bioassays were performed using the disc-diffusion bioassay method adapted from Salie *et al*., (1996). Sterile filter discs (9 mm, Macherey-Nagal) were impregnated with either broth supernatant (250 µL) or methanol extract (100 µL). Extracts were applied to the filter discs in 50 µL aliquots and allowed to air-dry between applications in a laminar flow bench before use. A single disc per sample was assayed against each test organism.

The bacterial test organisms used were *Escherichia coli*, *Micrococcus luteus* and *Bacillus subtilis*. For bioassay inoculum production each of these species were cultured overnight in 20 mL NB at 28°C with agitation at 120 rpm, before being diluted with sterile quarter-strength Ringer's solution to achieve a standardised A_{550} ~0.5 (UV Mini-1240, Shimadzu Corp., Kyoto, Japan). Aliquots of adjusted bacterial test organism culture (100 µL) were inoculated onto sterile NA plates using the standard spread plate procedure and spread with an ethanol-flamed glass hockey stick. Inoculated plates were left in a laminar flow cabinet for 30 minutes to allow excess moisture to evaporate. Filter discs (4 discs per plate) impregnated with either *Photorhabdus* broth supernatant or the methanol extracts were then aseptically transferred with flame-sterilised tweezers onto the respective Petri dishes. These were then incubated at 28°C for a period of 1 week with observations and measurements taken at 24 h, 48 h, 72 h, 120 h and 168 h intervals. The diameter of the zone of inhibition around each disc was measured at two locations perpendicular to each other and measurements averaged.

For the fungal bioassays *Botrytis cinerea* and *Rhizoctonia solani* were cultured and maintained on Potato Dextrose Agar (PDA) (Biolab) plates at 28°C for 7 d. Fungal inoculum was applied to sterile PDA plates in 5 mm x 5 mm plugs excised from the growing edge of a fungal culture on PDA (48–72 h old). Filter discs impregnated with culture supernatant or methanol extracts were applied as described previously. These were then incubated at 28°C for a period of 1 week with observations

and measurements taken at 24 h, 48 h, 72 h, 120 h and 168 h intervals. Zone of inhibition measurements were taken from the edge of the filter disc closest to the advancing fungal mycelium.

2.3.6.2. Effects of culture age of *Photorhabdus* Isolate 22 on antibacterial activity

An assessment of the effects of culture age on antibacterial activity was carried out using *Photorhabdus* Isolate 22. Isolate 22 was grown in seven replicates of 20 mL TSB over a period of two weeks at 28°C in the manner described previously for the screening bioassays (Section 2.3.6.1). A single flask was removed and processed at time intervals of 12 h, 24 h, 48 h, 72 h, 96 h, 186 h and 336 h after inoculation. Aliquots of 4.5 mL of culture were aseptically removed and placed in 1.5 mL microfuge tubes and centrifuged at 8000 x *g* for 5 minutes in a benchtop centrifuge. Supernatants were then transferred into sterile 1.5 mL microfuge tubes and stored at -20°C until use. Cell pellets were washed with sterile quarter-strength Ringer's solution and extracted with methanol as described previously (Section 2.3.6.1), with the additional step of a second methanol wash of the cell pellets. Disc-diffusion bioassays were then performed against *M. luteus* and *B. subtilis* as described previously (Section 2.3.6.1), and the bioassays periodically examined over 96 h to assess levels of antimicrobial activity.

2.3.6.3. Antimicrobial compound extraction

For production of putative antimicrobial compounds, single Phase one colonies of *Photorhabdus* Isolates 91, 161 and 173 were inoculated into 20 mL sterile TSB and incubated for 24 h at 28°C with agitation at 120 rpm. These starter cultures were then aseptically inoculated at a 5% (v/v) inoculum concentration (1 mL in 19 mL TSB) into multiple 100 mL flat-bottomed conical flasks of sterile TSB. These flasks were incubated at 28°C and 120 rpm for 48 h. Thereafter cultures of each isolate were combined in 500 mL PTFE centrifuge tubes (Beckman Coultier) and centrifuged at 11305 x *g* (Avanti centrifuge, Beckman Coultier). The supernatant was gently poured off the cell pellet and processed immediately and the cell pellet was discarded.

a) Ethyl acetate extraction

Cell-free culture supernatant (200 mL) was mixed with an equal volume of ethyl acetate before being left to settle in a separating funnel for 15 minutes, allowing the organic layer to separate from the aqueous layer (Eleftherianos *et al*., 2007). Bubbles between the aqueous layer and the organic layer were avoided by gentle mixing and the application of small volumes of ethanol (~1 mL) when required. After decanting the organic fraction, the extraction step was repeated on the aqueous layer with a further 200 mL of ethyl acetate. The two resultant organic extracts were then combined before evaporating at 45°C in a rotary evaporator under partial vacuum. The resultant yellow oily fraction was suspended in 10 mL methanol and transferred to glass polytop vials and allowed to evaporate to dryness in a fume hood and stored at -20°C until use.

Extracted cell-free supernatant and the combined ethyl acetate fractions (evaporated and suspended in methanol) were then assessed for antibacterial activity against *M. luteus* and *B. subtilis*. Aliquots of these extracts, namely: extracted cell-free supernatant at 250 µL per disc; or ethyl acetate fractions evaporated and suspended in methanol at 100 µL per disc, were applied in duplicate, using the disc-diffusion bioassay described previously (Section 2.3.6.1). A separate extraction was performed on 50 mL sterile TSB and used as a control. Additional controls comprised cell-free culture supernatant before ethyl acetate extraction (250 µL per disc).

b) Methanol extraction from lyophilised broth

A 200 mL aliquot of cell-free broth supernatant from each selected *Photorhabdus* sp. (*viz*. Isolates 91, 161 and 173) was frozen at -20°C overnight in sterile 500 mL beakers covered in a layer of aluminium foil. The foil was perforated and the beakers placed in a freeze-dryer until the contents were completely lyophilised. Residue arising from the lyophilised supernatant was resuspended in 15 mL methanol for 15 minutes before being decanted into 1.5 mL microfuge tubes, and centrifuged at 8000 x *g* for 5 minutes in a benchtop centrifuge. The methanol extraction step was then repeated for the remaining residue. Both methanol fractions were combined and stored in a glass polytop vial and the contents completely evaporated in a fume hood, thereafter the residues were stored at -20°C until use. The broth supernatant residue that remained after methanol extraction was

resuspended in 10 mL of sterile distilled water, decanted into 1.5 mL microfuge tubes and centrifuged at 8000 x *g* for 5 minutes. The resulting supernatants were stored in 1.5 mL microfuge tubes at -20°C until use. An extraction of sterile lyophilised TSB (200 mL) was also performed as a control.

Antibacterial activity of each methanol and aqueous extract (100 µL) was assessed using the disc-diffusion assay against *M. luteus* and *B. subtilis* as described previously (Section 2.3.6.1). An un-extracted broth supernatant (250 µL) from each isolate was also included for control purposes.

2.3.7. Analysis of *Photorhabdus* antibiotic compound extracts

2.3.7.1. TLC separation

Extract samples were chosen for TLC analysis based on the presence of antimicrobial activity. Initially, samples derived from methanol-extracted cell pellets (24 h and 168 h TSB) of *Photorhabdus* Isolate 22 (see Section 2.3.6.3) were tested using two mobile phase solvent mixtures 98.5 : 1.5 (v/v) chloroform : methanol and 50 : 50 (v/v) isopropanol : water. (Hu *et al.*, 1997).

Antibiotic extracts derived from Isolates 91, 161 and 173 using three different extraction techniques were also evaluated using TLC. Extraction techniques included ethyl acetate extraction of cell-free broth supernatant (Section 2.3.6.3); methanol extraction of cell-free lyophilised broth supernatant (Section 2.3.6.3); and, methanol extraction of cell pellet material (see Section 2.3.6.3). These samples were separated using a mobile phase solvent mixture of 98.5 : 1.5 (v/v) chloroform : methanol (Hu *et al.*, 1997).

TLC analyses were carried out on oven-dried (70°C, 18 h) TLC Silica Gel 60 F254 aluminium plates (5 cm x 10 cm) (Merck). Samples were spotted (total volume 50 µl) 1 cm from the bottom of the plate, and applied slowly to limit spot size. Impregnated plates were suspended in a TLC glass tank containing the mobile phase solvent mixture (~5 mm depth), and the tank lid sealed in place with petroleum jelly. Tanks were allowed to stand for 30 minutes to saturate the tank atmosphere and

the silica. Thereafter, the TLC plate was lowered into the mobile phase and the tank re-sealed. The plates were left to develop until the solvent front reached ~1 cm from the top of the plate. At this point the plate was removed and the solvent front marked with a pencil. The plates were allowed to air dry and then exposed to UV (~260 nm) light in order to visualise and mark any bands on the plate. Digital images of the plates were captured using a flat bed scanner. Retention factor (R_f) values of the observed bands were calculated using the formula (Kowalska *et al*., 2003):

R_f = Distance of chromatographic band centre from the spotted region (mm) / Distance between the spotted region and the solvent front (mm).

2.3.7.2. Antibacterial bioassays of TLC fractions obtained from ethyl acetate extracts

Fractions from ethyl acetate antibiotic extracts of *Photorhabdus* Isolates 91, 161 and 173 were separated using TLC and screened for antibacterial activity. Two hundred microlitres of extract (Section 2.3.6.3) was applied in a continuous horizontal line 1 cm from the bottom of a Silica Gel 60 F254 aluminium TLC plate (5 cm x 10 cm, oven-dried 70°C, 18 h) (Merck). The TLC plate was placed in a tank containing 98.5 : 1.5 (v/v) chloroform : methanol mobile phase and developed as described previously (Section 2.3.7.1). Bands were visualised with UV light, and R_f values calculated as described previously (Section 2.3.7.1).

Compounds associated with each band resolved were extracted by scrapping the silica from the associated band regions and resuspending in 500 µl of methanol for 30 minutes. These samples were centrifuged 8000 x *g* for 5 minutes in a benchtop centrifuge, and the supernatants transferred to new microfuge tubes. The supernatant fractions were then assessed for antibacterial activity in disc-diffusion bioassays against *M. luteus* and *B. subtilis* as described in Section 2.3.6.1. One hundred microlitre aliquots were applied onto sterile filter discs (9 mm, Macherey-Nagal), with each band sample performed as a single replicate. Bioassays were incubated at 28°C and observations recorded over a 7 d period.

2.3.7.3. Microplate dilution assay of ethyl acetate extracts

Antibacterial activity present in the ethyl acetate extracts of broth supernatant from Isolates 91, 161 and 173 (Section 2.3.6.3) were assessed using a microplate dilution assay adapted from Langfield *et al.* (2004). The extracts were diluted serially from 10% (v/v) to 0.002% (v/v) in TSB containing cultures of either *M. luteus* or *B. subtilis*. Using 96-well microtitre plates, wells were loaded with 50 µL aliquots of bacterial culture at A_{550} ~0.1. In Row 2 40 µL of broth culture was loaded and thereafter a 10 µL aliquot of each respective ethyl acetate extract suspended in methanol was added, to give a final concentration of 10% (v/v) antibiotic extract. Fifty microlitres was removed from each of these wells and added to Row 3, thereby generating a 5% (v/v) extract concentration. Further serial dilutions were performed in this manner up to extract concentration of 0.002% (v/v). Mixing of each dilution was achieved by repeated up-and-down pipetting. Control rows were also included. These comprised of a pure methanol control to evaluate the effects of the methanol solvent; a sterility control in Row 12 comprised of sterile TSB (50 µL); and an antibiotic-free control in Row 1 comprised of culture without antibiotic extract added. The microplates were incubated for 24 h at 28°C, thereafter 50 µl of a 0.01% (w/v) TTC solution was added to each well, with subsequent 18 h incubation at 28°C. Bacterial metabolism was indicated by the presence of a pink to red colouration in the well, and the absence of activity was denoted by colourless wells.

2.3.7.4. Fractionation of Isolate 91 aqueous extract exhibiting antimicrobial activity

The aqueous extract of Isolate 91 derived from lyophilised broth (post methanol extraction) (Section 2.3.6.3) was subjected to further purification steps using C18 column fractionation. A Sep-Pak Plus C18 syringe cartridge (Waters Corporation, Milford, Massachusetts, USA) was conditioned by passing 5 mL of pure methanol through it, followed by 5 mL of sterile distilled water. A 1 mL aliquot of aqueous extracted broth was loaded slowly into the column and the eluent collected. Thereafter 1 mL of water was passed through the column and the sample collected. This was followed by methanol-water mixtures (1 mL) commencing at 20% (v/v) methanol : water and increasing in 20% increments until 100% methanol was used to elute the column. Each fraction (~1 mL) was assayed for antibacterial activity using the disc-diffusion bioassay as described previously (Section 2.3.6.1). A 100 µL sample volume was applied per filter disc, and the bioassays performed in duplicate and observed after 48 h and 168 h of incubation.

2.3.7.5. Partial purification of methanol-extracted lyophilised broths

Crude methanol extracts of lyophilised broth for Isolates 91, 161 and 173 were processed with a Sep-Pak Plus C18 syringe cartridge (Waters Corporation) to remove non-active fractions. Methanol extract residues were resuspended in 5 mL methanol before processing. A Sep-Pak Plus C18 syringe cartridge was conditioned as described previously (Section 2.3.7.4). Thereafter, 0.5 mL of methanol extract supernatant mixed with 0.5 mL sterile distilled water was loaded slowly into the column and the eluent collected. One millilitre of water was then passed through the column and the sample collected, this was repeated with 1 mL of 60% (v/v) methanol : water, followed by 1 mL of pure methanol. The column was then reconditioned by washing with 5 mL methanol followed by 5 mL water and the process repeated with the remainder of the extract. Sep-Pak columns were replaced after 5 fractionation runs and when extracts from different isolates were processed. Each sample was tested for activity in a disc-diffusion procedure as described previously (Section 2.3.6.1). Purified extract obtained from the 100% methanol elution step was pooled in a polytop vial and evaporated to dryness in a fume hood before storage at -20°C.

2.3.7.6. Mass spectroscopy analysis of extracts

Mass spectroscopy analysis using UPLC-ESI-TOF-MS and GC-MS was carried out on C18-purified methanol extracts derived from lyophilised broth and ethyl acetate samples obtained from Isolates 91, 161 and 173 (Section 2.3.6.3).

a) UPLC-ESI-TOF-MS analysis of extracts

UPLC-ESI-TOF-MS analysis of extracts was carried out using a Waters Aquity UPLC linked to a Micromass LCT Premier mass spectrometer (Waters Corporation) run in electrospray positive/negative modes. Evaporated samples were dissolved in 0.5 mL HPLC-grade methanol.

Samples (2 µL) were separated using a BEH C18 column (2.1 mm x 100 mm, 1.7 µm) maintained at 35°C using Solvent A (0.1% acetic acid in water) and Solvent B (methanol) at 0.3 mL/minute flow rate with the following run parameters: Analysis was initiated with 90% Solvent A for 0.1 minutes, followed by a linear increase of Solvent B to 100% over a 50-minute period. The elution conditions were held at 100% Solvent B for 8 minutes before returning to 90% Solvent A for the remainder of the 60-minute run. A Photo diode array was used to detect compounds within a 220–400 nm range. Anthraquinone (Sigma Aldrich) and trans-stilbene (Sigma Aldrich) were included as reference standards.

b) GC-MS analysis of extracts

Evaporated samples (7 uL) were dissolved in 0.5 mL HPLC-grade methanol prior to analysis on a ThermoFinnigan Trace GC (Thermo Fisher Scientific, Waltham, USA) fitted with a BPX5 capillary column (30 m x 0.32 mmID) (SGE Analytical Science, Melbourne, Australia) and coupled to a Thermo PolarisQ mass spectrometer (Thermo Fisher Scientific) operating in electron impact positive mode (EI+). The run parameters were a 40°C start for 1 minute followed by a 12°C/minute ramp to 200°C with a final hold for 10 minutes. The carrier gas was helium with a constant flow rate of 1.0 mL/minute. Anthraquinone (Sigma Aldrich) and trans-stilbene (Sigma Aldrich) were included as reference standards.

CHAPTER THREE

Genetic, phenotypic and proteomic diversity amongst South African *Photorhabdus* spp. isolates

3.1. Introduction

Twenty presumptive *Photorhabdus* spp. isolates from the Freestate and Western Cape provinces of South Africa were analysed by a number of methods to confirm their identity and determine their diversity and relationships to previously characterised strains and to each other. Phenotypic traits were evaluated and compared using API 20E (bioMérieux) test strips in conjunction with a selection of supplementary tests. Genetic diversity was assessed by 16S rRNA gene sequence analysis as well as DNA fingerprinting using 16S rRNA gene PCR-RFLP analysis with the restriction endonucleases HAEIII, HHAI and ALUI. Whole genome fingerprinting analysis was performed using RAPD-PCR with the OPG 5, OPG 8, OPG 11 and OPG 16 primers. Lastly, proteomic diversity was evaluated using the Bruker Daltonics Biotyper system and cluster analysis of isolate mass spectra profiles.

3.2. Results

3.2.1. Phenotypic characteristics of *Photorhabdus* isolates

API 20E identification strips were employed to assess metabolic diversity amongst *Photorhabdus* isolates (Table 3.1). Isolates 165 and 178 were excluded on the basis of the 16S rRNA gene sequence findings excluding them as *Photorhabdus* sp. (Section 3.2.2.3). All isolates examined were positive for gelatinase, tryptophan deaminase activity and glucose fermentation; with Isolate 91 and *P. luminescens* DSM 3368 showing weak positive results for glucose fermentation. Varied results were obtained for the test of indole production where Isolates 22, 66, 96, 161, 172, 173, 175 and 180 exhibited a positive result; Isolates 32, 40, 50 and 151 showed weak positive results and reference strains *P. luminescens* DSM 3368 and *P. temperata* DSM 14550 together with Isolates 36, 91, 164, 170, 174 and 179 returned a negative result. The remaining biochemical tests were negative in all

cases, namely: mannitol, inositol, D-sorbitol, L-rhamnose, D-saccharose, D-melibiose, D-sucrose, amygdalin and L-arabinose fermentation/oxidation, citrate and urea utilisation, arginine dihydrolase, lysine and ornithine decarboxylase, production of H_2S, acetoin production (Voges Proskauer), production of NO_2 and reduction to N_2 gas.

Table 3.1. Phenotypic differentiation of *Photorhabdus* isolates based on API 20E biochemical tests.

Isolate	Gelatinase	Indole production	Tryptophan deaminase	Glucose fermentation
P. luminescens DSM 3368	+	-	+	+ (w)
P. temperata DSM 14550	+	-	+	+
22	+	+	+	+
32	+	+ (w)	+	+
36	+	-	+	+
40	+	+ (w)	+	+
50	+	+ (w)	+	+
66	+	+	+	+
91	+	-	+	+ (w)
96	+	+	+	+
151	+	+ (w)	+	+
161	+	+	+	+
164	+	-	+	+
170	+	-	+	+
172	+	+	+	+
173	+	+	+	+
174	+	-	+	+
175	+	+	+	+
179	+	-	+	+
180	+	+	+	+

+ = Positive reaction; + (w) = Weak positive reaction; - = Negative reaction

In addition to the API 20E test strips several supplementary biochemical tests were also included (Table 3.2). Results for starch degradation were negative for all isolates. Results obtained for mannitol fermentation, citrate utilisation and urea utilisation did not always match the results obtained from the corresponding test in the API strip. For example *Photorhabdus luminescens* DSM 3368, showed a positive result for mannitol fermentation, which was contrary to the API test result (Table 3.1). In the case of citrate utilisation, *P. luminescens* DSM 3368 and Isolate 91 showed a positive reaction, which contrasted with the negative API test result obtained (Table 3.2). The majority of isolates produced a positive result for the urea utilisation test with only Isolates 91, 165

and 178 showing a negative reaction (Plate 3.2). Findings for urea utilisation contrasted with the API data where negative results were recorded for all isolates.

From the trehalose fermentation assay it was noted that the majority of isolates screened produced a pinkish-red pigmentation in the inoculated agar medium, and examples of the various reactions observed assay are shown in Plate 3.1. The pigment was not detected in any of the trehalose-negative controls and was associated with a positive test for trehalose fermentation (Table 3.2). However Isolate 91 returned a negative result for trehalose fermentation yet still produced the red pigment.

Table 3.2. Supplementary phenotypic differentiation of metabolic characteristics of *Photorhabdus* isolates using biochemical tests.

Isolate	Catalase	Casein	Esculin	Trehalose	Mannitol	Citrate	Urea
P. luminescens (DSM 3368)	+	+	+	+	+	+	+
P. temperata (DSM 14550)	+	+	+	-	-	-	+
22	+	+	+	+	-	-	+
32	+	+	+	+p	-	-	+
36	+	+	+	+p	-	-	+
40	+	+	-	+p	-	-	+
50	+	+	+	+	-	-	+
66	+	+	-	+r	-	-	+
91	+	+	-	-r	-	+ (w)	-
96	+	+	+	+p	-	-	+
151	+	+	-	+p	-	-	+
161	+	+	+	+p	-	-	+
164	+	+	+	+	-	-	+
165	+	+	-	+	-	+	-
170	+	+	+	+r	-	-	+
172	+	+	+	-	-	-	+
173	+	+	+	+r	-	-	+
174	+	+	+	+p	-	-	+
175	+	+	-	+	-	-	+
178	-	+ (w)	-	+	-	-	-
179	+	+	+	-	-	-	+
180	+	+	+	+r	-	-	+

+ = Positive reaction; + (w) = Weak positive reaction; - = Negative reaction; r = Red pigment production; p = Pink pigment production

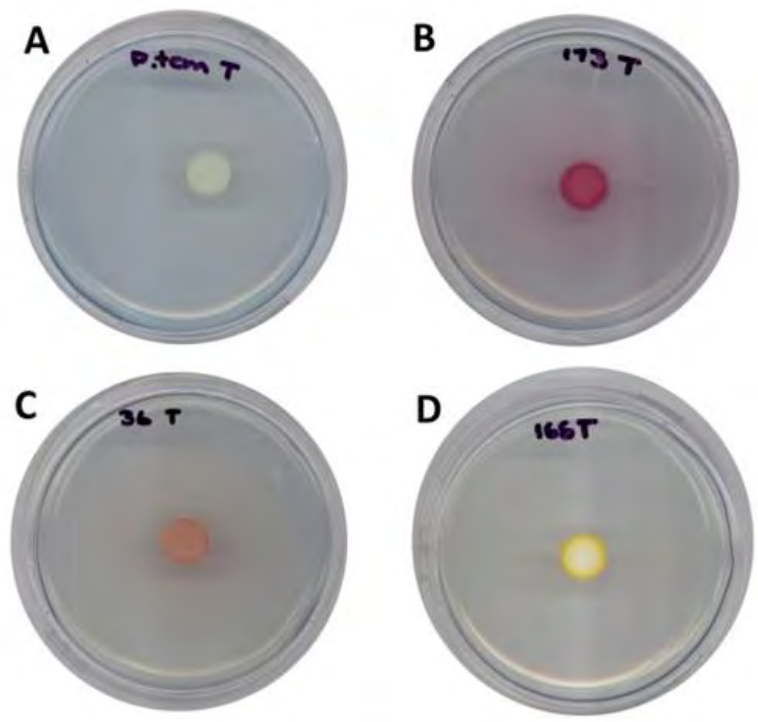


Plate 3.1. Visualisation of selected results of trehalose fermentation where: (A) trehalose negative result, (B) trehalose positive with red colouration, (C) trehalose positive with pink colouration and (D) trehalose positive with no red pigmentation.

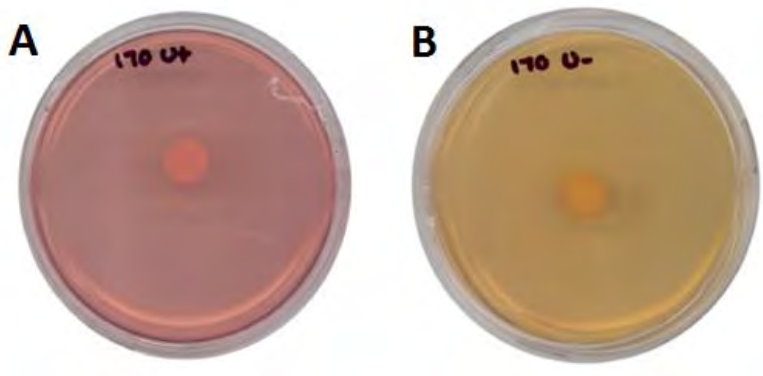


Plate 3.2. Visualisation of selected results of urea utilisation where: (A) denotes a urease positive and (B) urea negative control.

3.2.2. Genetic diversity amongst *Photorhabdus* isolates assessed using DNA fingerprinting and 16S rRNA partial gene sequence analysis

After PCR to amplify the 16S rRNA gene fragments, the concentration and purity of the 16S rRNA amplicons were determined (Table 3.3). Concentrations ranged from 244.83–458.2 ng/µL, with $A_{260:280}$ ratios greater than 1.8 indicating that the DNA was of high purity.

Table 3.3. DNA concentration and purity of amplified 16S rRNA gene fragment after purification.

Isolate	DNA Concentration (ng/µL)	Absorbance Ratio (260:280)
P. luminescens	411.82	1.88
P. temperata	417.13	1.89
22	317.12	1.86
32	425.69	1.89
36	358.64	1.89
40	221.24	1.88
50	414.79	1.88
66	371.01	1.87
91	364.87	1.9
96	385.37	1.91
151	458.2	1.86
161	363.05	1.88
164	315.27	1.88
165	341.83	1.88
170	351.89	1.88
172	428.71	1.87
173	230.77	1.89
174	315.67	1.89
175	244.83	1.82
178	334.66	1.88
179	363.83	1.88
180	350.98	1.89

3.2.2.1. 16S rRNA gene PCR-RFLP fingerprinting of *Photorhabdus* isolates using the restriction endonucleases ALUI, HAEIII and HHAI

Three restriction endonucleases (*viz*. ALUI, HHAI and HAEIII) were applied in this 16S PCR restriction fragment length polymorphism (PCR-RFLP) protocol to determine the degree of 16S rRNA gene fragment variability between the isolates. The 16S rRNA gene PCR-RFLP profiles for each of the endonucleases are shown in Figures 3.1, 3.3 and 3.5 respectively. Variability between the fingerprint profiles obtained from each respective endonuclease were determined by unweighted pair group method with arithmetic mean (UPGMA) analysis. Dendrograms showing relationships between isolates for each of the three endonucleases are shown in Figures 3.2, 3.4 and 3.6 respectively.

The profiles obtained from ALUI (Figure 3.1) showed that the majority of isolates displayed very similar profiles, but distinguished Isolate 165 by its distinct profile. *Photorhabdus luminescens* DSM 3368 also shows a unique fingerprint, while Isolates 91 and 178 form similar profiles. These observations were confirmed in the dendrogram generated from these profiles (Figure 3.2).

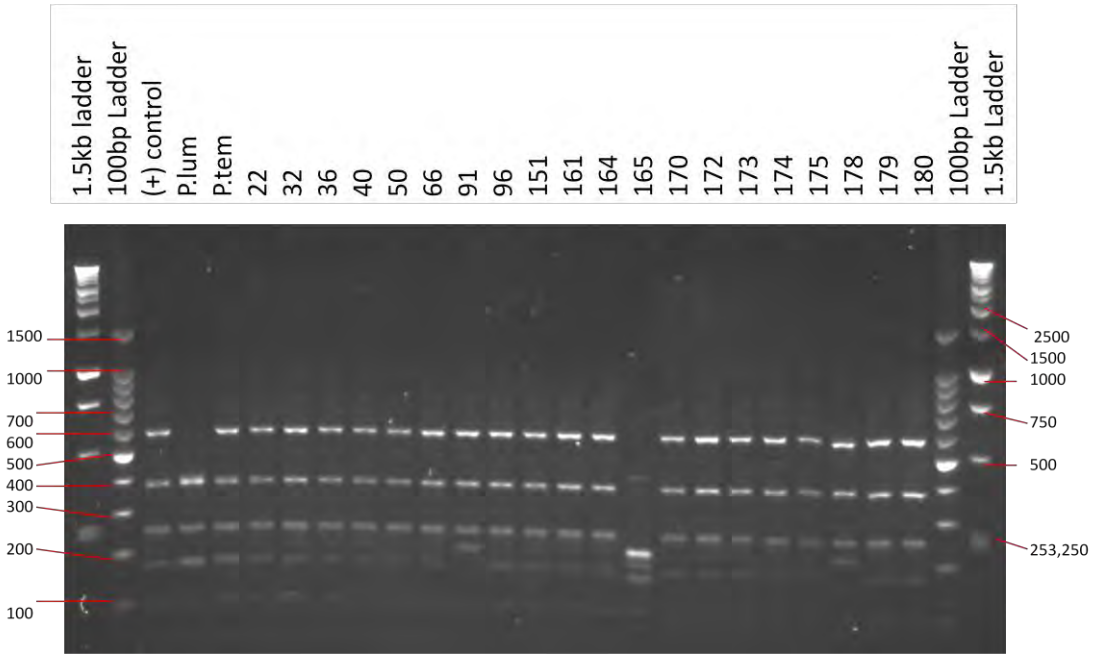


Figure 3.1. ALUI digest of 16S rRNA gene fragment amplified from *Photorhabdus* isolates.

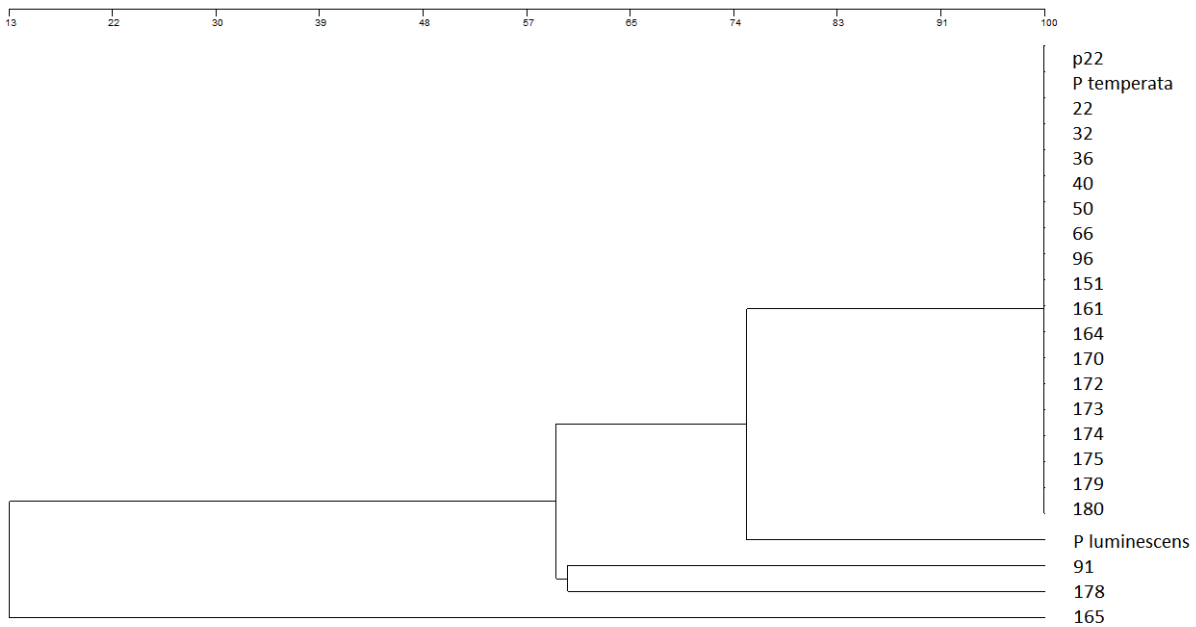


Figure 3.2. UPGMA dendrogram depicting relationships of *Photorhabdus* isolates based on the PCR-RFLP fingerprint profiles from the ALUI restriction endonuclease of the 16S rRNA gene fragment generated by Syngene GeneTools software (Version 4.01).

Restriction digests with the HHAI endonuclease yielded the most diverse banding patterns with six distinct profiles being differentiated (Figure 3.3). *Photorhabdus luminescens* DSM 3368, *P. temperata* DSM 14550 and Isolates 178 and 91 all gave rise to distinct patterns, although some band similarity to the other isolates were evident. Isolate 165 again displayed a unique profile with no matching bands to any of the other isolates. These observations were confirmed by the dendrogram generated from these profiles (Figure 3.4) which highlights the similarities of the two *Photorhabdus* reference strains, and the distinct profiles of Isolates 91, 165 and 178.

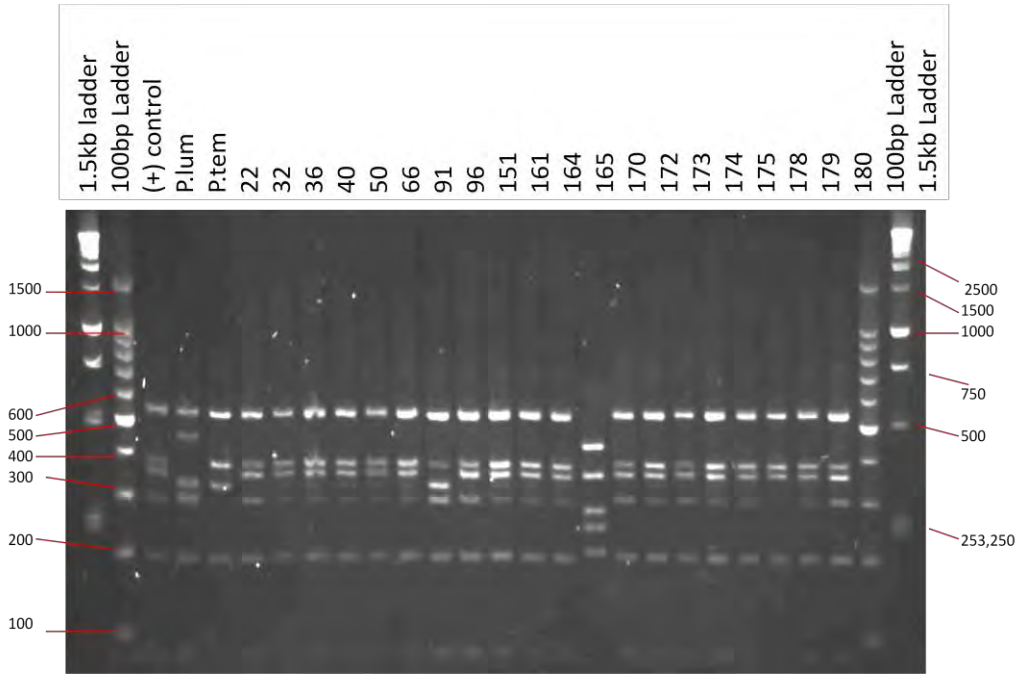


Figure 3.3. HHAI digest of 16S rRNA gene fragment amplified from *Photorhabdus* isolates.

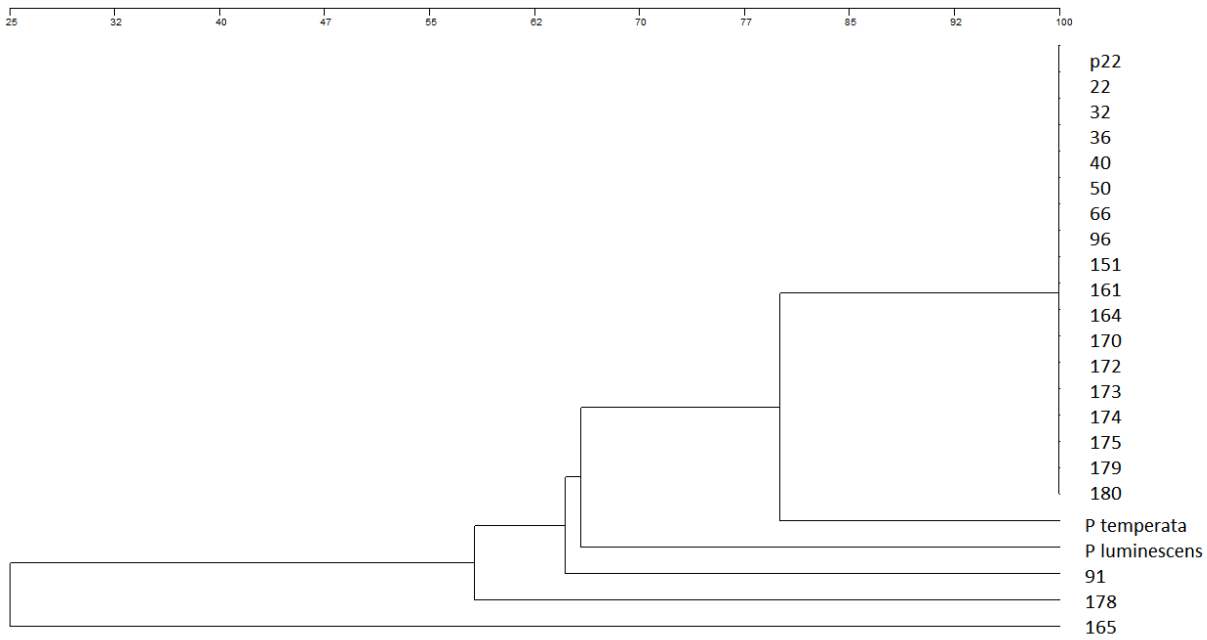


Figure 3.4. UPGMA Dendrogram depicting relationships of *Photorhabdus* isolates based on the PCR-RFLP fingerprint from the HHAI restriction endonuclease of the 16S rRNA gene fragment generated by Syngene GeneTools software (Version 4.01).

Restriction digestion with HAEIII endonuclease resulted in profiles which demonstrated the lowest degree of variance between isolates (Figure 3.5), with all isolates with the exception of Isolate 165 having highly similar 16S rRNA gene PCR-RFLP profiles. Isolate 178 showed additional small-sized faint bands appearing in an otherwise identical profile to the majority of isolates. Isolates 178 and 165 were clustered separately to the other isolates and reference strains in the band profile dendrogram generated (Figure 3.6).

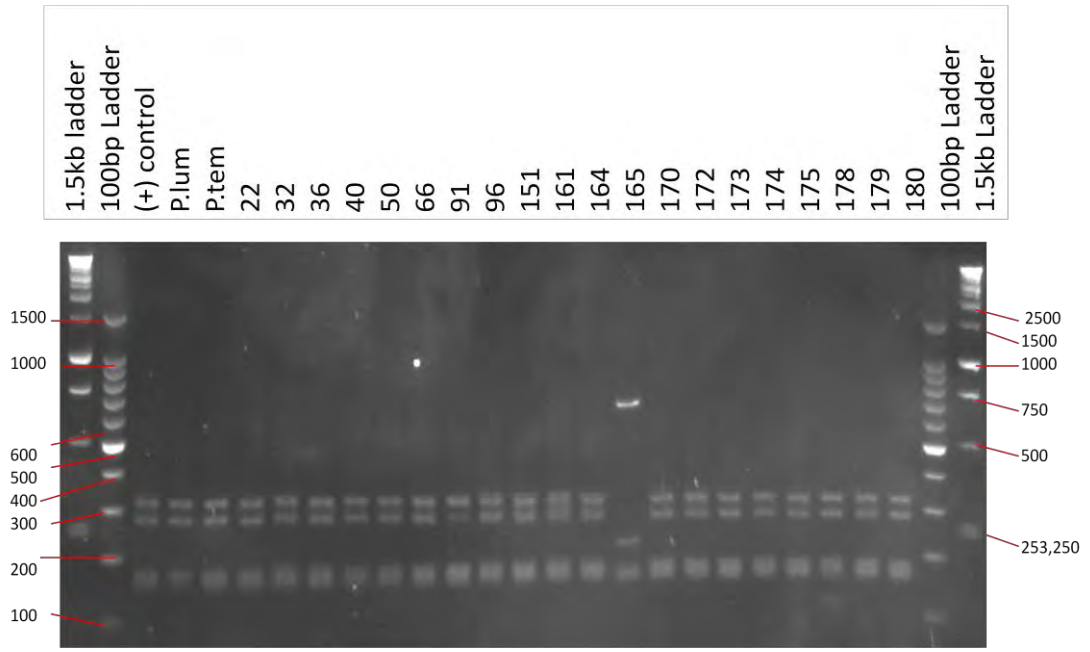


Figure 3.5. HAEIII digest of 16S rRNA gene fragment amplified from *Photorhabdus* isolates.

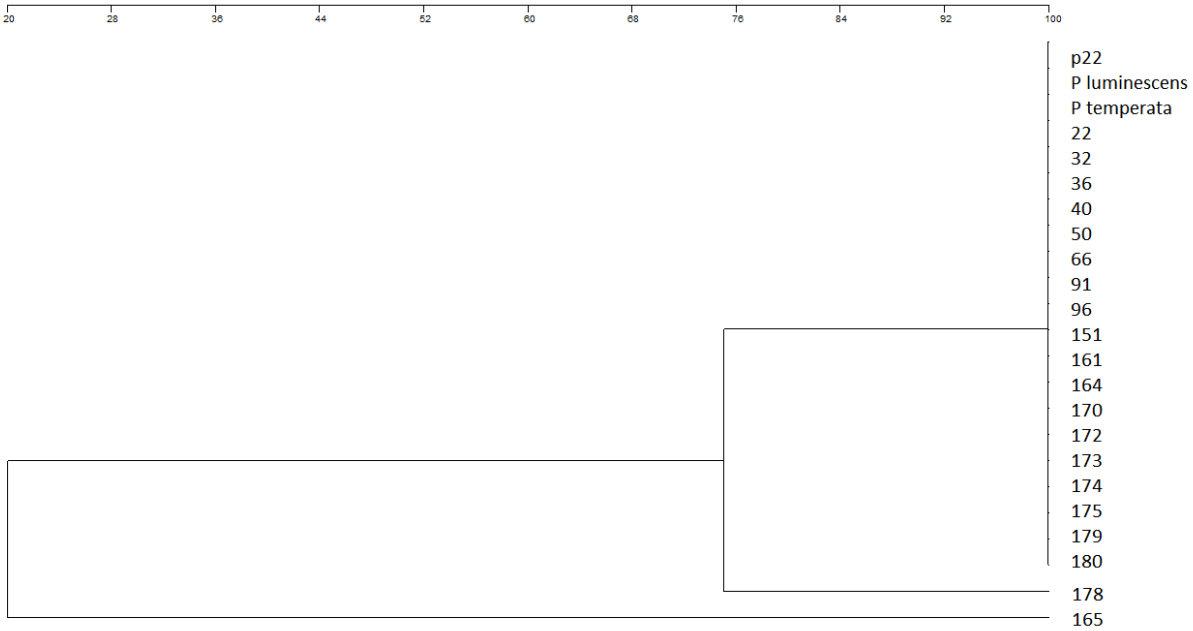


Figure 3.6. UPGMA dendrogram depicting relationships of *Photorhabdus* isolates based on the PCR-RFLP fingerprint from the HAEIII restriction endonuclease of the 16S rRNA gene fragment generated by Syngene GeneTools software (Version 4.01).

A summary of the representative banding patterns obtained for each of the three restriction endonucleases evaluated is presented in Figure 3.7. Most of the isolates formed 16S rRNA gene PCR-RFLP banding profiles that were consistent with a single group represented here by Isolate 22. Isolate 165 was consistently differentiated from the other strains with all of the endonucleases used. *Photorhabdus luminescens* DSM 3368 and Isolate 91 were differentiated with HHAI and ALUI but not with HAEIII. *Photorhabdus temperata* DSM 14550 and Isolate 178 were only differentiated by HHAI. The HHAI digest shows the highest level of diversity with six profiles being distinguished in total.

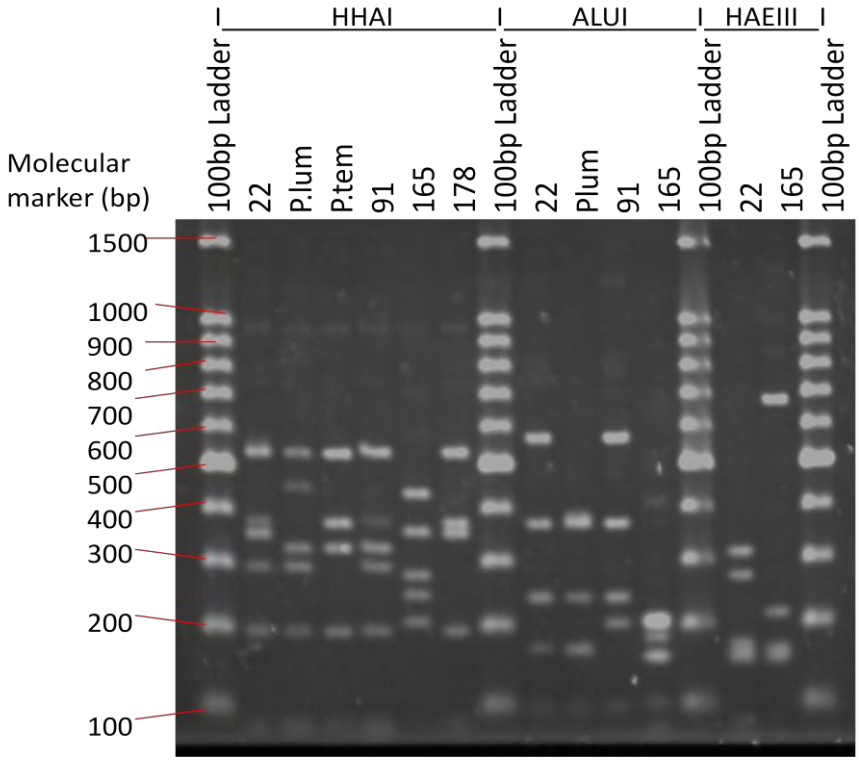


Figure 3.7. Comparison of all 16S rRNA gene PCR-RFLP group patterns of HHAI, ALUI and HAEIII restriction endonuclease digests of selected *Photorhabdus* isolates.

3.2.2.2. DNA fingerprinting of *Photorhabdus* isolates using RAPD-PCR

RAPD-PCR was evaluated using four primers (OPG 5, 8, 11 and 16). DNA fingerprint profiles between *Photorhabdus* isolates are shown in Figure 3.8.

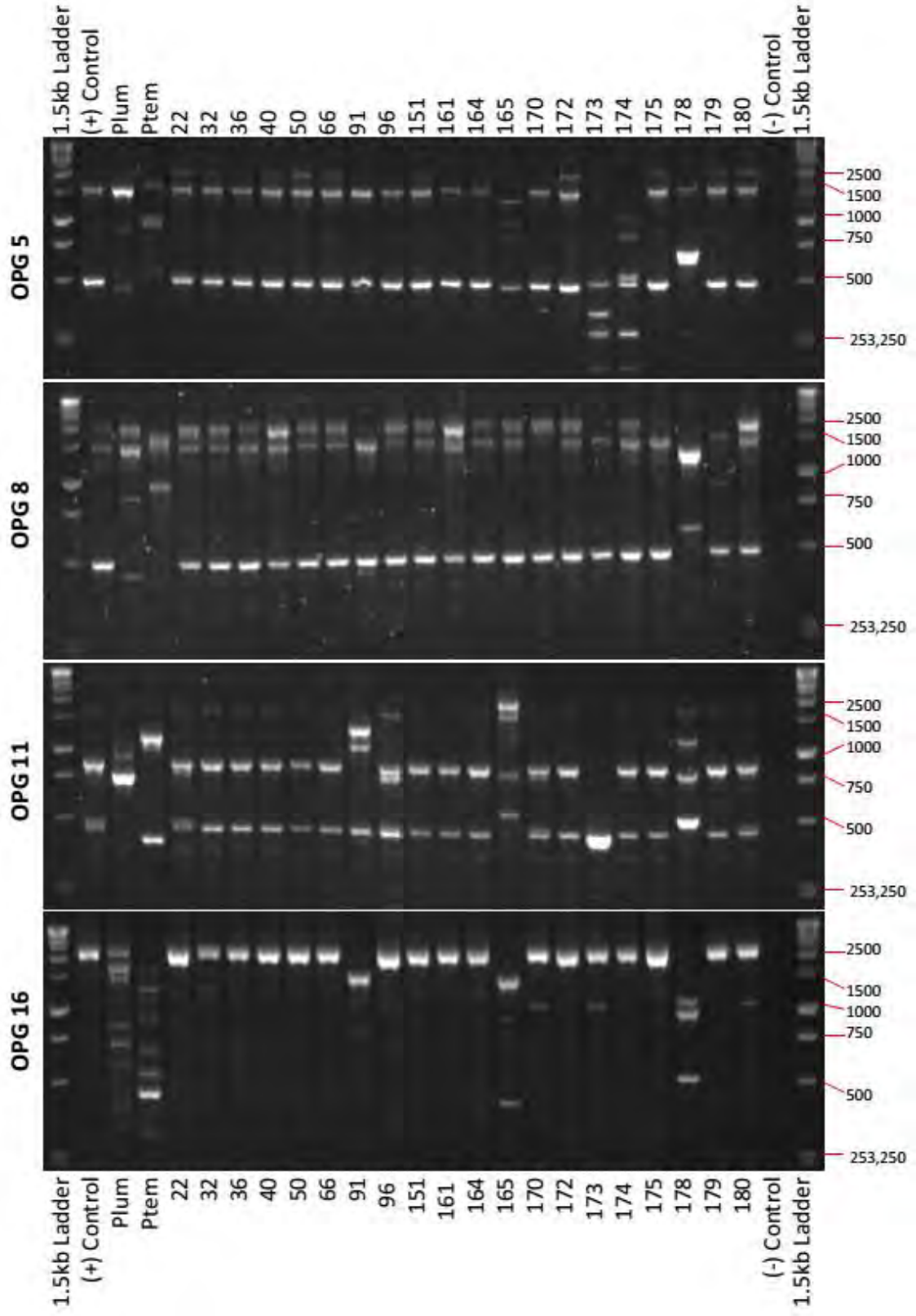


Figure 3.8. DNA fingerprint banding patterns for *Photorhabdus* isolates after RAPD-PCR using primers OPG 5, 8, 11, and 16.

The majority of the fingerprint profiles obtained with the OPG 5 primer show a similar banding pattern for Isolates 22, 32, 40, 50, 66, 151, 170, 172, 175, 179 and 180. Isolates 36, 96, 161 and 164 have a pattern comparable to the first grouping, differing only with the presence of an additional faint band ~2500 bp in size. *Photorhabdus luminescens* DSM 3368, *P. temperata* DSM 14550 and Isolates 165, 173, 174 and 178 have unique fingerprint profiles within the OPG 5 primer. Isolate 91 shows a similar profile to isolate 96 with the exception of an additional faint band ~500 bp in size.

For RAPD primer OPG 8 the majority of isolates form a similar banding pattern; namely Isolates 22, 32, 36, 50, 66, 96, 151, 164, 165, 172 and 174. A second grouping could also be distinguished which comprised Isolates 40, 161 and 180. These possess a similar pattern, but with some band size and intensity variations being evident when compared to the first grouping. *Photorhabdus luminescens* DSM 3368, *P. temperata* DSM 14550, and Isolates 91, 170, 173, 175, 178 and 179 each returned unique fingerprints for the OPG 8 primer.

OPG 11 revealed Isolates 32, 36, 40, 66, 151, 161, 164, 172, 174, 175, and 179 to have identical banding patterns. Isolates 22, 170 and 180 exhibited similar banding patterns, varied from the first grouping with extra bands at ~750 bp and ~400 bp. The profile for Isolate 50 is also similar to the majority but lacks a band at ~2000 bp. *Photorhabdus luminescens* DSM 3368, *P. temperata* DSM 14550, and Isolates 91, 96, 165, 173, and 178 each have unique fingerprints for this primer.

Primer OPG 16 detected great variation in band intensity between isolates. Based solely on the prominent banding, two groups are distinguishable. One grouping consists of Isolates 22, 96, 172 and 175; while the other group contains Isolates 32, 36, 40, 50, 66, 151, 161, 164, 170, 173, 174, 179 and 180. Isolates 170, 173 and 180 appear to have a similar faint band pattern. Isolates 91, 165, 178, and reference strains *P. luminescens* DSM 3368 and *P. temperata* DSM 14550, each have unique banding profiles.

Comparison between OPG primers revealed that the majority of isolates produced similar banding patterns. Isolates 91, 165, 173, 178, *P. luminescens* DSM 3368 and *P. temperata* DSM 14550 consistently demonstrated different banding patterns to the rest of the isolates for each of the four

primers evaluated. Isolates 96 (OPG 11), 179 (OPG 8) and 174 (OPG 5), also show unique profiles in one primer set while conforming to the other *Photorhabdus* isolates for the remaining primers.

3.2.2.3. Sequence analysis and phylogenetic relationships of the 16S rRNA gene fragment of selected *Photorhabdus* isolates

Based on the findings of 16S rRNA gene PCR-RFLP and RAPD-PCR fingerprinting, seven isolates representative of the groups distinguished were selected for 16S rRNA gene sequence analysis in addition to the two reference strains (*viz*. Isolates 91, 151, 161, 165, 173, 178 and 180). A sequence identity matrix of these sequences is shown in Table 3.4. A high degree of similarity between sequences of Isolates 151, 161, 173 and 180 (0.993–0.998 similarity) was observed. Isolate 165 was clearly distinguished from all the other isolates (0.821–0.831 similarity). Isolate 178 shows low similarity to the remaining isolates (0.941–0.946 similarity). Isolate 91 and the two reference strains exhibited lower similarity to each other and to Isolates 151, 161, 173 and 180 with approximately 0.96–0.98 similarity.

Table 3.4. Sequence identity matrix of 16S rRNA gene sequence fragments generated for *Photorhabdus* isolates and two reference strains.

	Isolate 91	***P. luminescens* DSM 3368**	**Isolate 151**	**Isolate 161**	**Isolate 173**	**Isolate 180**	***P. temperata* DSM 14550**	**Isolate 178**	**Isolate 165**
Isolate 91	-	0.971	0.975	0.976	0.977	0.977	0.961	0.941	0.821
***P. luminescens* DSM 3368**	0.971	-	0.979	0.981	0.982	0.981	0.976	0.941	0.827
Isolate 151	0.975	0.979	-	0.998	0.996	0.993	0.971	0.946	0.825
Isolate 161	0.976	0.981	0.998	-	0.997	0.995	0.971	0.946	0.826
Isolate 173	0.977	0.982	0.996	0.997	-	0.996	0.972	0.946	0.827
Isolate 180	0.977	0.981	0.993	0.995	0.996	-	0.973	0.944	0.825
***P. temperata* DSM 14550**	0.961	0.976	0.971	0.971	0.972	0.973	-	0.941	0.83
Isolate 178	0.941	0.941	0.946	0.946	0.946	0.944	0.941	-	0.831
Isolate 165	0.821	0.827	0.825	0.826	0.827	0.825	0.83	0.831	-

The sequence identity matches for the selected isolates are presented in Table 3.5. Sequences were aligned using MAFFT and shortened to equivalent lengths (~1440 bp) prior to submission to NCBI BLAST-N database. Isolates 91, 151, 161, 173 and 180 were all matched most closely to *Photorhabdus luminescens* subsp. *laumondii* strains. The reference strains *P. luminescens* DSM 3368 and *P. temperata* DSM 14550 correlate to their expected species designation with high similarity scores. Isolates 165 and 178 were not matched to strains of *Photorhabdus*, but were found to be highly similar to strains of *Pseudomonas* spp. and *Xenorhabdus* spp. respectively.

Despite Isolates 151, 161, 173 and 180 showing highest similarity (~98%) to *P. luminescens* subsp. *laumondii* TT01, they did not group closely with this reference strain when phylogenetic relationships were inferred from this sequence data (Figure 3.9). Isolates 151, 161, 173 and 180 clustered closely together in a distinct sub-cluster within the *P. luminescens* species clade. Isolate 91, exhibited a higher degree of sequence relatedness to *P. luminescens* subsp. *laumondii* TT01, and clustered nearer to this particular sequence. The reference strains both clustered with their corresponding database sequences in the phylogenetic tree. Isolates 165 and 178 grouped with representatives of their respective genera, as expected from 16S rRNA gene sequence matches (Table 3.5).

Table 3.5. 16S rRNA gene fragments sequences after comparison to NCBI GenBank BLAST Nucleotide database of sequenced isolates (Accessed 14 October 2014).

Isolate	Top three NCBI BLAST matches	Query cover (%)	Similarity (%)	Max Score	Total Score	Accession No.
91	*Photorhabdus luminescens* subsp. *laumondii* TT01	100	98.90	2586	2586	BX571859.1
	Photorhabdus luminescens subsp. *laumondii* TT01	100	98.76	2575	2575	BX571863.1
	Photorhabdus luminescens subsp. *laumondii* DSM 15195	100	98.76	2575	2575	AJ560633.1
151	*Photorhabdus luminescens* subsp. *laumondii* ARG	100	98.41	2545	2545	AY278650.1
	Photorhabdus luminescens subsp. *laumondii* TT01	100	98.34	2540	2540	NR 112706.1
	Photorhabdus luminescens subsp. *laumondii* FR42	100	98.34	2540	2540	EU190980.1
161	*Photorhabdus luminescens* subsp. *laumondii* ARG	100	98.41	2545	2545	AY278650.1
	Photorhabdus luminescens subsp. *laumondii* TT01	100	98.41	2545	2545	BX571863.1
	Photorhabdus luminescens subsp. *laumondii* TT01	100	98.34	2540	2540	NR 112706.1
165	*Pseudomonas* sp. HMPB1	100	99.86	2647	2647	AM745260.1
	Pseudomonas sp. CMR5c	99	99.93	2643	2643	FJ652623.1
	Pseudomonas sp. CMR12a	99	99.58	2621	2621	FJ652622.1
173	*Photorhabdus luminescens* subsp. *laumondii* Brecon	100	98.34	2540	2540	AY278647.1
	Photorhabdus luminescens subsp. *laumondii* TT01	100	98.34	2540	2540	BX571863.1
	Photorhabdus luminescens subsp. *laumondii* TT01	100	98.34	2540	2540	BX571859.1
178	*Xenorhabdus* sp. MY8 KsSu155	100	98.13	2519	2519	AB5057812.1
	Xenorhabdus sp. MY8 NJ	100	98.13	2519	2519	AB507811.1
	Xenorhabdus ishibashi	100	98.13	2519	2519	AB243427.1
180	*Photorhabdus luminescens* subsp. *laumondii* Brecon	100	98.21	2529	2529	AY278647.1
	Photorhabdus luminescens subsp. *laumondii* TT01	100	98.21	2529	2529	BX571863.1
	Photorhabdus luminescens subsp. *laumondii* TT01	100	98.21	2529	2529	BX571859.1
P. luminescens DSM 3368	*Photorhabdus luminescens* Hb	100	99.93	2669	2669	NR 0307074.1
	Photorhabdus luminescens subsp. *luminescens* Hm	100	99.86	2663	2663	AY278641.1
	Photorhabdus luminescens Hb	100	99.79	2663	2663	NR 115190.1
P. temperata DSM 14550	*Photorhabdus temperata* UK211	100	100.00	2675	2675	AB355871.1
	Photorhabdus temperata NLH-A97	100	100.00	2675	2675	AB355870.1
	Photorhabdus temperata XINach	100	100.00	2675	2675	NR 028871.1

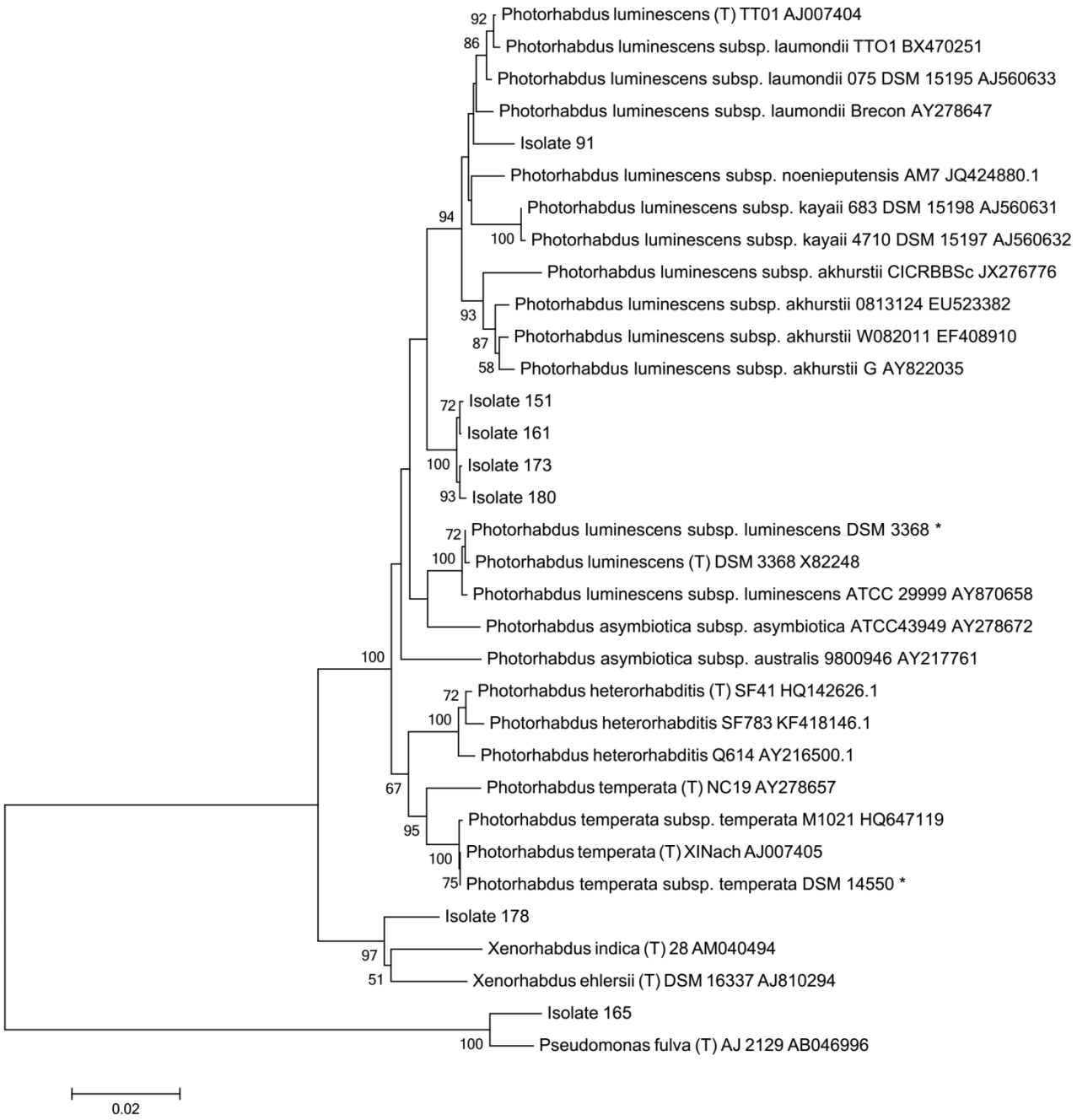


Figure 3.9. Neighbour-Joining phylogenetic tree inferring the evolutionary relationship between *Photorhabdus* isolates based on partial 16S rRNA gene sequence analysis. Only bootstrap values >50% are included. Bacteria names with (T) denote type strain sequences while * represents type strains sequenced in this study.

3.2.3. MALDI-TOF-MS profiling of *Photorhabdus* isolates

Diversity amongst the *Photorhabdus* isolates was also assessed on a proteomic basis using whole-cell matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). The spectra generated for each *Photorhabdus* isolate after MALDI-TOF-MS was compared to the Bruker Daltonics database of reference strains for identification purposes. Dendrograms were subsequently generated from mass spectra data in order to visualise the relationships between isolates and in relation to library strains.

Mass spectra generated from selected isolates were compared with those contained in the BDAL library and ascribed identity score values (Table 3.6). Of the two reference strains only *P. temperata* DSM 14550 was assigned a species level score value to the corresponding type strain in the database. *Photorhabdus luminescens* DSM 3368 was only identified with confidence to genus level, and a probable designation as a *luminescens* species. Isolate 165 was identified with confidence as a *Pseudomonas* species, while Isolate 178 was designated to probable *Xenorhabdus* genus level. Isolates 96 and 173 returned confident scores for *P. luminescens*; while Isolates 151, 161, 170, 175 and 180 obtained score values associated with a secure genus and probable species as *P. luminescens*. Isolate 91 returned a score suggesting probable *Photorhabdus* genus.

Table 3.6. MALDI-TOF-MS identification score values of selected isolates as compared to the Bruker Daltonics Biotyper spectra database (BDAL).

Isolate	Highest scored BDAL match	Score Value
P. luminescens DSM 3368	*P. luminescens* subsp. *akhurstii* DSM 15138T HAM	2.006
P. temperata DSM 14550	*P. temperata* subsp. *temperata* DSM 14550T HAM	2.304
91	*P. luminescens* subsp. *kayaii* DSM 15194T HAM	1.908
96	*P. luminescens* subsp. *laumondii* DSM 15139T HAM	2.304
151	*P. luminescens* subsp. *laumondii* DSM 15139T HAM	2.227
161	*P. luminescens* subsp. *laumondii* DSM 15139T HAM	2.243
165	*Pseudomonas corrugata* DSM 7228T HAM	2.014
170	*P. luminescens* subsp. *laumondii* DSM 15139T HAM	2.279
173	*P. luminescens* subsp. *laumondii* DSM 15139T HAM	2.311
175	*P. luminescens* subsp. *laumondii* DSM 15139T HAM	2.216
178	*Xenorhabdus ehlersii* DSM 16337T HAM	1.923
180	*P. luminescens* subsp. *laumondii* DSM 15139T HAM	2.265

Identification Score values: 2.300–3.000 = Highly probable genus and species identification; 2.000–2.299 = Secure genus and probable species identification; 1.700–1.999 = Probable genus identification; 0.000–1.699 = No consistency in genus or species.

Mass spectra profiles (MSP) were generated from the selected isolates and two reference strains for the generation of a dendrogram which included reference strains from the BDAL library (Figure 3.10). Isolates 165 and 178 each form their own clade in the dendrogram, which is reflective of their Biotyper database matches as *Pseudomonas* sp. and *Xenorhabdus* sp. respectively. All of the remaining isolates showed high levels of spectral homology, forming a distinct grouping that clustered closely with *P. luminescens* subsp. *laumondii* DSM 15139T. Isolate 91 is distinct but clustered close to the other isolates. The reference strains used in this study (*P. luminescens* DSM 3368 and *P. temperata* DSM 14550) do not cluster with their contemporaries in the BDAL reference library. Two very similar subgroups of the *Photorhabdus* isolates are distinguishable, with Isolates 32, 66, 50, 179, 175, 36, 164, 40 and 151 grouping separately from Isolates 174, 96, 172, 22, 170, 173, 161 and 180.

For visual comparison between the isolate MSPs the presence and relative intensity of mass spectra peaks were expressed in a gel view using mMass software (Figure 3.11). Isolate 165 has a very

distinct profile across the *m/z* range in comparison to the other isolates. Profiles for Isolate 178 and the two reference strains are distinct from the remainder of the *Photorhabdus* isolates. The remaining isolates have many peaks in common; however there are some differences in intensity (expressed as a function of greyscale) and presence of certain peaks.

These observations are confirmed by an additional comparison of the individual mass spectra generated for Isolates 161, 165 and 178 and *P. luminescens* DSM 3368 as shown in Figure 3.12. The similarities between Isolate 161 and *P. luminescens* DSM 3368 are apparent from the overall peak profile as well as relative peak intensities. The mass spectra of Isolates 178 and 165 demonstrate greater differences to the majority of isolates in mass ranges of prominent peaks and peak intensities.

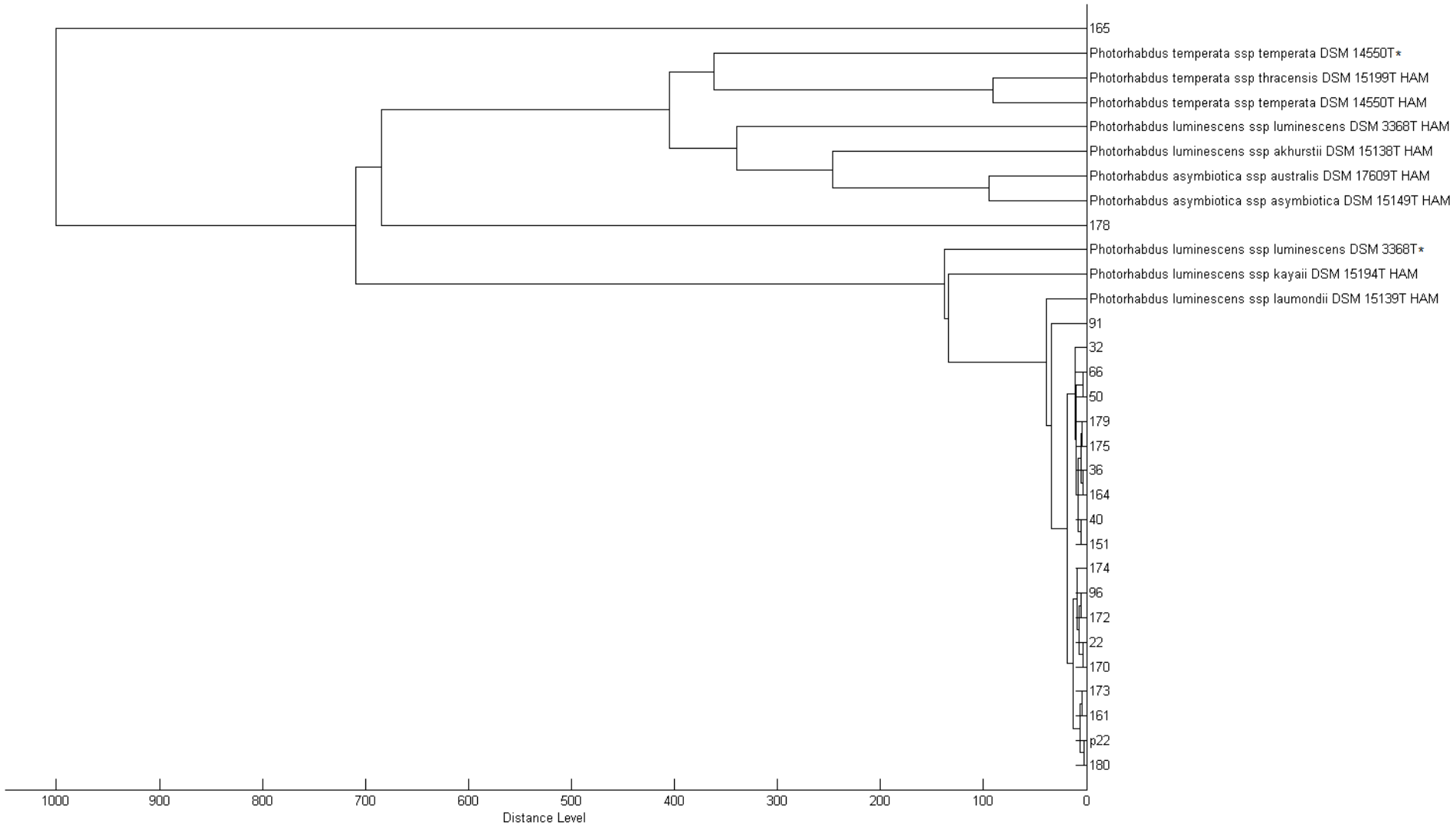


Figure 3.10. Dendrogram of *Photorhabdus* isolates and reference strains compared to the Bruker Daltonics Biotyper spectra database (BDAL) reference strains.

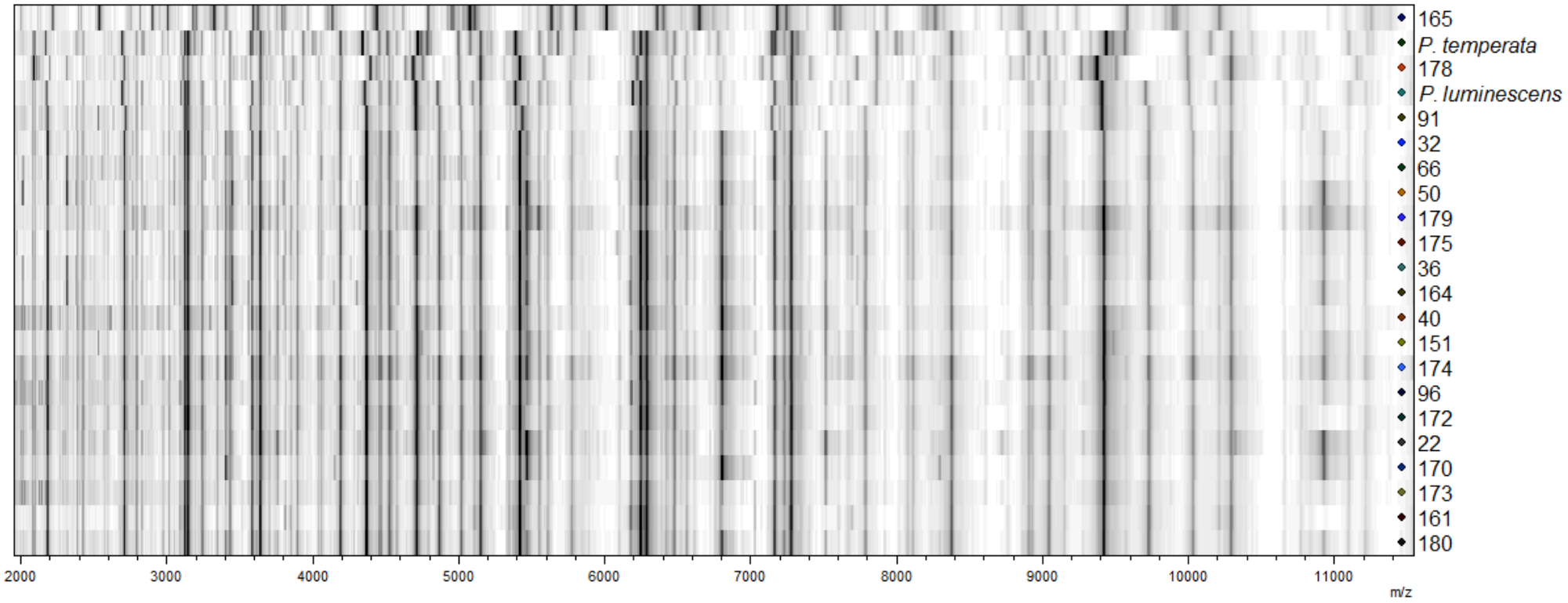


Figure 3.11. MALDI-TOF-MS mass spectra profiles of *Photorhabdus* isolates and reference strains represented as a gel view using mMass software (*m/z* 2000–11500).

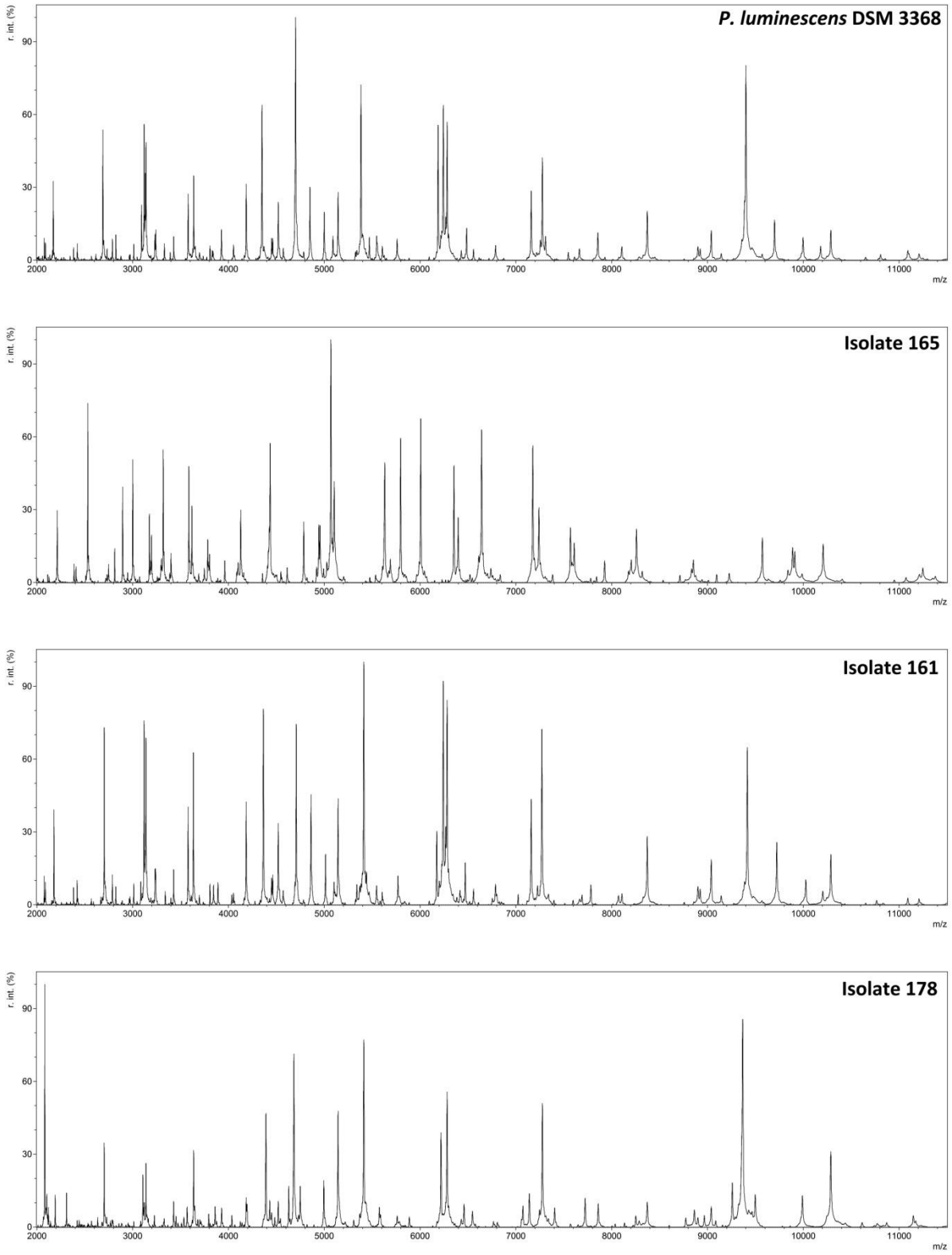


Figure 3.12. Mass spectra profiles of reference strain *Photorhabdus luminescens* DSM 3368 and representative Isolates 161, 178 and 165 (*m/z* 2000–11500).

3.3. Discussion

Previous studies have been carried out to assess the diversity within isolates of the genus *Photorhabdus* from Australia, North America, Europe and the Caribbean (Hazir *et al.,* 2004; Akhurst *et al.,* 2004; Fischer-Le Saux *et al.,* 1999; Fischer-Le Saux *et al.,* 1998). Comparatively little is known of the species- and strain-level diversity amongst South African *Photorhabdus* spp.. However, recently published research has seen the isolation and description of a new South African *P. luminescens* subspecies *noenieputensis* and a new species *Photorhabdus heterorhabditis* (Ferreira *et al.,* 2014; Ferreira *et al.,* 2013).

3.3.1. Phenotypic characteristics

Variations in phenotypic traits have long been the standard approach to bacterial species identification and differentiation, and have been used to describe and characterise known species of *Photorhabdus* (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005). However, phenotypic approaches can be limited and often encounter reproducibility issues; hence in many modern studies genotyping is often applied in place of phenotyping owing to its lower cost and greater accuracy and discriminating power at the strain level (Li *et al.,* 2009). This research aimed to determine levels of diversity amongst a subset of *Photorhabdus* isolates, using phenotypic characteristics, DNA fingerprinting, gene sequence analysis and applying MALDI-TOF-MS as a proteomic approach to characterisation.

Despite limitations in reproducibility and low throughput compared to genetic methods, phenotyping is still relevant to modern microbiology for identification and characterisation of unknown organisms, with literature such as *Bergey's Manual* and *The Prokaryotes* still widely used (Bochner, 2009). Phenotypic data from a range of *Photorhabdus* species and subspecies have been published as supplementary means of identification and classification in studies relying on DNA-based techniques (Ferreira *et al.,* 2014; Ferreira *et al.,* 2013; Boemare and Akhurst, 2005; Fischer-Le Saux *et al*., 1999). Differentiation of *Photorhabdus* spp. from closely related *Xenorhabdus* and *Proteus* genera is possible using phenotypic data such as bioluminescence, catalase, annular haemolytic ability, urease, indole production, H_2S production, nitrate reductase and acid production

from D-mannose (Boemare and Akhurst, 2005). Several phenotypic traits have been identified as useful for differentiating between *Photorhabdus* species. These include: maximum growth temperature, indole production, DNase and urease (Christensen's) activity, esculin hydrolysis, tryptophan deaminase activity, Simmons citrate utilisation, annular haemolysis (sheep or horse blood agar), acid production from mannitol and trehalose and utilisation of L-fucose, DL-glycerate, L(-)histidine, myo-inositol, DL-lactate and D-mannitol (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005).

A number of phenotypic characteristics of the *Photorhabdus* isolates were assessed in the current study using the API 20E test strip and several supplementary test media (Tables 3.1 and 3.2). Identification of isolates within this subset to species level proved difficult with the traits targeted. This was chiefly due to low variability in target characteristics between isolates, and the multiple negative results obtained for the majority of the API 20E reactions. Furthermore, resource limitations forced the narrowing of the range of characteristics that could be evaluated, which further reduced the ability to ascribe any isolate to a particular species with sufficient confidence.

All isolates with the exception of Isolate 178 were catalase positive, suggesting that this isolate may not be of the *Photorhabdus* genus. The catalase test is an important characteristic which differentiates *Photorhabdus* spp. from the closely-related *Xenorhabus* spp. (Boemare and Akhurst, 2005). Casein hydrolysis and gelatinase test both returned positive results for all isolates which is characteristic of *Photorhabdus* spp. and unsurprising considering their ecological niche and role in the processing of insect cadavers (Eleftherianos *et al.,* 2007; ffrench-Constant *et al.,* 2007; Boemare and Akhurst, 2006; Boemare and Akhurst, 2005). All isolates were confirmed to ferment glucose, which is expected positive for *Photorhabdus* spp. (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005). Although Isolate 91 and the reference strain *P. luminescens* subsp. *luminescens* DSM 3368 produced a weak reaction possibly due to a slower growth rate. Esculin hydrolysis is a common trait associated with members of *Photorhabdus*, and all isolates, with the exception of Isolates 40, 66, 91, 151, 165, 175 and 178, tested positive for this trait (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005).

The absence of mannitol fermentation is common within the *Photorhabdus* genus with only *P. luminescens* subsp. *akhurstii* producing a positive reaction for most strains (Boemare and Akhurst,

2006; Boemare and Akhurst, 2005). There was a slight disparity between the results gathered from the API 20E test and those obtained from the supplementary test medium for acid production from mannitol. The majority of isolates were negative on both media while *P. luminescens* subsp. *luminescens* DSM 3368 showed a positive reaction on the supplementary medium. *Photorhabdus luminescens* is described in literature as variably positive for acid production from mannitol, hence the observed positive reaction is not unexpected (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005). However, the negative reaction in the API 20E is in conflict with the observations from the supplementary test. It is possible that differences in media constituency and the respective experimental procedures between the two tests are responsible for this disparity (Akhurst *et al*., 2004).

Photorhabdus spp. are generally positive for acid production from utilisation of trehalose although a degree of strain variability and weak reactions to this test have been reported (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005). The absence of acid production from trehalose in the case of *P. temperata* subsp. *temperata* DSM 14550 was therefore unexpected (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005). Isolates 91, 151, 165, 175 and 178 were also negative for acid production from trehalose while all other isolates demonstrated positive results. Noted during trehalose data collection was the development of a red or pinkish pigmentation within the test media by certain isolates (Plate 3.1). For the most part, colouration corresponded to a positive test for acid production, except in the case of Isolate 91 where no acid production was noted. Production of a red pigment by *Photorhabdus* has been described as a common occurrence (Mahar *et al.,* 2005; Forst and Nealson, 1996). Nematode-infected insect larvae have been reported to take on a “red brick” colouration; hence the production of a red pigment by Isolates in the presence of an insect carbohydrate is noteworthy (Castillo *et al*., 2012; Thompson, 2003). This pigmentation may be a result of the production of red anthraquinone pigments which have been identified from *Photorhabdus* (Richardson *et al.,* 1988).

Utilisation of citrate is found to be highly variable amongst the currently described *Photorhabdus* subspecies (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005). *Photorhabdus asymbiotica* and *P*. *luminescens* subsp. *luminescens* appear to be the only species that consistently produce positive results for most strains. This was corroborated by the positive result obtained for *P. luminescens* subsp. *luminescens* DSM 3368 in the supplementary citrate test. However the API 20E

reaction for citrate utilisation was negative for this organism. *Photorhabdus temperata* strains exhibit highly variable citrate utilisation profiles; hence the negative result obtained for *P. temperata* subsp. *temperata* DSM 14550 is not unexpected (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005). Isolates 91 and 165 were the only others to show positive reactions for this test on supplementary media; however, they also returned a negative result for citrate utilisation on the API 20E strip. All other isolates had a negative reaction on both media. Positive results observed for citrate were only noted on the supplementary test media after 7 days of incubation. This slow reaction could possibly be attributed to slow growth rates and could explain the absence of any positive reactions API 20E strip due to the shortened incubation period (48 h) used.

The API 20E and the supplementary media for urea utilisation produced contradictory results, with the API test being collectively negative whereas the majority of isolates displayed a positive reaction on the medium used for the supplementary test of urea utilisation. Only Isolate 91 displayed a negative urease reaction on both media. Strains of *P. luminescens* subsp. *luminescens* and *P. temperata* subsp. *temperata* are classified as negative to mostly negative for urease activity (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005), therefore a positive result as obtained for the two reference strains was unexpected.

Bergey's Manual indicates that most *Photorhabdus* subspecies display a negative result for tryptophan deaminase activity. Exceptions include strains of *P. luminescens* subsp. *laumondii*, which displays variable reactions, and strains of and *P*. *luminescens* and *P. temperata* which exhibit very low frequency positive reactions (Boemare and Akhurst, 2005). A positive result is expected for 25–75% of strains of *P. luminescens* subsp. *laumondii* and *P. temperata* subsp. *temperata*, and >75% for strains of *P. temperata* (Boemare and Akhurst, 2006). Hence the positive result for reference strain *P. temperata* subsp. *temperata* DSM 14550 is not unexpected; yet the positive result for reference strain *P. luminescens* subsp. *luminescens* DSM 3368 is unanticipated.

Strains of *P. luminescens* subsp. *luminescens* are usually associated with indole production, however this result was not observed for *P. luminescens* subsp. *luminescens* DSM 3368 (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005). For *P. temperata* subsp. *temperata* DSM 14550 the negative result obtained corresponds with findings previously reported in the literature. The negative reactions obtained for Isolates 36, 91, 164, 170, 174 and 179 suggests closer similarity to *P.*

temperata, *P. asymbiotica* or *P. luminescens* subsp. *akhurstii*. Positive results for the remaining isolates in this test are consistent with a result for a *P. luminescens* subspecies.

All isolates returned a negative response for the utilisation of inositol. This finding is in contrast to data presented in *Bergey's Manual*, in which the utilisation of inositol has been commonly observed within the genus *Photorhabdus,* with only *P. temperata* subsp. *temperata* and *P*. *asymbiotica* demonstrating variable results (Boemare and Akhurst, 2006; Boemare and Akhurst, 2005). While this result is possibly correct for the reference strain *P. temperata* subsp. *temperata* DSM 14550, it contradicts the expected positive result for the *P. luminescens* subsp. *luminescens* strain DSM 3368.

The API 20E kit includes a number of reactions which were universally negative for the tested isolates and were therefore of little use in differentiating strains. Similarly in *Bergey's Manual* these reactions have also been reported as being uniformly negative within the genus *Photorhabdus* (Boemare and Akhurst, 2005). These frequently negative tests include: utilisation of D-sorbitol, L-rhamnose and D-sucrose, L-arabinose fermentation/oxidation, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, acetoin, production of NO_2 and reduction to N_2 gas, and production of H_2S gas. The tests for utilisation of D-saccharose, D-melibiose and amygdalin in the API 20E strip were all negative for all isolates; however *Bergey's Manual* does not offer data on these traits for known *Photorhabdus* species and strains. The supplementary starch hydrolysis assay was also negative for all of the isolates tested; although here again no literature data is available for this characteristic in *Photorhabdus*.

In *Bergey's Manual* the metabolic characteristics presented for the species of *Photorhabdus* described are very similar (Boemare and Akhurst, 2005). The high strain variability for certain characteristics (i.e. 25–75% variability) complicates differentiation based on these traits. In addition, reproducibility issues are frequently associated with phenotypic differentiation of bacteria (Li *et al.,* 2009). Conflicting phenotypic data for *Photorhabdus* have been reported for characteristics such as citrate, DNase, urease and acid production from maltose (Akhurst *et al.,* 2004). The effectiveness of API strips in these instances may therefore be limited. For example, in the current study different results were obtained with supplementary media compared to the API 20E equivalent, as was in the case of acid production from mannitol, citrate utilisation tests, and most notably for the urease test. Results for some of the API 20E tests were contrary to those reported in the literature (e.g. citrate

utilisation, tryptophan deaminase, indole production and inositol utilisation). Similar variations between test methodology and phenotypic characteristic results for the genus *Xenorhabdus* have been reported by Somvanshi *et al.* (2006), with variation between BIOLOG GN results and API 50CH and 20NE outcomes. Findings for these *Photorhabdus* isolates highlight the limitations and poor reliability of phenotypic characterisation. However these problems are not insurmountable; researchers have reported success using API kits, though supplementation of the media with growth factors is often reported (Ferreira *et al.,* 2013; An and Grewal, 2011; Somvanshi, *et al.,* 2006; Hazir, *et al.,* 2004; Grimont, *et al.,* 1984).

3.3.2. Genetic diversity assessment

DNA fingerprinting using 16S PCR-RFLP has been established as a fast, cost-effective means for the differentiation of *Photorhabdus* and *Xenorhabdus* strains (Fischer-Le Saux *et al*., 1998; Brunel *et al*., 1997). This method has been used to differentiate *Photorhabdus* at the species and subspecies level using a minimum of three restriction endonucleases (namely ALUI, HAEIII and HHAI) (Fischer-Le Saux *et al*., 1998). For all restriction digests Isolate 165 are found to be significantly different from the other isolates (Figure 3.7). Isolate 178 was also differentiated in all digests but it was found to have some bands in common with the remaining isolates. Within the sample set only Isolates 91, 165 and 178 are unique; the remaining isolates produced identical fingerprint profiles for all three digests evaluated. Restriction endonuclease HHAI discerned the most banding profiles, with Isolates 91, 165, 178 and the two reference strains being differentiated from the majority which displayed identical band profiles (Figures 3.3 and 3.4). Restriction endonuclease HAEIII produced profiles exhibiting the least diversity between isolates with only Isolates 165 and 178 being differentiated (Figures 3.5 and 3.6). Restriction endonuclease ALUI distinguished Isolates 165 and 178 and Isolate 91 and *P. luminescens* subsp. *luminescens* (Figures 3.1 and 3.2). The ALUI digest band profile of *P. temperata* subsp. *temperata* was not differentiated from the other isolates. These data suggest that there is very little diversity within these *Photorhabdus* sp. isolates 16S rRNA gene that is resolvable using these three restriction endonucleases.

The use of RAPD-PCR analysis on *Photorhabdus* isolates appears to be largely unexplored. To the best of our knowledge only one study using this technique on entomopathogenic nematode symbionts has been previously published (Kumar *et al.,* 2011). The four RAPD primers applied in the

current study consistently distinguished the two references strains as well as Isolate 178 from the remaining isolates (Figure 3.8). Isolate 165 formed unique profiles when using OPG 5, 11 and 16, but exhibited a similar band profile when compared to the majority of isolates for OPG 8. Isolate 91 produced similar band profiles to the majority of isolates for OPG 5, 8 and 11, while being unique when OPG 16 was used. Isolate 173 has a unique profile for primers OPG 5 and 11; however its profile appears similar to the majority of isolates when OPG 8 and 16 were used. Other isolates which conformed to the majority for three of the four primers tested gave unique profiles for a single primer, these included Isolate 96 (OPG 11), Isolate 174 (OPG 5), Isolate 175 (OPG 8) and Isolate 179 (OPG 8). Isolates 32, 50, 66, 151 and 172 showed identical profiles to each other across the four RAPD primers. Isolates 36 and 164 are also identical to each other in all four primer sets. There were cases of Isolate banding patterns being consistent with the majority in some primers, but not in others (namely Isolates 22, 40, 161, 170 and 180). In contrast to the more narrowed view offered by 16S rRNA PCR-RFLP, RAPD-PCR fingerprinting techniques offer resolution of genome-wide differences between isolates with high throughput, low material costs and high sensitivity to strain level variations (Li *et al.,* 2009). However, the varied banding pattern relationships discerned between the RAPD primer highlights the importance of primer choice, and the use of a suite of primers, in order to accurately differentiate between isolates.

While DNA fingerprinting techniques can give some indication as to the diversity amongst a set of isolates, gene sequence analysis is considered to represent a more reliable means of identifying bacterial isolates. A similarity matrix of the partial 16S rRNA gene sequences obtained from the selected isolates shows Isolates 151, 161, 173 and 180 are very similar (Table 3.4). These achieved >99.3% similarity between them over the 1445 bp aligned sequence (Table 3.5). Isolate 165 is clearly very different to all other isolates with ~82–83% sequence similarity. Isolate 178 is more closely related but is only 94% similar to the *Photorhabdus* isolates. The highest-scoring similarities of the isolates to the type strains was Isolate 173 (98.2% similarity to *P. luminescens* subsp. *luminescens*), and Isolate 180 (97.3% similarity to *P. temperata* subsp. *temperata*). Isolate 91 is significantly different to the other isolates (151, 161, 173 and 180) with only ~97% similarities.

16S rRNA partial gene sequence data was submitted to NCBI BLAST to search for similar sequences within the database (Table 3.5). From this data it is clear that Isolate 165 is closely related to *Pseudomonas* spp. (*Pseudomonas* sp. HMPB1 and CMR5c with 1–2 bp difference), while Isolate 178

could be matched to *Xenorhabdus* spp. (98% similarity to a *Xenorhabdus* strain MY8 KsSu155). Neither of these two isolates had any close matches to published *Photorhabdus* strain sequences. The two reference strain sequences correspond to their respective NCBI database sequences with very high sequence similarity (*P. temperata* subsp. *temperata* 100%, and *P. luminescens* subsp. *luminescens* 99.93%). Sequences for Isolates 91, 151, 161, 173 and 180 were all matched closest with strains of *Photorhabdus luminescens* subsp. *laumondii* with ~98% sequence similarity.

The phylogenetic tree generated from the partial 16S rRNA gene sequence data and reference strains clustered Isolates 178 and 165, as expected, with *Xenorhabdus* and *Pseudomonas* sequences respectively (Figure 3.9). Likewise the two reference strains group within their respective subspecies clusters, and cluster closely to their corresponding sequence data contained in the GenBank database. All of the remaining isolates sequenced were grouped within the *P. luminescens* cluster, with Isolates 180, 173, 151 and 161 clustered closely in a single group which was distinct from any of the defined *P. luminescens* subspecies. Isolate 91 aligned between *P. luminescens* subsp. *laumondii* and *kayaii* subspecies clusters and near the recently described South African *P. luminescens* subsp. *noenieputensis* with which it has a partial 16S rRNA gene sequence similarity of 98.4%. 16S rRNA sequence similarity between the *Photorhabdus* isolates in this study and previously described subspecies are as dissimilar as those figures noted in the description of the new subspecies *P. luminescens* subsp. *noenieputensis* (Ferreira *et al.,* 2013). Analysis of alternative gene sequences such as *rec*A, *gyr*B, *dna*N, *glt*X and *inf*B have been used in previous studies to define taxonomic relationships within *Photorhabdus* spp. (Ferreira *et al.,* 2013).

3.3.3. Whole-cell MALDI-TOF-MS analysis

MALDI-TOF-MS represents a rapid method for bacterial identification based on protein biomarker peaks and mass spectra profiles (Welker and Moore, 2011). Library-based systems offer identification of bacterial isolates based on comparisons with a reference strain database, which was accomplished to varying degrees of confidence within the *Photorhabdus* isolates studied (Table 3.6). In corroboration with the previous DNA fingerprinting and 16S rRNA gene sequence analysis results, Isolate 165 was identified to as *Pseudomonas* sp., and Isolate 178 as a *Xenorhabdus* sp.. The remaining isolates were identified using Biotyper software as member of the genus *Photorhabdus* and with varying confidence levels (scores ranging from secure to probable) to species level as *P.*

luminescens. The two reference strains included in this study were present in the BDAL library; however the confidence values for their identification with Biotyper in this study were lower than expected. *Photorhabdus temperata* subsp. *temperata* was correctly identified to the genus and species level (score 2.304), while *P. luminescens* subsp. *luminescens* was only identified with genus-level confidence (score 2.006). This variation in confidence level scores may be ascribed to subtle differences in the mass spectra profiles, possibly caused by variations in culture age, medium constituency, or the presence of some cells in a state of phase variation (Šedo et al., 2011; Valentine *et al.*, 2005; Wunschel *et al.*, 2005). Overall, characterisation of isolates using the MALDI Biotyper returned results similar to 16S rRNA gene sequence analysis.

The dendrogram comparing isolate MSPs to BDAL library reference strains highlights the similarities between the majority of this subset of *Photorhabdus* isolates (Figure 3.10). Interestingly the reference strains did not cluster closely with their equivalents in the BDAL library, which as mentioned above, could be ascribed to some variations in culturing practice or sample handling between this study and the generation of the library profiles. The gel view of the MSPs also reveals differences in peak intensity and occurance amongst the remaining *P. luminescens* isolates (Figure 3.11). A visual comparison of Isolate 161 and *P. luminescens* DSM 3368 in Figure 3.12 shows few significant differences, while Isolate 178 shows broad similarity but with some differences. Isolate 165 shows a profile clearly different from the other isolates.

3.3.4. Conclusions

The phenotypic tests used in this study provided limited information that could be used to differentiate isolates. However, these tests proved insufficient to identify isolates. The catalase test was able to differentiate Isolate 178 as a non-*Photorhabdus* sp., however none of the other tests performed could confirm this. The selection of phenotypic tests also failed to distinguish Isolate 165 as an unrelated member of the genus *Pseudomonas*. Additionally, the conflicting outcomes between the API test strip and supplementary protocols for several traits raised some concerns as to phenotypic data reliability and reproducibility.

In contrast to phenotypic characterisation methods, a higher degree of differentiation amongst isolates was achieved using DNA fingerprinting techniques. 16S rRNA gene PCR-RFLP distinguished the reference strains from the *Photorhabdus* isolates screened, and clearly distinguishing Isolate 165 as being significantly different. Isolate 178 showed higher similarity to the majority of the isolates, but its profiles were still distinct from those of the *Photorhabdus* isolates. Overall levels of diversity within the confirmed *Photorhabdus* isolates were found to be low based on 16S rRNA gene PCR-RFLP, with only Isolate 91 being distinguished from the majority using this method. The RAPD-PCR fingerprint profiles were able to detect broad similarities between the isolates, while also distinguishing the reference strains, as well as Isolates 91, 165 and 178. Relatively few isolates produced identical banding profiles for each of the four RAPD-PCR primer sets evaluated. This contrasted with the 16S rRNA gene PCR-RFLP results, which showed limited diversity amongst the majority of isolates. The RAPD profiles indicate greater levels of diversity between these isolates than indicated by either 16S rRNA PCR-RFLP or gene sequence analysis. This is not unexpected, as the RAPD-PCR targets sites throughout the whole bacterial genome, whereas the gene sequencing and 16S rRNA gene PCR-RFLP were limited to the more highly conserved 16S rRNA gene.

MALDI Biotyper and 16S rRNA gene sequence data concur in assigning isolates as *P. luminescens* and confirming the high degree of relatedness amongst these isolates. Both techniques further concur on the identification of Isolates 165 and 178 as strains of *Pseudomonas* sp. and *Xenorhabdus* sp. respectively. Overall, the mass spectra gel view image shows strong similarities in MALDI-TOF-MS peak profiles amongst *Photorhabdus* spp. isolates; yet differences in band intensity and band presence allude to the underlying differences between these isolates that were observed with RAPD-PCR data. Furthermore, MALDI-TOF-MS and RAPD-PCR data suggest that the isolates are not as homogenous as 16S rRNA sequence analysis suggests, hence it may be of interest to analyse alternative housekeeping genes to further characterise genetic diversity in this group. Sequence analysis of the 16S rRNA gene remains the gold standard for bacterial identification, with MALDI-TOF-MS being touted as a close rival (Welker and Moore, 2011; Mellmann *et al.,* 2008; Olsen and Woese, 1993). MALDI-TOF-MS offers a similar expense per run to the PCR-based fingerprinting techniques, and a faster turnaround time. Results obtained with MALDI-TOF-MS were found to be in line with results achieved with 16S rRNA sequence data.

The results of this diversity study showed that the majority of the isolates evaluated belonged to a single species, *P. luminescens*. The data indicates that most of these isolates could be placed into a single closely related cluster, with Isolate 91 being related but grouping separate from the rest. However, neither of these possible groupings could be confidently ascribed to any currently-described *P. luminescens* subspecies. Hence, additional study of these isolates with the aim to differentiate them further at the subspecies, or even the strain level, is a future possibility for this research.

CHAPTER FOUR

Antimicrobial compound diversity amongst South African *Photorhabdus* spp. isolates

4.1. Introduction

Preliminary antimicrobial activity assessment of *Photorhabdus* isolates applied disc-diffusion bioassays with selected fungal and bacterial species. Isolate 22 was chosen as a representative isolate for the evaluation of compound production over a two-week period. Extractions were carried out at several time points to compare antimicrobial compounds contained in both cell-free broth supernatant and within the cell pellet. Isolates 91, 161 and 173 were selected for further study on the basis of their performance in the preliminary bioassays, and as representatives of *Photorhabdus* isolates showing the greatest level of genetic and phenotypic variation within this subset. These underwent compound extraction comparing three approaches: ethyl acetate extraction of the cell-free broth supernatant, methanol extraction of lyophilised cell-free broth supernatant and methanol extraction of cell pellet material. These extracts were then separated into their component fractions using thin-layer chromatography (TLC), and the bands assayed for antibacterial activity. Thereafter, sample extracts from the three isolates were analysed using ultra-performance liquid chromatography electrospray ionisation time-of-flight mass spectrometry (UPLC-ESI-TOF-MS) and gas chromatography mass spectrometry (GC-MS).

4.2. Results

4.2.1. Antimicrobial screening bioassays

Antimicrobial activity of *Photorhabdus* isolates was evaluated using cell-free supernatants and methanol extracts of cellular material in order to determine the spectrum of antimicrobial activity present. The results of the disc-diffusion bioassays against bacterial test organisms *M. luteus* and *B. subtilis* are shown in Tables 4.1 and 4.2. The incidence and extent of antifungal activity towards *B.*

cinerea and *R. solani* is shown in Tables 4.3 and 4.4. With the exception of Isolate 178, none of the isolates showed activity against *E. coli*.

All isolates, with the exception of Isolate 165, demonstrated activity against *M. luteus* (Table 4.1). This activity was associated with both the methanol extracts derived from cell pellets as well as with the cell-free culture supernatants. A trend common to most of the *Photorhabdus* isolates evaluated was the progressive shrinking of each zone of inhibition over the course of a 168 h observation period. During the course of observation the inhibition zone was progressively colonised by *M. luteus*. Typically, the growth of *M. luteus* within the inhibition zone was associated with reduced cell density compared to the control plates. Cell-free supernatant taken from 24 h cultures of Isolates 36, 40, 91 and 178 all demonstrated sustained antibacterial activity over the 168 h observation period. In general, cell-free supernatants sampled on 7 d show a marked decrease in antimicrobial activity compared to samples taken on 1 d or 2 d; whereas the activity associated with methanol extracts from cell pellets was retained at each of the sampling intervals evaluated.

All isolates showed antibacterial activity against *B. subtilis* with the exception of Isolate 165 (Table 4.2). Activity against *B. subtilis* in the methanol extraction of cell pellets is more prevalent in cells harvested at 7 d than those from 1 d. Overall, activity associated with the methanol extracts were observable for most isolates with the exception of Isolates 165, 170 and 180. Activity associated with the 7 d cell-free supernatant sample was greatly diminished compared to the 1 d and 2 d samples.

The assessment of antifungal potential of the samples against *B. cinerea* (Table 4.3) revealed that extract from all isolates demonstrate a possible fungistatic effect whereby changes in gross mycelial morphology at most sample ages were observed. Morphological changes detected include colour change of the fungal mycelia and/or a gross variation in the fungal mat appearance in comparison to the control plates. Isolate 165 was the only organism that displayed distinct fungal growth inhibition as evidenced by a defined area which the fungal mycelia did not colonise. Mycelial changes that were visible to the naked eye appear to be more common in the samples derived from broth supernatant than those from the methanol extraction of cell pellet material. A pink colour change in the *B. cinerea* mycelia was noted for most isolates in the 1 d old broth supernatant assay; and was also observed in a few instances for other samples. The pink colour reaction appeared to be

transient in the case of Isolate 165, whereas for the other isolates the colour developed progressively over time.

Photorhabdus extracts against *R. solani* (Table 4.4) revealed that only Isolate 165 showed inhibition of fungal mycelia. Inhibition was limited and associated with the 1 d old broth supernatant and the 7 d cell pellet methanol extract. The remaining isolates produced no zones of inhibition but instead were associated with changes to the mycelia morphology, often resulting in a darkening or "browning" of the fungal mycelia compared to the control plates.

Table 4.1. Disc-diffusion assay of *Photorhabdus* isolate interactions with *Micrococcus luteus.*

Sampling interval:	Cell-free supernatant									Methanol extract of cell pellet					
	Day 1			Day 2			Day 7			Day 1			Day 7		
	Diameter of inhibition zone (mm)														
Isolate	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**
P.luminescens	18	18	18.5	17.5	13	14.5	13.5	*	*	11.5	*	*	23	*	*
P.temperata	17	*	*	17.5	*	*	*	*	*	10	*	*	24	14.5	*
22	25	16.5	*	26	*	*	*	*	*	13.5	*	*	20	*	*
32	24.5	20	*	23.5	*	*	12.5	*	*	12.5	15	*	19.5	*	*
36	22.5	20	15	23.5	*	*	*	*	*	12.5	*	*	17.5	*	*
40	24	21.5	13.5	23.5	*	*	11	*	*	17.5	*	*	18.5	*	*
50	26.5	23.5	*	23.5	*	*	11.5	*	*	13.5	*	*	22	*	*
66	24	17	*	25	*	*	*	*	*	14.5	*	*	18.5	*	*
91	21.5	22	21	23	23	21.5	13	10.5	10.5	-	-	-	19.5	*	*
96	24	18	*	24	15.5	*	*	*	*	11.5	*	*	19	*	*
151	25.5	15.5	*	22.5	*	*	*	*	*	15.5	*	*	18.5	*	*
161	25.5	22.5	*	24	15	*	*	*	*	18	12.5	*	20.5	*	*
164	25.5	22	*	24	*	*	*	*	*	15.5	*	*	20	*	*
165	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-
170	23	15.5	*	23.5	*	*	*	*	*	19	*	*	19.5	*	*
172	25.5	*	*	23.5	*	*	12	*	*	21.5	*	*	22.5	*	*
173	24	15.5	*	23	*	*	13	*	*	24.5	13	*	20.5	*	*
174	25	20	*	25.5	*	*	12	*	*	24	*	*	19	*	*
175	25.5	*	*	20	*	*	-	*	*	18	*	*	16.5	12.5	*
178	28.5	27	28	33.5	32.5	32.5	29	29	30	22.5	22.5	23	-	-	-
179	25	21.5	*	24.5	*	*	*	*	*	21.5	*	*	19	*	*
180	24	*	*	24.5	*	*	*	*	*	15.5	*	*	19	*	*
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Day 1, day 2 and day 7 = Time at which culture was sampled and processed for disc-diffusion bioassay; 48h, 96h and 168h = Bioassay observations at 48, 96 and 168 hours post inoculation

* = Weak antibacterial activity; - = No inhibition visible

Table 4.2. Disc-diffusion assay of *Photorhabdus* isolate interactions with *Bacillus subtilis*.

Sampling interval:	Cell-free supernatant									Methanol extract of cell pellet					
	Day 1			Day 2			Day 7			Day 1			Day 7		
	Diameter of inhibition zone (mm)														
Isolate	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**
P.luminescens	14.5	14.5	14	13	12	12.5	-	-	*	-	-	-	12.5	13	12.5
P.temperata	*	*	*	*	-	*	-	-	*	-	-	-	11	11.5	11
22	14	*	*	14	*	*	-	*	*	-	-	-	11	10.5	11
32	13.5	11	11	13	10.5	*	-	*	*	-	-	-	11	10	*
36	12	*	*	12	10.5	*	-	*	*	-	-	-	11	*	*
40	12.5	*	*	12.5	11.5	*	-	*	*	-	-	-	12	11.5	12
50	12.5	*	*	14	13	*	-	*	*	-	-	-	12	11	11.5
66	13	*	*	13	*	*	-	*	*	-	-	-	-	*	*
91	17	17	17	17	16	16.5	11	*	*	-	-	-	11	10.5	10.5
96	12	*	*	13.5	12	*	-	*	*	-	-	-	11	10.5	*
151	12	*	*	13.5	12	*	-	*	*	-	-	-	10.5	*	*
161	12	*	*	13	13	*	-	*	*	-	-	-	12	11.5	12
164	11.5	*	10.5	13	13	*	-	*	*	-	-	-	*	10.5	*
165	-	*	*	-	-	*	-	*	*	-	-	-	-	-	-
170	12.5	*	*	14.5	12	*	-	*	*	-	-	-	-	*	*
172	12	*	*	12	11.5	*	-	*	*	11.5	12	12	12	12	12.5
173	12.5	10.5	*	14.5	13.5	*	-	*	*	11	12	*	13	13	13
174	12	*	*	13.5	12.5	*	-	*	*	11	10.5	*	13	13	12
175	11.5	*	*	*	*	*	-	*	*	10	*	*	*	10.5	10.5
178	21	22.5	22.5	21.5	23.5	25.5	23	24.5	22.5	13.5	15	15	-	-	-
179	13.5	11.5	11	13.5	11.5	*	-	*	*	-	-	-	10.5	11	*
180	13	*	*	11.5	11	*	-	*	*	-	-	-	-	-	*
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Day 1, day 2 and day 7 = Time at which culture was sampled and processed for disc-diffusion bioassay; 48h, 96h and 168h = Bioassay observations at 48, 96 and 168 hours post inoculation; * = Weak antibacterial activity; - = No inhibition visible.

Table 4.3. Disc-diffusion assay of *Photorhabdus* isolate interactions with *Botrytis cinerea*.

Sampling interval:	Cell-free supernatant									Methanol extract of cell pellet					
	Day 1			Day 2			Day 7			Day 1			Day 7		
	Diameter of inhibition zone (mm)														
Isolate	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**
P.luminescens	-	* P	* P	-	*	*	-	*	*	-	-	- P	+	*	-
P.temperata	*	* P	* P	* P	*	*	-	-	-	-	-	-	-	*	-
22	*	* P	* P	-	*	*	-	*	-	-	-	-	-	*	-
32	*	* P	* P	+	*	*	*	*	*	-	-	-	*	*	-
36	*	* P	* P	*	*	*	*	*	*	*	-	-	-	-	-
40	*	* P	*	*	*	*	-	*	*	-	-	-	-	*	-
50	*	* P	* P	-	*	*	*	*	*	-	-	-	-	*	-
66	*	* P	* P	*	*	*	*	*	*	-	-	-	-	-	-
91	-	* P	* P	*	*	*	*	*	*	-	-	-	+	*	-
96	*	* P	* P	*	*	*	-	*	*	*	-	-	-	*	-
151	*	*	*	*	*	*	-	*	*	-	-	-	-	-	-
161	*	* P	* P	*	*	*	-	*	*	-	-	-	-	*	-
164	*	* P	* P	*	*	*	-	*	*	*	-	-	+	*	-
165	++	* P	* P	+++	++ P	++	+	*	*	-	-	-	+++	+++	+
170	-	* P	* P	*	*	*	-	*	*	-	-	- P	*	-	-
172	-	*	*	*	*	*	-	*	*	*	-	- P	*	*	-
173	*	*	* P	*	*	*	*	*	*	*	-	- P	*	*	-
174	*	*	*	*	*	*	*	*	*	*	-	- P	*	*	-
175	-	*	*	*	*	-	-	*	*	*	-	- P	*	-	-
178	*	-	-	*	*	*	*	*	*	*	-	-	-	-	-
179	*	* P	* P	*	*	*	*	*	*	*	-	- P	*	*	-
180	*	* P	* P	*	*	*	*	*	-	*	-	-	*	*	-
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Day 1, day 2 and day 7 = Time at which culture was sampled and processed for disc-diffusion bioassay; - = No inhibition visible, filter dic covered with mycelia; * = Growth to edge of filter disc with morphology changes; + = 1-3 mm between filter disc and Fungal mycelia; ++ = 3-10 mm between filter disc and fungal mycelia; +++ = 10 mm or greater between filter disc and fungal mycelia; P = Mycelia associated with filter disc shows pink colouration.

Table 4.4. Disc-diffusion assay of *Photorhabdus* isolate interactions with *Rhizoctonia solani*.

Sampling interval:	Cell-free supernatant									Methanol extract of cell pellet					
	Day 1			Day 2			Day 7			Day 1			Day 7		
	Diameter of inhibition zone (mm)														
Isolate	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**
P.luminescens	-	-	- Br	-	- Br	- Br	-	- Br	- Br	-	-	-	*	-	- Br
P.temperata	*	-	- Br	*	- Br	- Br	*	- Br	-	-	- Br	- Br	*	*	* Br
22	*	-	- Br	*	- Br	- Br	-	- Br	-	-	-	-	*	-	- Br
32	*	-	- Br	*	* Br	- Br	* Br	-	- Br	-	- Br	- Br	*	-	- Br
36	*	-	- Br	*	* Br	- Br	* Br	- Br	- Br	-	- Br	- Br	*	-	- Br
40	*	-	- Br	*	-	- Br	* Br	- Br	- Br	-	-	-	*	*	* Br
50	*	-	- Br	*	*	- Br	* Br	- Br	- Br	-	- Br	- Br	*	-	- Br
66	*	-	- Br	*	* Br	- Br	* Br	- Br	- Br	-	- Br	- Br	-	-	- Br
91	*	-	- Br	-	* Br	- Br	-	- Br	- Br	-	-	-	*	-	- Br
96	*	-	- Br	*	* Br	- Br	-	- Br	- Br	-	- Br	- Br	-	-	- Br
151	*	-	- Br	*	* Br	- Br	*	- Br	- Br	-	- Br	- Br	*	-	- Br
161	-	-	- Br	*	* Br	- Br	-	- Br	- Br	-	- Br	- Br	*	-	- Br
164	*	-	- Br	*	* Br	- Br	-	- Br	- Br	*	- Br	- Br	-	*	* Br
165	+	* Br	- Br	-	-	- Br	-	-	- Br	-	- Br	- Br	++	+	*
170	*	-	- Br	*	-	- Br	-	- Br	- Br	-	- Br	- Br	-	-	- Br
172	*	-	- Br	*	-	- Br	*	-	- Br	*	- Br	- Br	*	*	* Br
173	*	-	- Br	*	* Br	- Br	*	-	- Br	*	- Br	- Br	*	*	* Br
174	*	-	- Br	*	-	- Br	*	-	- Br	-	- Br	- Br	*	-	- Br
175	*	-	- Br	-	-	- Br	*	-	- Br	-	- Br	- Br	*	-	- Br
178	*	-	-	-	-	-	*	-	-	*	-	-	-	-	- Br
179	*	*	- Br	-	- Br	- Br	* Br	* Br	- Br	-	-	-	*	-	- Br
180	*	*	* Br	*	- Br	- Br	* Br	* Br	- Br	-	- Br	- Br	*	*	* Br
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Day 1, day 2 and day 7 = Time at which culture was sampled and processed for disc-diffusion bioassay; 48h, 96h and 168h = Bioassay observations at 48, 96 and 168 hours post inoculation;
- = No inhibition visible, filter disc covered with mycelia; * = Growth to edge of filter disc with morphology changes; + = 1-3 mm between filter disc and Fungal mycelia; ++ = 3-10 mm between filter disc and Fungal mycelia; Br = Browning of mycelia in close proximity to the disc.

4.2.2. Effects of culture age on antibacterial activity for *Photorhabdus* Isolate 22

Isolate 22 was chosen as a representative isolate for the evaluation of *Photorhabdus* compound production over the course of a growth cycle. The selection of a single representative isolate afforded the opportunity for higher sampling frequency than was possible with multiple isolates. Methanol extraction of compounds from the cell pellet was also performed to ascertain whether there were differences in the profile of antimicrobial compound production over the two-week culture period. The efficacy of the methanol extraction procedure was also evaluated with the inclusion of a subsequent methanol extraction.

Antibacterial activities of samples taken from batch culture of Isolate 22 were noted from the first sample interval (12 h) against both bacterial test organisms *M. luteus* and *B. subtilis* (Table 4.5). Broth supernatant showed no activity at 336 h against either bacterial species; activity was noted at 168 h against *M. luteus* only. Methanol washes of the cell pellet material retained activity up to 336 h against both *M. luteus* and *B. subtilis*. Generally, the antibacterial activity observed between the first and second methanol washes was similar with a slight reduction in the zones of inhibition being associated with the second wash. In all three treatments the highest levels of activity were observed in extracts taken from the culture at 24 h, 48 h and 72 h of incubation. Activity in the broth supernatant decreases with culture time at a greater rate than either of the methanol washes of the cell pellet for both *M. luteus* and *B. subtilis* (Figure 4.1).

Table 4.5. Disc-diffusion assay of *Photorhabdus* Isolate 22 culture supernatant and sequential methanol washes of the cell pellet obtained from cultures grown over a 2 week period.

		Micrococcus luteus			***Bacillus subtilis***		
		Diameter of inhibition zone (mm)					
		Observation Times					
	Culture age	**24 h**	**48 h**	**96 h**	**24 h**	**48 h**	**96 h**
Supernatant	12 h	25(±0.71)	24.63(±1.03)	22.63(±0.63)	15(±0.41)	14.63(±0.75)	13.88(±0.63)
	24 h	27(±1.08)	25.25(±2.72)	26.63(±0.25)	17.75(±0.5)	17.25(±0.5)	17.25(±0.65)
	48 h	24.5(±0.41)	26.63(±1.89)	26.5(±1.55)	18(±0.71)	17.88(±0.48)	17.75(±0.87)
	72 h	24(±2.48)	23(±1.0)	21.13(±0.75)	15.88(±1.11)	15.5(±0.82)	15.25(±1.26)
	96 h	18.88(±1.49)	18.5(±1.35)	18.63(±2.25)	12(±0.58)	10.88(±0.63)	11.25(±0.29)
	168 h	15.38(±1.43)	14.63(±1.31)	11.88(±0.48)	0(±0.0)	0(±0.0)	0(±0.0)
	336 h	0(±0.0)	0(±0.0)	0(±0.0)	0(±0.0)	0(±0.0)	0(±0.0)
Cell pellet 1st Methanol wash	12 h	24.75(±3.89)	25.25(±2.47)	21.5(±2.12)	18.5(±0.0)	13.75(±3.18)	17.25(±0.35)
	24 h	27(±2.12)	26.75(±0.35)	28.25(±1.06)	19.25(±0.35)	18.5(±1.41)	19.5(±1.41)
	48 h	30.5(±0.71)	31.75(±1.06)	30.75(±0.35)	19.75(±0.35)	20.25(±2.48)	21.25(±1.06)
	72 h	29.5(±2.83)	29.5(±0.71)	29.5(±0.0)	21(±2.83)	20.75(±2.48)	21.25(±1.77)
	96 h	28(±4.95)	34.75(±3.90)	29.5(±2.83)	21.25(±0.35)	20.75(±0.35)	20.75(±0.35)
	168 h	27(±0.71)	26(±2.83)	23.5(±1.41)	19.25(±0.35)	18(±0.71)	19.25(±0.35)
	336 h	23.25(±0.35)	22.25(±0.35)	21.5(±0.0)	15.5(±0.0)	15.75(±0.35)	15.75(±1.06)
Cell pellet 2nd Methanol wash	12 h	20.5(±0.71)	17.5(±2.12)	12.5(±4.24)	13.5(±0.71)	12.5(±0.71)	12(±2.12)
	24 h	24.5(±2.12)	16(±0.71)	25.5(±0.71)	19(±0.71)	19(±2.12)	19.5(±2.12)
	48 h	27.5(±0.71)	27(±0.71)	27(±0.71)	19.5(±2.12)	19.5(±2.12)	19.5(±0.71)
	72 h	29.5(±2.83)	29.5(±0.71)	28.5(±2.83)	19(±0.71)	20(±0.71)	20(±2.83)
	96 h	26(±4.24)	27(±2.12)	25.5(±0.71)	20.5(±2.12)	19.5(±2.83)	20(±0.71)
	168 h	26.5(±0.71)	26(±0.71)	25(±2.12)	17.5(±0.71)	18.5(±0.71)	18(±2.12)
	336 h	21(±2.83)	18(±2.12)	15(±2.12)	15(±0.71)	15(±4.24)	15.5(±2.83)

24 h, 48 h and 168 h = Bioassay observations at 48, 96 and 168 hours post inoculation; - = No inhibition visible; zone of inhibition = Average of two replicate samples, Standard deviation in parenthesis

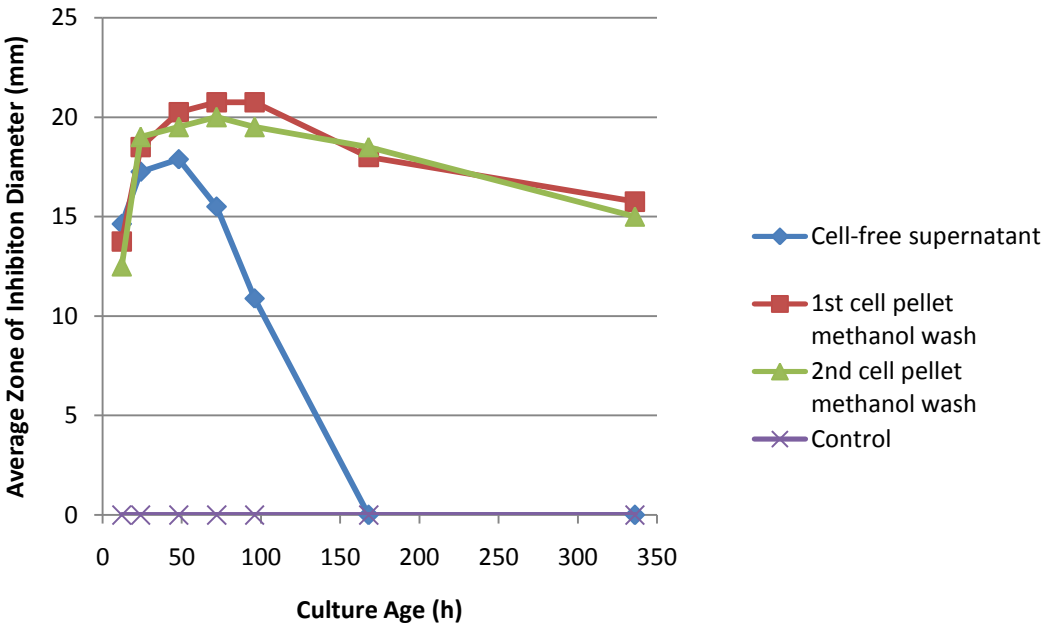


Figure 4.1. Antibacterial activity associated with cell-free supernatant and cell pellet methanol extract derived from a culture of *Photorhabdus* Isolate 22 against *B. subtilis*.

4.2.3. Extraction of antibacterial compounds from *Photorhabdus* Isolates 91, 161 and 173

Isolates 91, 161 and 173 were selected for further study based on their antibacterial activity in the initial screening and as representatives of genetically diverse isolates (Chapter 3). Fungal test organisms were not included in this assay due to low antifungal activity in the initial screening bioassays. Various methods of active compound extraction and purification were assessed. Resultant extracts were assayed against *M. luteus* and *B. subtilis* using a disc-diffusion assay.

4.2.3.1. Ethyl acetate extraction of cell-free broth supernatant

Ethyl acetate fractions from each *Photorhabdus* isolate all showed activity against the bacterial test species (Table 4.6). Generally, *M. luteus* showed a higher level of sensitivity than *B. subtilis.* The extracts showed an apparent concentration effect whereby higher levels of activity were associated with the ethyl acetate extract in comparison to the cell-free supernatant before extraction. Isolate 91 was an exception whereby the ethyl acetate fraction exhibited reduced activity against *M. luteus*. For all three isolates some degree of antibacterial activity was retained in the aqueous phase even after two washes (Table 4.6). Anomalously the extracted supernatant of Isolate 173 appears more active against both test organisms than the unextracted broth control. A similar result was observed for Isolate 91 against *B. subtilis*. No antibacterial activity was evident in the sterile tryptic soy broth (TSB) control extraction.

Table 4.6. Antimicrobial activity associated with cell-free supernatant from *Photorhabdus* isolates cultured in TSB before and after ethyl acetate liquid-liquid extraction.

	Micrococcus luteus			***Bacillus subtilis***		
	Diameter of inhibition zone (mm)					
Sample	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**
91 EthAc	19 (±1.82)	20 (±1.31)	20 (±0.85)	11.5 (±0.58)	12 (±0.63)	12 (±0.41)
91 ExS	17 (±0.82)	16.5 (±0.58)	16 (±0.63)	18 (±0.25)	19 (±0.5)	18 (±1.26)
91 CFS	22.5 (±1.29)	21.5 (±1.68)	17 (±1.03)	*	*	*
161 EthAc	20 (±0.62)	19 (±1.11)	18 (±0.63)	14 (±0.82)	12.5 (±0.58)	12.5 (±1.29)
161 ExS	15.5 (±0.58)	13 (±0.82)	*	*	*	*
161 CFS	17 (±0.65)	15 (±0.41)	14.5 (±0.58)	*	*	*
173 EthAc	20 (±0.82)	20 (±0.82)	20 (±0.25)	15 (±0.5)	13.5 (±1.29)	14 (±0.85)
173 ExS	18 (±0.82)	15 (±0.82)	*	12.5 (±0.48)	*	*
173 CFS	14.5 (±1.29)	13.5 (±0.91)	13 (±0.25)	*	*	*
TSB EthAc	-	-	-	-	-	-

* = Weak antibacterial activity; - = no inhibition visible; EthAc = Ethyl acetate fraction concentrated in methanol; ExS = Extracted supernatant; CFS = Cell free supernatant before extraction; 48 h, 96 h and 168 h = Bioassay observations time intervals (hours); Data presented as an average of duplicate assays; Standard deviation in parenthesis

4.2.3.2. Microplate dilution assay of ethyl acetate extracts from Isolates 91, 161 and 173

The results for a microplate assay investigating the effects of dilution on antibacterial activity of ethyl acetate extracts from Isolates 91, 161 and 173 is presented in Plate 4.1. Bacterial growth inhibition was determined by visual examination of wells; the conversion of triphenyltetrazolium chloride (TTC) to red triphenylformazan was considered an indicator of metabolising cells (Burdock *et al.,* 2011; Eloff, 1998). Isolate 173 yielded the highest antibacterial activity with inhibition of *M. luteus* demonstrated at 1 : 40 dilution and in a single replicate against *B. subtilis* at this dilution. The extract from Isolate 161 inhibited bacterial growth at dilutions up to 1 : 20 against *B. subtilis* and *M. luteus*. The initial dilution of extract (1 : 10) from Isolate 91 did not demonstrate activity against *B. subtilis* and did not completely inhibit *M. luteus;* although a lower colour intensity was evident in these wells compared to the control wells. The extract-free controls for both *B. subtilis* and *M. luteus* showed consistent growth, whereas the sterile broth control showed that none of the test wells were influenced by medium constituents. The control treatment comprising a dilution of pure methanol also showed no influence on viability of *B. subtilis* or *M. luteus*. This indicated that methanol at the concentration present in the antibiotic extracts was not responsible for observed inhibition.

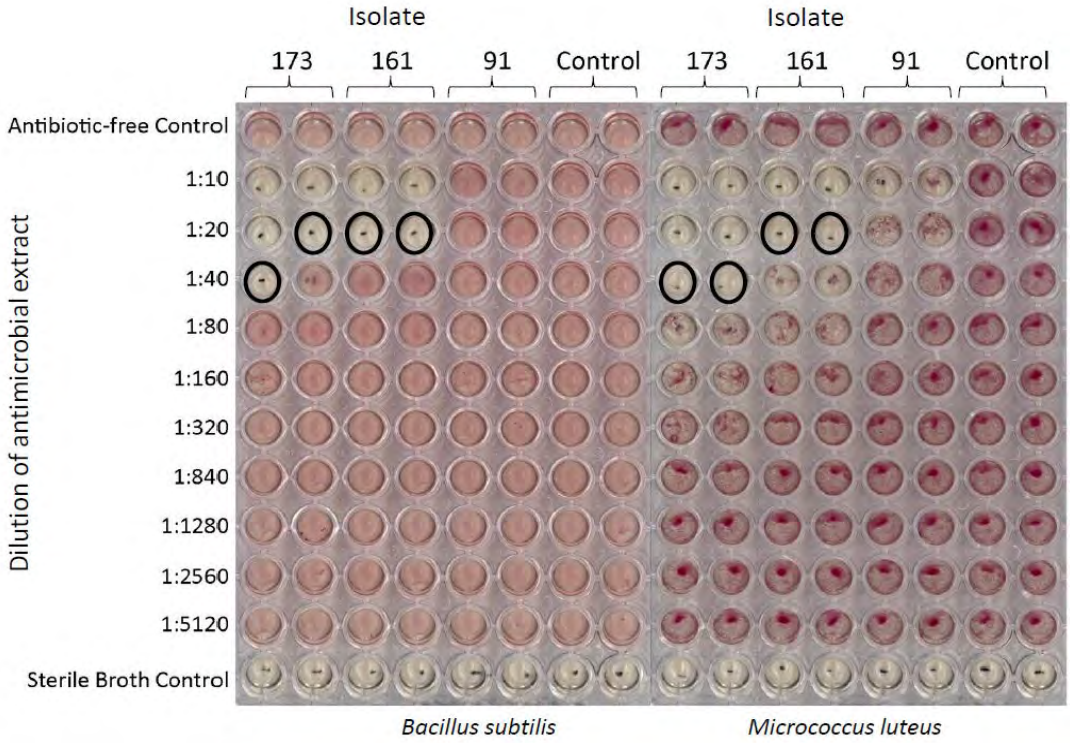


Plate 4.1. 96-well microplate assay demonstrating the effects of dilution on antimicrobial extracts from *Photorhabdus* Isolates 91, 161 and 173 against *M. luteus* and *B. subtilis*. Circled wells indicate the highest dilution exhibiting complete inhibition.

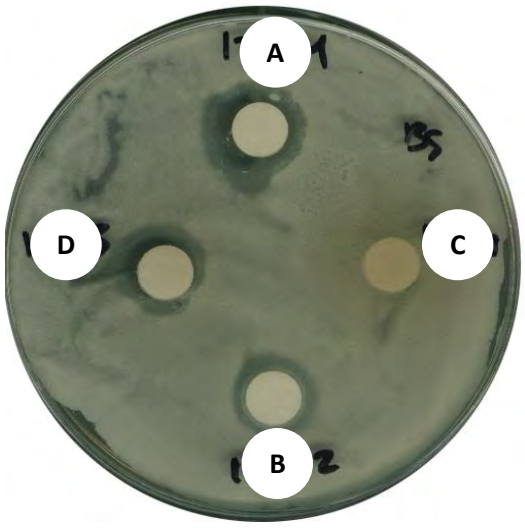


Plate 4.2. Methanol extraction of lyophilised TSB broth for Isolate 173 after 168 h incubation, where: (A) is the first methanol extraction of the wash of the lyophilised broth supernatant, (B) shows the second methanol wash, (C) the remaining lyophilised broth resuspended in water after 168 h incubation, and (D) the broth supernatant pre-extraction.

4.2.3.3. Methanol extraction of lyophilised cell-free broth supernatant

Antibacterial activity was detected in the methanol extract from lyophilised broth culture and is presented in Table 4.7. Successive extractions with methanol resulted in decreasing levels of antibacterial activity. No activity was detected in aqueous suspensions of the extracted lyophilised broth for Isolates 161 and 173, whereas activity was still evident in the aqueous fraction of Isolate 91.

Table 4.7. Antimicrobial activity associated with a methanol extraction of lyophilised cell-free supernatant from *Photorhabdus* isolates cultured in TSB.

	Micrococcus luteus		***Bacillus subtilis***	
	Diameter of inhibition zone (mm)			
Sample	**48 h**	**168 h**	**48 h**	**168 h**
91 1st extraction	15(±1.41)	12.5(±2.12)	12(±0.0)	12(±0.0)
91 2nd extraction	11.5(±0.71)	*	-	-
91 aqueous	12(±0.0)	11.5(±0.71)	13.5(±0.71)	14(±0.0)
91 broth	16(±1.41)	14(±0.71)	17(±0.71)	14(±0.71)
161 1st extraction	14(±0.0)	*	11.5(±0.71)	11.5(±0.71)
161 2nd extraction	12(±0.0)	*	-	-
161 aqueous	-	-	-	-
161 broth	19(±0.71)	17(±1.41)	16(±0.71)	15(±1.41)
173 1st extraction	16.5(±0.71)	14.5(±0.71)	15.5(±0.71)	14.5(±0.71)
173 2nd extraction	13.5(±0.71)	11.5(±0.71)	11(±0.0)	*
173 aqueous	-	-	-	-
173 broth	20(±1.41)	18(±1.41)	19(±0.71)	16(±1.41)
Methanol control	-	-	-	-

* = Weak antibacterial activity; - = No inhibition visible; 1st Extraction = First wash of lyophilised broth with methanol; 2nd Extraction = Second wash of lyophilised broth with methanol; Aqueous = Lyophilised remainder re-dissolved in water; Broth = Cell-free broth supernatant prior to extraction; 48 h and 168 h = Bioassay observation time intervals (hours); Methanol control = Pure methanol control; Data presented as an average of duplicate assays with standard deviation in parenthesis.

Antibacterial activity decreased with successive methanol extractions of lyophilised TSB broth from Isolate 173, with the 1st extraction (Plate 4.2.A) showing greater activity than the second (Plate 4.2.B). Evidence of weak antibacterial activity is noted in Plate 4.2.B with growth over the initial inhibition zone (>168 h). Also evident is the absence of activity in the remaining broth residue after being resuspended in water (Plate 4.2.C). These trends were also observed for the extracts from Isolates 91 and 161.

After two successive methanol extraction steps, the lyophilised broth of Isolate 91 retained residual antibacterial activity when resuspended in water. Subsequent fractionation of the aqueous broth suspension was attempted using Sep-Pak Plus C18 syringe cartridge eluted with increasing concentrations of methanol. Disc-diffusion assays were performed to assess fractions for antibacterial activity (Table 4.8). Antibacterial activity was associated with the eluent resulting from the initial loading of the Sep-Pak column as well as in the first elution with pure water. No activity was noted in any of the methanol elutions hence it appears that the active fraction was not retained in the column, and passed through the column either during loading or in the first elution with water.

Sep-Pak Plus C18 syringe cartridges were used to separate and partially purify the active fractions of methanolic extractions of lyophilised cell-free supernatant from Isolates 91, 161 and 173. Samples of column eluent were assayed using the standard disc-diffusion assay against *M. luteus* and *B. subtilis* (Table 4.9). Activity for all isolates was predominantly associated with the 100% methanol eluent. In all three *Photorhabdus* isolates, there was evidence of minor antibacterial effect with the initial water wash of the loaded column against *M. luteus*; and against *B. subtilis* for Isolate 91. No activity was noted in the 60% (v/v) methanol : water eluent in all cases.

Table 4.8. C18 Sep-Pak separation of active fractions remaining in lyophilised broth after methanol extraction of Isolate 91.

	Micrococcus luteus			*Bacillus subtilis*		
	Diameter of inhibition zone (mm)					
Eluent Water : Methanol	**48 h**	**96 h**	**168 h**	**48 h**	**96 h**	**168 h**
Sample eluent	14.5(±0.71)	14.5(±0.71)	14(±0)	14(±0)	13(±0)	13(±0)
100 : 0	*	*	*	13(±0)	12(±0)	12(±0.71)
80 : 20	-	-	-	-	-	-
60 : 40	-	-	-	-	-	-
40 : 60	-	-	-	-	-	-
20 : 80	-	-	-	-	-	-
0 : 100	-	-	-	-	-	-

* = Weak antibacterial activity; - = No inhibition visible; 0–100 = Sequential elutions with water: methanol applied in increasing concentration (percentage v/v); 48 h, 96 h and 168 h = Bioassay observations time intervals (hours); Data presented as an average of duplicate assays with standard deviation in parenthesis.

Table 4.9. Activity present in methanol extracts of lyophilised broth after separation using a C18 Sep-Pak syringe column protocol.

		Micrococcus luteus		*Bacillus subtilis*	
		Diameter of inhibition zone (mm)			
Isolate	**Eluent Water : Methanol**	**48 h**	**168 h**	**48 h**	**168 h**
91	Sample eluent	*	0	*	0
	100 : 0	*	0	0	0
	40 : 60	0	0	0	0
	0 : 100	13(±1.41)	11.5(±0.71)	12(±0.0)	11(±0.0)
161	Sample eluent	*	0	0	0
	100 : 0	*	0	0	0
	40 : 60	0	0	0	0
	0 : 100	14.5(±0.71)	*	11.5(±0.71)	11(±0.0)
173	Sample eluent	*	0	0	0
	100 : 0	*	*	0	0
	40 : 60	0	0	0	0
	0 : 100	16(±0.0)	14(±0.71)	14.5(±0.71)	13.5(±0.71)

* = Weak antibacterial activity; - = No inhibition visible; H_2O flush = Sample of water used to flush the column after sample loading; 100 : 0, 40 : 60 and 0 : 100 = Ratio of water to methanol elution ; Data presented as an average of duplicate assays with standard deviation in parenthesis.

4.2.4. TLC analysis of antibacterial extracts from *Photorhabdus* isolates

Results for the TLC analysis of antibiotic extracts from Isolate 22 cell pellet material are shown in Plate 4.3 and Table 4.10. The chloroform : methanol mobile phase was found to be more effective at separating the extract constituents than isopropanol : water; producing better fraction separation as well as resolving a higher number of bands (Plate 4.3). The findings also showed that the 24 h sample resolved a greater number of distinct bands than the 168 h sample which produced an indistinct smear when the chloroform : methanol mobile phase was used.

Table 4.10. R_f values for TLC bands resolved from an Isolate 22 cell pellet extract as detected under UV light.

1.5 : 98.5 Chloroform : Methanol		50 : 50 Isopropanol : Water	
24 h	**168 h**	**24 h**	**168 h**
0.94		0.88	
0.82	0.82	0.84	0.84
0.52		0.81	0.81
0.37			
0.33			

Based on the results from TLC of Isolate 22 the chloroform : methanol mobile phase mobile phase was chosen for subsequent TLC assays. Results from the TLC separations of extracts from Isolates 91, 161 and 173 are shown in Plate 4.4 and Table 4.11. For Isolate 91, each of the extraction techniques evaluated yielded bands with similar R_f values. The exception being the methanol extraction from cell pellet which lacked bands corresponding to R_f 0.78 and R_f 0.74. For Isolates 161 and 173 the ethyl acetate extract and methanol extraction from cell pellet produced banding profiles with very similar R_f values. The methanol extract of lyophilised broth of Isolate 161 included an additional band at R_f 0.78 but lacked bands at approximately

R_f 0.31 and 0.23. Similarly, the methanol extract of lyophilised broth of Isolate 173 also resolved a band at R_f 0.78 and lacked bands corresponding to R_f 0.84 and 0.30.

Table 4.11. R_f values for TLC bands separated from Isolates 91, 161 and 173 extracts.

Isolate 91			Isolate 161			Isolate 173		
EthAc	**MeL**	**MeP**	**EthAc**	**MeL**	**MeP**	**EthAc**	**MeL**	**MeP**
0.78	0.77		0.88	0.87	0.86	0.88	0.88	0.88
0.75	0.73		0.84	0.84	0.84	0.84		0.85
0.42	0.40	0.46		0.78			0.78	
0.14	0.14	0.17	0.52	0.48	0.48	0.47	0.47	0.50
			0.31		0.30	0.29		0.34
			0.25		0.22			
					0.14			

EthAc = Ethyl acetate extraction of cell-free broth supernatant; MeL = Methanol extraction of cell-free lyophilised broth supernatant; MeP = Methanol extraction of cell pellet material.

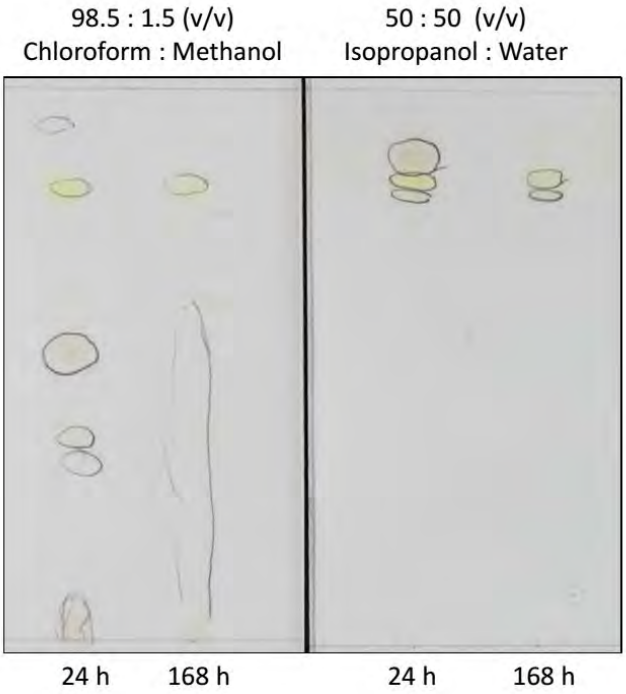


Plate 4.3. TLC plates of cell pellet methanol extracts of Isolate 22 performed with 98.5 : 1.5 (v/v) chloroform : methanol and 50 : 50 (v/v) isopropanol : water mobile phase.

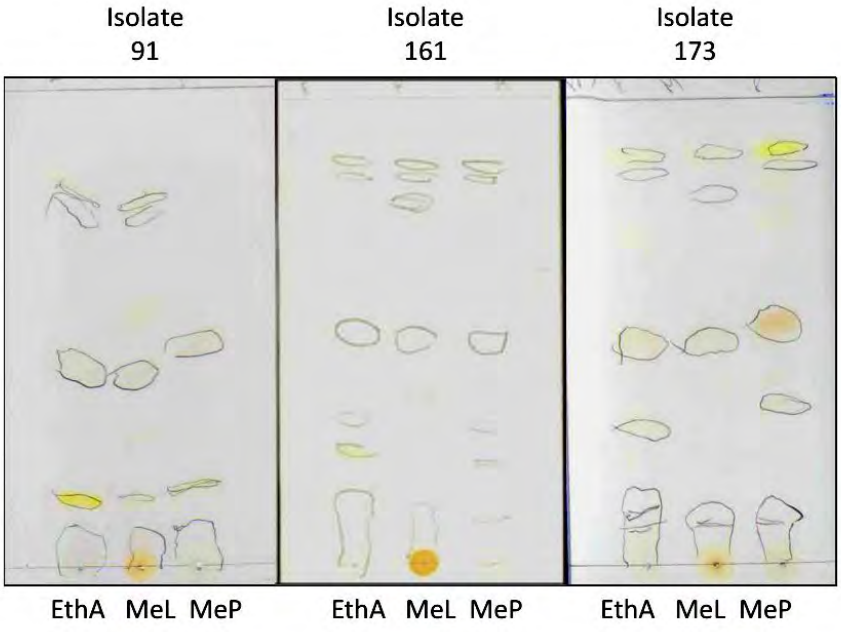


Plate 4.4. TLC plates of ethyl acetate extracts (EthA), methanol extracts of cell-free lyophilised broth (MeL) and cell pellet methanol extracts (MeP) from Isolates 91, 161 and 173 developed with 98.5 : 1.5 chloroform : methanol mobile phase.

Disc-diffusion bioassays were performed to assess antibacterial activity of different TLC fractions of ethyl acetate extracts of cell-free supernatant from Isolates 91, 161 and 173 (Table 4.12). Activity for Isolates 161 and 173 were primarily associated with ultraviolet (UV) visible bands corresponding to R_f 0.52/0.47 and 0.31/0.29. For Isolate 91 only the UV visible band at R_f 0.46/0.42 exhibited some evidence of weak antibacterial activity against *M. luteus*. Activity for Isolates 161 and 173 was observed primarily against *M. luteus* with limited activity against *B. subtilis* being evident. Isolates 161 and 173 both resolved similar banding patterns and R_f values; a similar band profile was obtained for Isolate 91 but at lower R_f values for each band resolved.

Table 4.12. Disc-diffusion bioassays used to detect antimicrobial activity in ethyl acetate extracts from *Photorhabdus* Isolates 91, 161 and 173.

Isolate	R_f	Observation (UV)	Inhibition Zone (mm)					
			Micrococcus luteus			*Bacillus subtilis*		
			48 h	96 h	168 h	48 h	96 h	168 h
91	0.78	Yellow	-	-	-	-	-	-
	0.75	Orange	-	-	-	-	-	-
	0.42	Purple	*	-	-	-	-	-
	0.14	Orange/Purple	-	-	-	-	-	-
	0	Purple	-	-	-	-	-	-
161	0.88	Yellow	-	-	-	-	-	-
	0.84	White	-	-	-	-	-	-
	0.52	Purple	17.5	16.5	16	*	*	*
	0.31	Dark purple	15.5	14.5	15	*	*	*
	0.25	White	-	-	-	-	-	-
	0	Purple	-	-	-	-	-	-
173	0.88	Yellow	-	-	-	-	-	-
	0.84	White	-	-	-	-	-	-
	0.47	Purple	19.5	19	*	10.5	*	*
	0.29	White	11.5	*	*	-	-	-
	0	Purple	-	-	-	-	-	-

* = Weak antibacterial activity; - = No inhibition visible

4.2.5. ESI-TOF-MS analysis of antibacterial extracts from *Photorhabdus* isolates

Antibacterial fractions derived from ethyl acetate extracts and methanol extracts of lyophilised broth of Isolates 91, 161 and 173 were analysed using UPLC-ESI-TOF-MS. (Figure 4.2 and 4.3). The UPLC chromatograms for each ethyl acetate extract displayed very similar profiles comprising multiple peaks (Figure 4.2). A dominant peak resolved at ~26.6 minutes was common to all extracts. The smaller peaks also displayed similar distributions between the isolates. The ESI mass spectra determined for each of the common peaks eluted at 3.95, 4.8, 10.85, 17.15, 19.8, 24.7 and 26.6 minutes were highly similar between isolates and extraction techniques (Appendix 1). The ESI mass spectra for the peaks resolved at 26.6 minutes were identical for all three isolates, and is shown for Isolates 161 and 91 in both positive and negative ion modes (Figures 4.4 and 4.5).

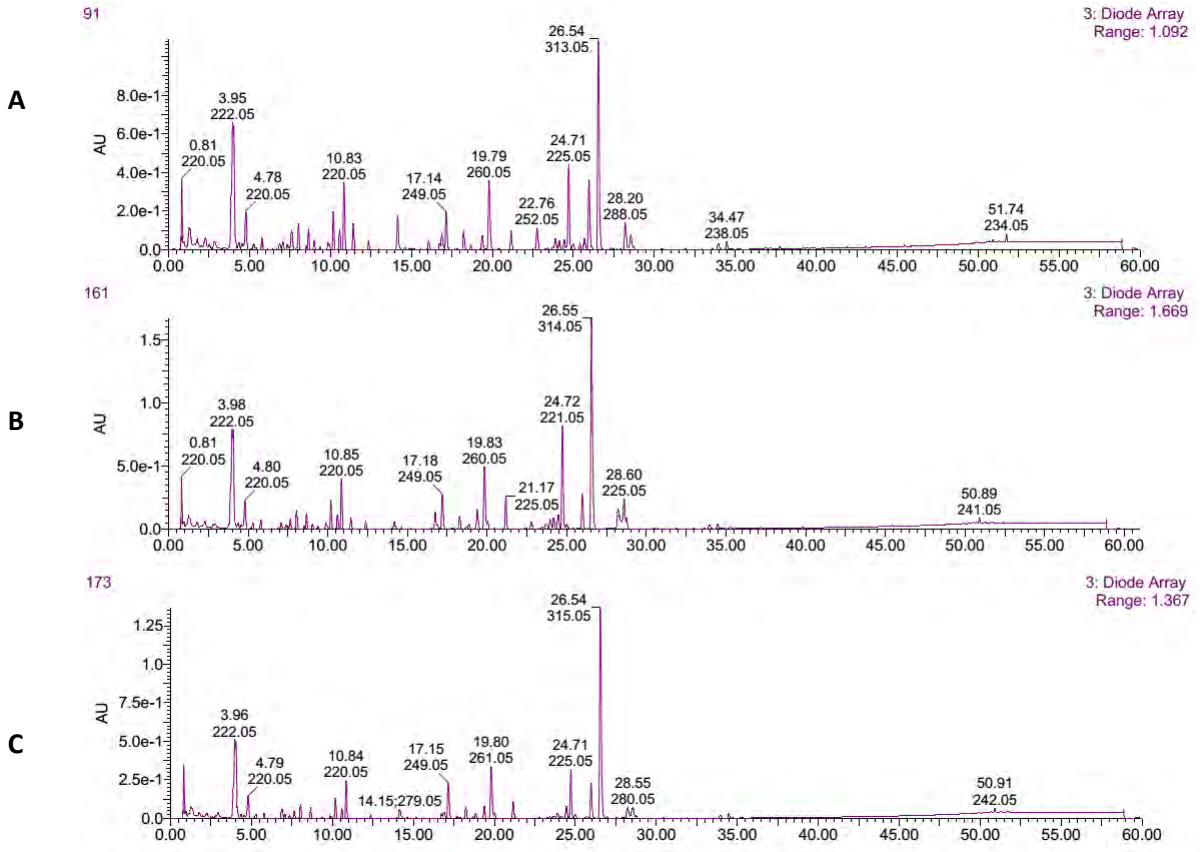
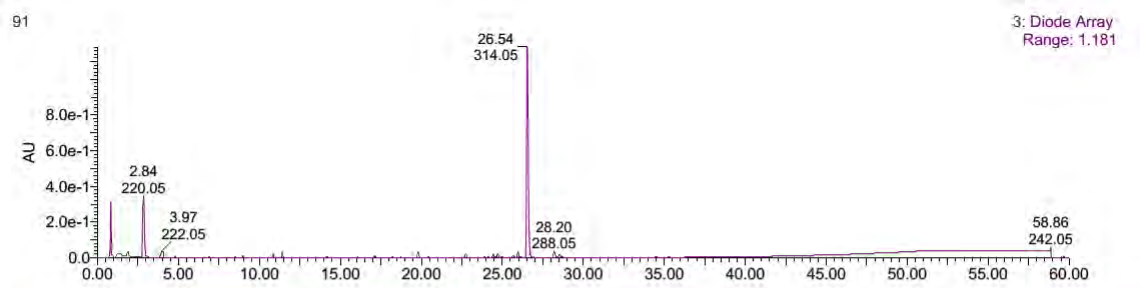


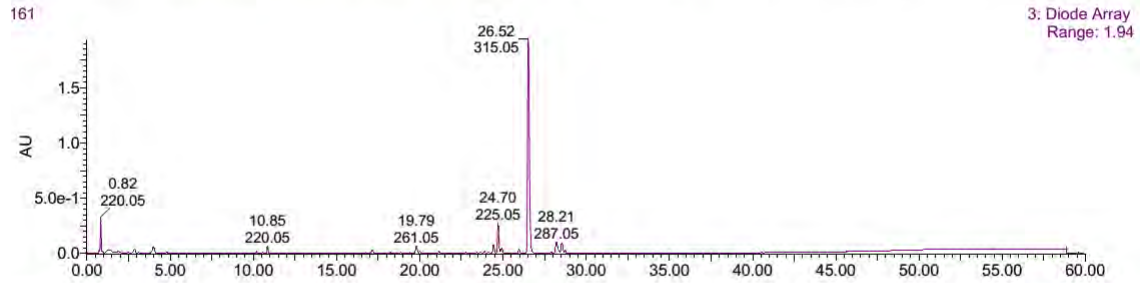
Figure 4.2. UPLC trace of ethyl acetate extractions of supernatant from cultures of Isolates 91 (A), 161 (B) and 173 (C).

Chromatograms for the partially purified methanol extracts displayed very similar profiles with a single dominant peak being resolved at 26.6 minutes (Figure 4.3). The ESI mass spectra for this peak was identical for all three isolates and matched the spectra of the equivalent peaks resolved from the ethyl acetate extracts (Figure 4.4 and 4.5). Several minor peaks were evident in the partially purified methanol extract chromatogram, however these were greatly diminished compared to those present in the ethyl acetate extracts (Figure 4.2). The mass spectrum of the dominant peak (~26.6 minutes) revealed the presence of major mass fragment with an approximate mass of 254 g/mol in both the negative and positive ESI modes. A secondary mass fragment of approximately 211 g/mol was also prominent in both ESI spectra (Figures 4.4 and 4.5).

A



B



C

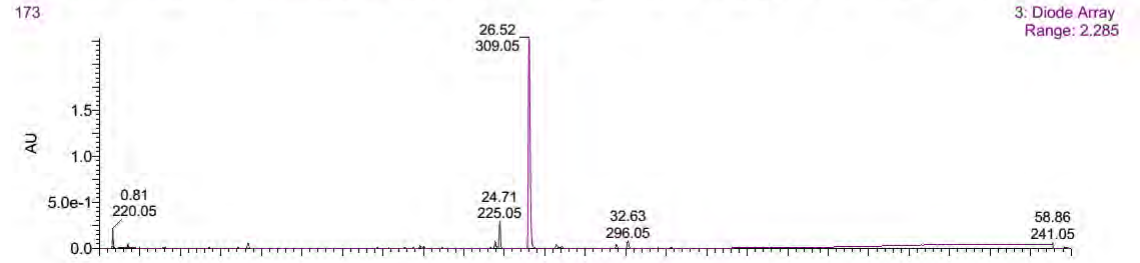


Figure 4.3. UPLC trace of lyophilised broth extractions cleaned with a Sep-Pak column of Isolates 91 (A), 161 (B) and 173 (C).

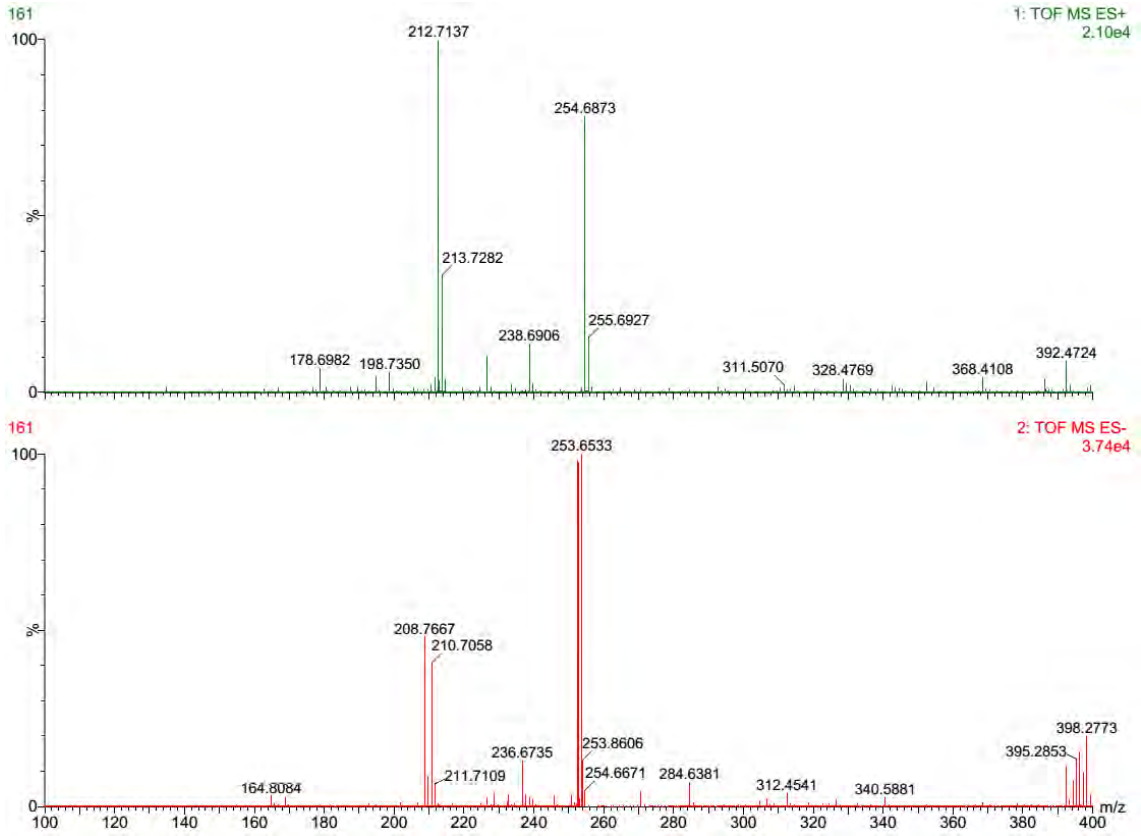


Figure 4.4. Mass spectra of the dominant UPLC peak (~26.6 minutes) resolved from an ethyl acetate extract of Isolate 161 culture supernatant.

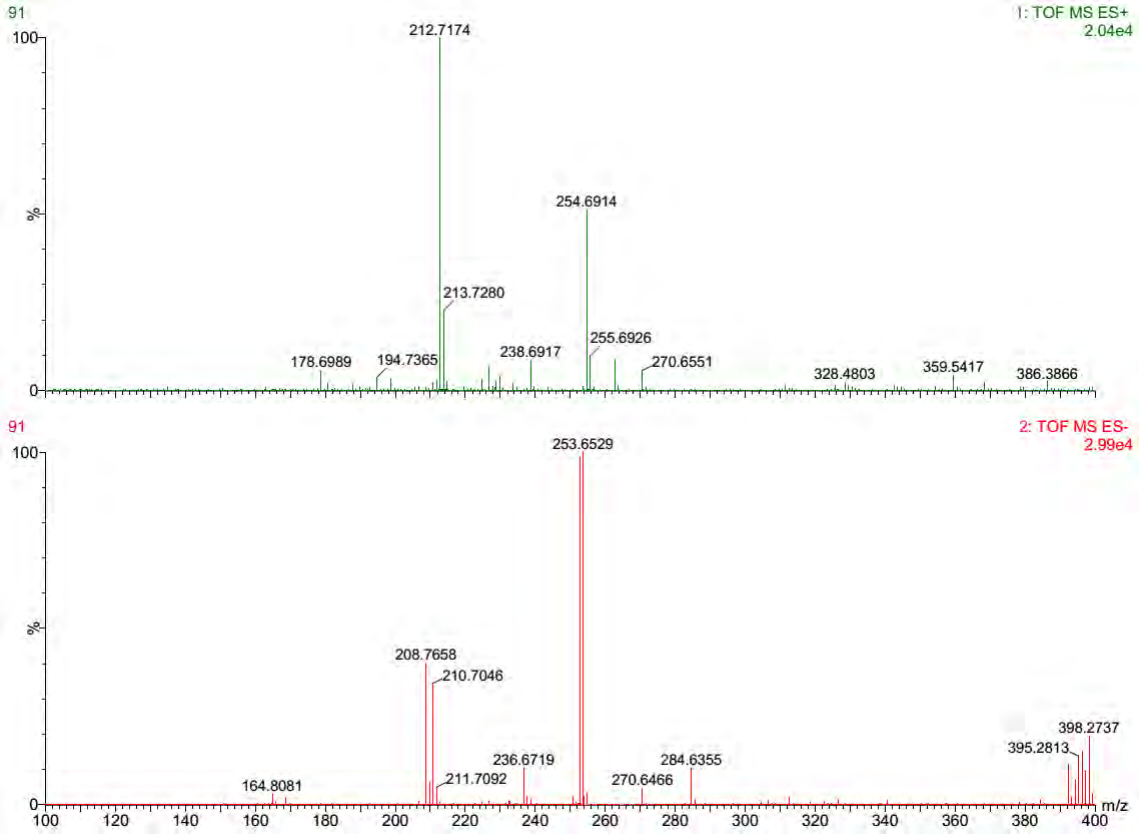


Figure 4.5. Mass spectra of the dominant UPLC peak (~26.6 minutes) resolved from methanol extract of lyophilised broth from Isolate 91.

4.2.6. ESI-TOF-MS and GC-MS analysis of anthraquinone and trans-stilbene reference standards

UPLC chromatograms for anthraquinone and trans-stilbene reference standards are shown in Figure 4.6. Both standards produced a single dominant peak, with anthraquinone appearing at 26.7 minutes and trans-stilbene at 35.5 minutes. A few minor peaks of low intensity are noted, predominantly within the anthraquinone trace. Neither of the dominant peaks produced spectra of the anticipated mass fractions in either the positive or negative ion mode.

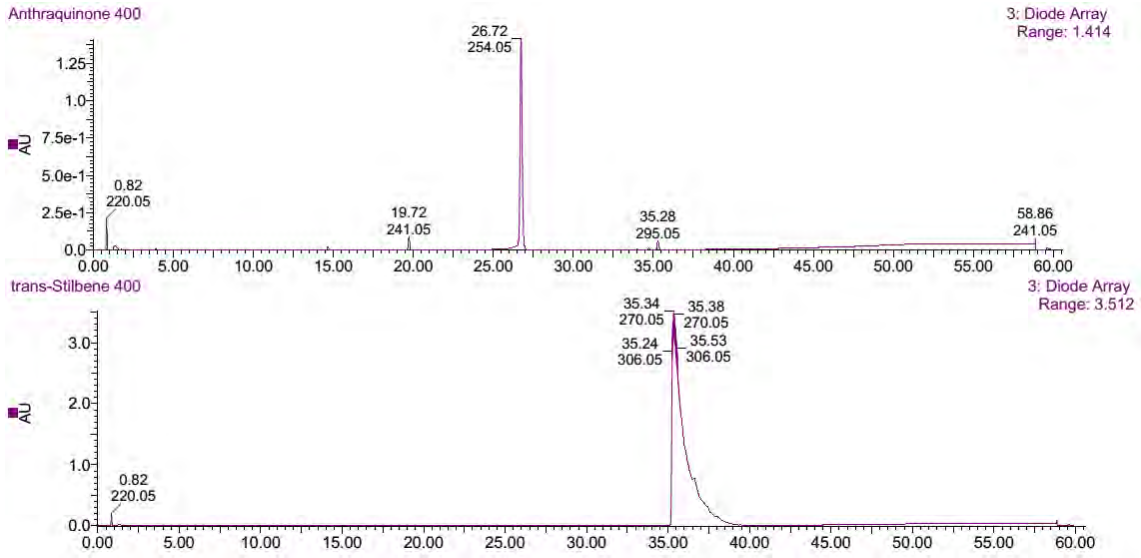


Figure 4.6. UPLC trace of anthraquinone and trans-stilbene reference standards.

The two reference standards were also included in GC-MS analysis (Figure 4.7). The mass spectra of anthraquinone corresponded to the expected spectra in the NIST Mass Spectral Search Program database (Version 2.0 d, 2005) with a major peak of 208 g/mol and subsequent peaks at 180 g/mol and 152 g/mol. Data for the expected mass spectra for trans-stilbene is absent from the database, however the dominant peak detected corresponds to the molecular mass of trans-stilbene of 180.25 g/mol (Sigma Aldrich). The GC-MS of the ethyl acetate extracts of Isolates 91, 161 and 173 as well as the partially purified methanol extracts from lyophilised broth supernatant produced no detectable mass spectra.

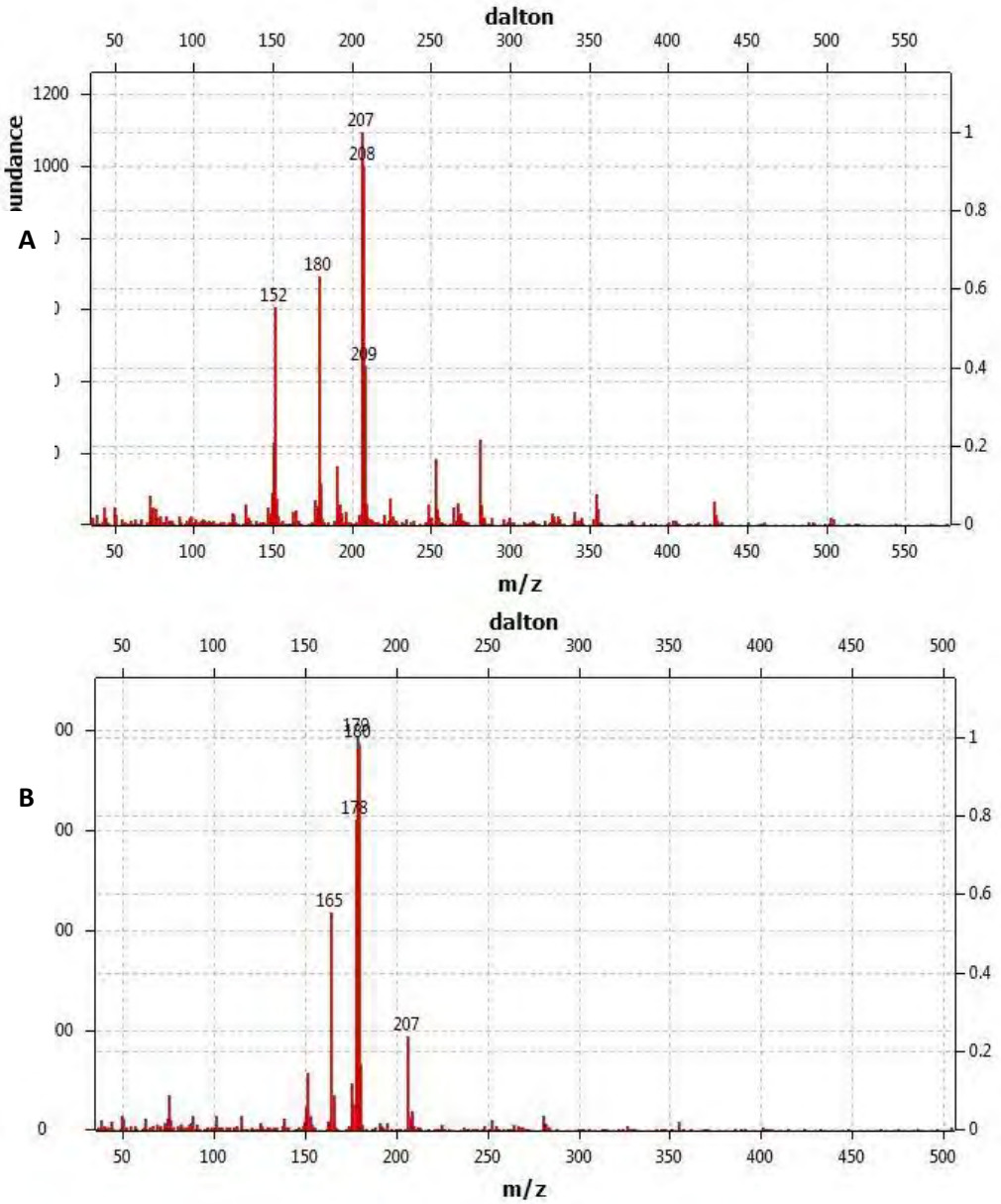


Figure 4.7. GC-MS Mass spectra of anthraquinone (A) and trans-stilbene (B) reference standards.

4.3. Discussion

Photorhabdus spp. are known to produce a range of compounds that exhibit antimicrobial and/or insecticidal activity (Piel, 2004). Stilbenes and anthraquinones are the most commonly reported bioactive compounds produced by *Photorhabdus* spp. (Bode, 2009; Boemare and Akhurst 2005; Piel, 2004; Hu and Webster, 2000; Hu *et al.,* 1998). In this study, preliminary assessment of the antimicrobial potential present within a subset of South African *Photorhabdus* isolates was conducted using *in vitro* disc-diffusion bioassays against selected bacterial and fungal test organisms. These bioassays evaluated activity in cell-free culture supernatant and methanol extractions of cell pellets. Initial analyses of antimicrobial compounds produced over the course of a two-week incubation was carried out with Isolate 22 in order to optimise appropriate extraction procedures. Three isolates (*viz*. Isolates 91, 161 and 173) were selected for compound extraction and assay using ethyl acetate extraction of broth supernatant as well as methanolic extractions of lyophilised broth supernatant. Purification of methanolic extractions of lyophilised broth were performed using C18 Sep-Pak syringe columns. Antimicrobial extracts derived from ethyl acetate extracts and extracts from lyophilised broth underwent analysis using TLC, UPLC-ESI-TOF-MS and GC-MS.

4.3.1. Antimicrobial screening bioassays

All isolates demonstrated activity in disc-diffusion bioassay against *M. luteus* and *B. subtilis*; excepting Isolate 165, which showed little to no activity against either of these species. Isolate 178 was the only isolate to demonstrate any activity against *E. coli*. Isolates 165 and 178 were identified as genera other than *Photorhabdus* (Chapter 3), which accounts for the disparity in their antimicrobial activity compared to the other isolates. The antibiotic activity of cell-free culture media was most prominent in samples taken after 1 or 2 days of incubation. A decrease in antibacterial activity was noted in the samples taken from one-week old broth, particularly against *B. subtilis*. These observations were attributed to the growth stage at which the biosynthesis of the active compounds occurs, or possibly due to progressive instability of the active metabolites produced (Hu *et al*., 1999; Li *et al*., 1995).

Photorhabdus antibiotics have been previously reported to show activity against both Gram positive and negative bacteria; however activity against Gram positive bacteria is more commonly observed (Boemare, and Akhurst, 2006). The findings from this study were in agreement with these observations where antibacterial activity was evident exclusively against the Gram positive bacterial species evaluated. Zones of inhibition measured in the bioassays were generally larger for *M. luteus* than *B. subtilis,* suggesting the former organism is more sensitive to the compounds produced by these *Photorhabdus* isolates. Inhibition zones produced by the two type strains followed a similar pattern to the isolates screened but were generally smaller in diameter. To our knowledge studies identifying compounds produced by either of the reference strains have not been published. Over the course of the disc-diffusion bioassays zones of inhibition decreased in diameter often becoming overgrown. This weakening antibacterial effect was attributed to various factors such as the degradation or diffusion of the active compounds resulting in below-active concentrations, or the ability of the test organisms to overcome the inhibitory effects of these compounds.

In most instances activity against *M. luteus* and *B. subtilis* was noted in both cell-free supernatants and methanol extractions of the *Photorhabdus* cell pellet (Tables 4.1 and 4.2). Exceptions include Isolate 91 against *M. luteus*; and Isolates 66, 170 and 180 against *B. subtilis*. This suggests that active compounds were released into the broth media but a portion is retained within the bacterial biomass. Reasons for this could include an antimicrobial compound that is sparingly soluble in the broth media but retaining higher solubility in cellular components, or the presence of two or more compounds exhibiting different solubilities.

Interestingly, all isolates with the exception of Isolates 66, 170 and 180 demonstrated antibacterial activities against *B. subtilis* in methanol extracts derived from cell pellets after 7 d culture, whereas the corresponding cell-free broth supernatant showed weak or no activity (Table 4.2). A similar pattern of antimicrobial activity was also noted against *M. luteus* with greater activity apparent in the methanol extract from 7 d cell pellets compared to the corresponding cell-free supernatant (Table 4.1). In contrast, Isolate 178 (*Xenorhabdus* sp.) is the notable exception to this with activity detected in the 7 d broth culture supernatant but not cell pellet methanol extract. These findings suggest that after one week of growth the

active compounds are primarily found in association with cell matter and their concentrations were diminished in the broth medium itself. This should correspond to late stationary or death phase in the culture. Evidence for this hypothesis is supported by Isolate 22 (Table 4.5 and Figure 4.1) where a clear decrease in the presence of antibacterial metabolites in the broth supernatant was noted, yet a high level of activity is retained in the cell pellet material. This may be due the degradation of a broth-soluble compound while a cell-material associated compound retained activity, or a change in the broth chemistry resulted in a lower solubility of an antimicrobial compound. Broth pH was noted to increase after several days of incubation to > 9.0 (data not shown) and it is possible this is responsible either for the change in solubility or compound degradation.

Antibiosis against the fungal species *B. cinerea* and *R. solani* was not conclusively established for any of the *Photorhabdus* isolates screened. Isolate 165 (*Pseudomonas* sp.) was the only isolate to demonstrate clearly defined inhibition of both fungi (Tables 4.3 and 4.4). Although conclusive inhibition was not observed, extracts from most isolates induced morphological changes in both fungal species that were visible to the naked eye. It is unclear whether these responses were fungistatic due to low concentration of antifungal compounds, or due to the effects of media components in the extract. Media components present in the broth supernatant could be responsible for some of the morphological changes and the appearance of pink colouration on the *B. cinerea* assay as this is predominantly associated with the broth supernatant bioassays. However a similar pattern of morphological changes between broth supernatant and cell pellet extract is not observed with regards to “browning” in *R. solani*. Antifungal compounds belonging to the stilbene class of compounds are known to be produced by strains of *Photorhabdus* (Boemare, and Akhurst, 2006). However, the apparent low sensitivity of the fungal test organisms to either treatment suggests that, if synthesised, these compounds were most likely not produced at sufficient concentrations to affect inhibition of fungal growth using this bioassay method.

4.3.2. Antibacterial compound extractions from Isolates 91, 161 and 173

Isolates 91, 161 and 173 were chosen for further characterisation of antibacterial compounds produced on the basis of their phenotypic diversity, antimicrobial activity and DNA fingerprinting profiles (Chapter 3). Extraction of antimicrobial compounds using ethyl acetate is a commonly-used technique that has been successfully applied to the extraction and concentration of active compounds from *Photorhabdus* spp. and hence was used in this study (Eleftherianos *et al.,* 2007; Hu *et al*., 1997; Li *et al.,* 1995; Richardson *et al.,* 1988). Additionally, an alternative methanol extraction technique was assayed which utilised lyophilisation to concentrate active metabolites.

4.3.2.1. Evaluation of antimicrobial activity of ethyl acetate extract of cell-free broth supernatant

Broth supernatant extraction using ethyl acetate was considered successful in that antimicrobial activity was noted in the final extract (Table 4.6). However, significant activity was still retained in the extracted broth after two washes. The levels of activity associated with the extracts are also lower than expected. If a 100% extraction efficiency had been achieved the concentration of the original broth supernatant (200 mL into 10 mL) would have resulted in a 20-fold increase in activity. This lowered activity may in part be attributed to the inefficiency of the extraction, and may have also resulted from degradation of active compounds during evaporation at an elevated temperature (45 °C). Activity in the extracted broth was found to be less than that of the pre-extracted broth, except in the case of Isolate 173 where increased activity was evident after the extraction step. Whilst this result is unexpected, it is possible that traces of ethyl acetate (solubility of 80 g/L in water) still remained in the extracted broth and a synergistic effect was responsible for the observed inhibition of the test organisms (Banerjee, 1984). Isolate 173 also demonstrated the largest zones of inhibition, while Isolate 91 showed the smallest. Since the extraction protocol used was identical for each isolate this data indicates that Isolate 173 may either be producing

higher concentrations of an active compound, or produce a compound which is more active than those produced by the other two isolates.

A 96-well microplate dilution assay evaluated the antibacterial activity of ethyl acetate extracts against *B. subtilis* and *M. luteus* ranked Isolate 173 as the most antibacterial, followed by Isolate 161 and lastly Isolate 91 (Plate 4.1). This finding suggests that the extract from Isolate 173 either has higher concentrations of the antibacterial compound(s) or produces a more bioactive component. The extract from Isolate 91 demonstrated the lowest levels of activity showing no complete inhibition at the highest concentration. As observed in the disc-diffusion bioassays, *M. luteus* appeared to be the more sensitive of the two bacterial species. *Micrococcus luteus* showed partial inhibition effects indicated by lower intensity colouration in wells with lower than minimum inhibitory concentration (MIC) of the extract. In contrast *B. subtilis* was either completely inhibited or not visibly distinct from control wells. Determining the MIC of these extracts is difficult without quantification of the initial concentration of the extracted compound(s). Hence, comparison between isolates gives only basic information as to relative production and activity of active compound(s) in these extracts.

4.3.2.2. Evaluation of antimicrobial activity of methanol extraction of lyophilised cell-free broth supernatant

For Isolates 161 and 173 the methanol extraction of lyophilised broth was found to be efficient in that no antibacterial activity was evident in the post-extraction broth (Table 4.7). A second methanol wash of the lyophilised broth from Isolate 91 showed low antibacterial activity, indicating that the first methanol wash significantly reduced the available concentration of methanol-soluble antibacterial compounds. However, activity was still noted in the extracted lyophilised broth when resuspended in water. Interestingly, the activity of this resuspended extracted broth appears biased toward *B. subtilis*, which is in contrast to the previously observed trend of larger zones of inhibition noted against *M. luteus*. It is possible that this represents a separate antimicrobial compound insoluble in methanol but soluble in water.

An attempt to purify the active compound(s) remaining in the extracted broth of Isolate 91 using a C18 Sep-Pak column separation protocol proved unsuccessful (Table 4.8). Active compounds failed to bind to the C18 column and were eluted during priming of the column and/or during the first elution with water. No antibacterial activity was noted in any subsequent fraction when eluted with increasing concentrations of methanol. This suggests that the compound of interest is more soluble in a polar solvent such as water than in methanol. The fact that it did not adsorb onto the C18 column matrix suggested that the active compound is a polar molecule with low hydrophobicity (McDonald, 2001). In future studies separation of this active compound might be achieved by modifying the separation protocol, either by using a less hydrophobic column packing during reverse phase chromatography, or with normal phase chromatography by optimising separation parameters such as pH or choice of solvents (McDonald, 2001).

Clean-up of lyophilised broth methanol extracts was performed due to the likelihood of medium residue and non-active metabolites being co-extracted from the lyophilised broth supernatant. Methanol-extracted samples were run through a C18 column, and the active compound was associated with the 100% methanol eluted fraction (Table 4.9). Residual activity was noted for all isolates in the "waste" fraction from the column during loading and during the water elution of the column. However, this activity did not result in a clearly defined inhibition zone during subsequent bioassays, and was found to be significantly less active than the final elution sample or the extract prior to separation. The low levels of antibacterial activity can be attributed to either low concentrations of an active compound that did not bind to the column. Further purification and/or characterisation was not attempted due to material and time constraints. However this may represent a novel target for future study as the observed characteristics of the hydrophilic compound(s) associated with Isolate 91 do not correlate with previously described antibiotics produced by *Photorhabdus* spp.

4.3.3. TLC analysis of antimicrobial extracts

TLC analysis of extracts was undertaken with the aim to establish the presence of active antibacterial fractions and as a means of comparing extraction techniques and antimicrobial activity between selected *Photorhabdus* isolates. The chloroform : methanol mobile phase achieved better separation of constituent compounds in the *Photorhabdus* Isolate 22 extract than did the isopropanol : water mobile phase (Plate 4.3). This result is consistent with published findings of TLC separation of *Photorhabdus* extracts which specifically targeted a stilbene antibiotic from *Photorhabdus sp.* (Hu *et al.*, 1997). The banding profile obtained from extract taken from a 168 h old culture of Isolate 22 was indistinct owing to smearing, whereas the 24 h sample yielded discrete bands. Band smearing may be attributed to a greater diversity of compounds produced during the later phases of bacterial growth or due to the accumulation of degradation products. This correlates with a longer period of growth demonstrating an increased variety of compounds produced in extracts taken from 2 d and 5 d old infected insect cadavers (Hu *et al.*, 1997). In general, fewer bands were noted in the TLC plates from this study than had been previously reported by Hu *et al.* (1997). This may be due to the differences in compound production between TSB culture and infected insect cadavers. The fact that antibacterial activity of the 24 h sample was found to be greater than that of the 168 h sample (Table 4.5), suggests that the active fraction should appear at a higher intensity in the 24 h sample. In the chloroform : methanol mobile phase bands unique to the active 24 h extract include those at R_f 0.94, 0.52, 0.37 and 0.33 and are potential candidates as the active fraction (Table 4.10). In the isopropanol : water mobile phase a single band at R_f 0.88 is unique in the 24 h sample (Table 4.10). Loss of activity with time suggests that the active fractions produced may not be stable under the existing culture conditions or are degraded over time.

Comparisons of ethyl acetate extractions from broth culture, methanol extraction of lyophilised broth and methanol extraction from cell pellet material for Isolates 91, 161 and 173 revealed several common bands, with some variation in band profiles between each extraction technique (Table 4.11). However, the exact nature of these band differences observed on TLC between the three extraction methods cannot be determined without further analysis which was not possible for this study. The TLC band profiles of Isolates 161 and 173 are quite similar,

with the extracts of Isolate 91 showing the most differences. Antibacterial activity was associated with bands in the R_f 0.40–0.52 range for Isolates 91, 161 and 173 as well as R_f 0.29–0.31 for Isolates 161 and 173 (Table 4.12). Although Isolate 91 R_f values are different to Isolates 161 and 173, they show very similar patterns of resolution in terms of band number, activity, appearance and resolution. It is possible that the differences in R_f values are due to TLC run conditions rather than the physical properties of the extract constituents. Variations in R_f values between intra-laboratory runs is a possibility when performing TLC (Sherma, 2003). Bands exhibiting strong yellow colouration under visible and UV light were not found to be associated with antibacterial activity, however it is possible that these compounds represent anthraquinone pigments known to be produced by *Photorhabdus* spp. (Boemare and Akhurst, 2005).

For Isolates 161 and 173 antibacterial activity was also noted for the fraction resolved at approximately R_f 0.30, which suggests a second active fraction is present in these extracts. Interestingly, this fraction was not detected in the extracts from lyophilised broth for either isolate when TLC was performed, and was not detected in any extract from Isolate 91. The antibacterial activity associated with extracts from Isolate 91 was extremely weak when bioassayed. Hence, if this additional fraction was present in extracts from Isolate 91, it may have been present at concentrations too low to be detected by TLC.

TLC fractions were not taken forward to ESI-TOF-MS analysis due to difficulty in producing sufficient volume of extracts for analysis from the small TLC plates available. In addition the bands of interest were noted to brown quickly after plate air-drying, which raised concerns as to the possibility of compound degradation.

4.3.4. Mass spectrometry

In an attempt to identify and compare active compounds present in the extracts from each isolate, extract samples were analysed using UPLC-ESI-TOF-MS. Ethyl acetate extracts from all three isolates produced highly similar UPLC chromatograms, with a dominant peak being resolved at ~26.6 minutes (Figure 4.2). Several minor common peaks were also observed between isolates. Mass spectra for these minor common peaks, were found to be virtually identical between isolates and did not demonstrate size ranges consistent with either stilbene or anthraquinone antibiotics previously described for *Photorhabdus* (Boemare and Akhurst, 2006; Piel, 2004). UPLC-ESI-TOF-MS analysis of lyophilised broth methanol extracts after Sep-Pak purification also demonstrated highly similar UPLC chromatograms with relatively few peaks (Figure 4.3). This suggests that lyophilised broth extracts contained a less diverse range of compounds compared to the ethyl acetate extracts although the dominant peak was common between extractions. The absence of any other significant peaks from the lyophilised broth extraction suggests that this single peak represents a compound exhibiting antibacterial activity. As antibacterial activity was associated with both extraction methods and only this peak was common (26.6 minutes) it is likely that this represents an active compound. A comparison of the mass spectra generated at this peak for all six samples was found to be highly similar; suggesting that the active compound responsible for antimicrobial activity is the same for each isolate (Figures 4.4 and 4.5, Appendix 1). The mass associated with this compound is 254 g/mol, which corresponds to the expected mass of 3,5-dihydroxy-4-isopropyistilbene (ST), a stilbene antibiotic known to be synthesised by *Photorhabdus spp.* (Hu *et al*., 1997). This finding concurs with a recently published mass value for this compound ($[M+H]^+$ *m/z* 255) which was detected using ESI-TOF-MS in positive ion mode and is a known stilbene produced by *Photorhabdus* spp*.* under culture in TSB (Park and Crawford, 2015; Boemare and Akhurst, 2006; Hu *et al,.* 1998; Forst and Nealson, 1996).

ST previously isolated from *Photorhabdus* spp. has been described as having a TLC R_f of 0.59 using the 98.5 : 1.5 (v/v) chloroform : methanol mobile phase (Hu *et al.*, 1997). When assayed the most active fraction of Isolates 91, 161 and 173 was noted at an R_f of 0.40–0.52. If this compound is indeed ST this suggests that factors in the TLC run parameters must have affected

the band separation. Parameters known to affect R_f outcome include: differences between development tanks, the equilibration time and volume of mobile phase, environmental conditions and any sample preparation variations (Sherma, 2003). Furthermore, the degree of separation of this compound has been found to be sensitive to the concentration of methanol in chloroform and it is possible that this was responsible for the decrease in R_f noted (Hu *et al.*, 1997). The inclusion of a ST reference standard would be definitive in confirming the constituency of these bands of interest, both for TLC purposes and in UPLC-ESI-TOF-MS. A comprehensive list of ST-producing *Photorhabdus* species is not currently available; however this compound does appear to be synthesised by a range of *Photorhabdus* strains (Boemare and Akhurst, 2005).

For comparative purposes two compounds were included as reference standards, namely anthraquinone and trans-stilbene. The UPLC chromatograms for anthraquinone produced one dominant peak at 26.7 minutes and a few minor peaks at 19.7 and 35.3 minutes (Figure 4.6). The trans-stilbene produced a single peak which was present over a significant time interval of ~4 minutes (Figure 4.6). Both anthraquinone and trans-stilbene did not produce any predicted ESI mass spectra data at these peak intervals in either positive or negative ion mode. The presence of polar or acidic groups on a molecule is responsible for good response using ESI-TOF-MS, hence the absence of any such groups on these two reference standards could be responsible for the absence of mass spectra noted (Banerjee and Mazumbar, 2012).

In addition to UPLC-ESI-TOF-MS, GC-MS analysis was also included in order to analyse the *Photorhabdus* extracts and reference standards (Figure 4.7). The anthraquinone reference compound produced a mass spectrum consistent with the library entry for this compound in the NIST Mass Spectral Search Program database (Version 2.0 d, 2005) with a mass of 208 g/mol. The GC-MS spectrum for trans-stilbene was lacking from this database, however it had a primary peak at the expected mass of 180 g/mol which is consistent with the reference sample molecular mass (Sigma Aldrich). Ethyl acetate and lyophilised broth extracts from the *Photorhabdus* isolates were both subjected to GC-MS; unfortunately no mass spectra peaks were detected from any of these analyses. The absence of spectra for any isolate with both extraction protocols may be due to the quantity of extract available for the GC-MS analysis.

Only a small quantity of extract was produced and the majority was utilised in bioassay, TLC and in the UPLC-ESI-TOF-MS analysis. In previous studies anthraquinone pigments have been shown to produce spectra under GC-MS (Li *et al.* 1995). However, data on *Photorhabdus* stilbene compounds analysed using GC-MS is lacking. For GC-MS, the related compound 3,5,4-trihydroxystilbene (Resveratrol) typically involves derivatisation with bis[trimeth-ylsilyl]trifluoroacetamide due to low compound volatility (Jerkovic *et al.,* 2008; Flamini, 2003; Sanders *et al.,* 2000).

4.3.5. Conclusions

All of the *Photorhabdus* isolates analysed exhibited activity against Gram positive bacteria and negligible antifungal activity. The observation that higher levels of activity was noted in extracts taken directly from cell pellet compared to broth supernatant extracts indicates that the cell pellet may be a preferable extraction target for *Photorhabdus* spp. compounds. It has been demonstrated that extraction procedures performed directly on *Photorhabdus* spp. infected insect cadavers has resulted in a greater range of antimicrobial compounds being isolated as compared to that achieved from cell-free media extractions taken from *in vitro* culture of *Photorhabdus* sp. (Boemare and Akhurst, 2006). This process would by necessity involve extraction of *Photorhabdus* cells in conjunction with the colonised insect cadaver. This suggests that the extraction of whole broth—including *Photorhabdus* cells—may provide increased diversity of antimicrobial compounds. Further study comparing active compounds extracted from the cell pellet, those extracted from broth supernatant and extractions of insect cadavers merits consideration in light of known differences in antibiotic expression previously described (Boemare and Akhurst, 2006; Hu *et al,.* 1998; Forst and Nealson, 1996).

Within the set of *Photorhabdus* isolates studied only Isolates 165 and 178 were found to have a unique range of antimicrobial activity. This is not unexpected as these two isolates were identified as a *Pseudomanas* sp. and a *Xenorhabdus* sp. respectively (Chapter 3). An active compound purified from each *Photorhabdus* strain (*viz*. Isolates 91, 161 and 173) was considered to be identical between isolates and was putatively identified as ST (254 g/mol). ST

was reported be to antagonistic towards *B. subtilis* but not *E. coli* (Park and Crawford, 2015); this finding corresponds with the activity spectrum noted in this study. While previous studies have reported that ST is active against *B. cinerea*; in this study antifungal activity was not noted for any of the *Photorhabdus* isolates evaluated (Li *et al.* 1995). This may be due to compound concentration in the screening bioassays being insufficient to produce a positive result, which could be clarified by utilising concentrated antimicrobial extract in a disc-diffusion bioassay.

Observations of activity over time showed that antibacterial activity peaked relatively quickly (24–48 h) after inoculation before decreasing (Section 4.2.1 and 4.2.2). This finding is consistent with earlier studies evaluating ST production throughout the bacterial growth cycle and the presence of this compound in broth supernatant (Hu *et al.,* 1999; Li *et al.* 1995). Additionally the reported low solubility of ST in water could account for antibacterial activity being associated with methanol extractions from pelleted cells (Section 4.2.1 and 4.2.2) (Zhang *et al,* 2011).

The presence of an antibacterially active band on the TLC of *Photorhabdus* extracts in a similar range to ST suggests this compound to be the primary source of activity due to its abundance in the purified lyophilised broth extracts. However, TLC revealed a second active band noted only in extracts from ethyl acetate and methanol extracts of the cell pellet. No peaks were noted in the UPLC chromatogram of the ethyl acetate extract which would produce mass spectra consistent to the size of currently described *Photorhabdus* antibiotics; hence direct analysis of this second fraction from TLC would be required for identification. This TLC band was not noted in any of the extractions from lyophilised broth but was present in ethyl acetate extractions of Isolates 161 and 173. Hence this compound may have been detected but not identified by UPLC-ESI-TOF-MS of the ethyl acetate extracts. Further analysis is warranted to characterise this compound and determine whether it is produced by one grouping of the *Photorhabdus* isolates studied (*viz.* Isolates 161 and 173) and not Isolate 91.

Anthraquinones are commonly described products of *Photorhabdus;* however these compounds were not detected in this study. While pigmented compounds were noted in TLC, identification of these would require mass spectral data. It is possible anthraquinone concentrations or levels of activity were too low to identify activity in comparison to the putative ST compound isolated. Additionally, medium composition has been reported to affect compound production in *Photorhabdus* spp. Only TSB was assayed in this study, yet a greater variety of anthraquinone compounds have been described when isolated from the more nutritionally complex insect cadaver (Boemare and Akhurst, 2006). In future studies the effect of medium composition on antibiotic production could be assessed along with whether extraction from cell pellet vs. broth supernatant results in a greater range of antimicrobials produced.

CHAPTER FIVE

General overview and conclusions

Members of the genus *Photorhabdus* are known to produce a range of antimicrobial compounds linked to killing and preservation of insects for *Heterorhabditis* nematodes (Hu *et al*., 2006; Fischer-Le Saux *et al.,* 1999). These compounds are of interest because of their potential role in the biological control of microbial and insect pests (Hu *et al.,* 1999; Hu *et al.,* 1998). Relatively little is known about the levels of diversity present within *Photorhabdus* spp. strains extant in South Africa. Furthermore, to the best of our knowledge, an evaluation of the antimicrobial compounds produced by representative South African *Photorhabdus* spp. has not been reported on previously. This study was undertaken to assess species and strain diversity amongst a subset of *Photorhabdus* isolates sourced from various geographical locations throughout South Africa; additionally, evaluation of the diversity in the antimicrobial compounds synthesised by these isolates was also undertaken.

Differentiation between these *Photorhabdus* isolates on the basis of phenotypic characteristics was complicated by the low level of phenotypic diversity observed between isolates. Several inconsistencies between parallel methods were also observed. However, testing of phenotypic traits did allow some level of differentiation amongst isolates to be made. The catalase test in particular distinguished Isolate 178 from the rest of the *Photorhabdus* isolates, and this isolate was subsequently determined to be a member of the genus *Xenorhabdus*. In another instance, the phenotypic tests performed were unable to distinguish Isolate 165—a *Pseudomonas* sp.—from the *Photorhabdus* isolates. Phenotypic data generated for the remaining isolates was consistent with results expected for *Photorhabdus* species but in itself was insufficient for identification purposes. In order for phenotypic testing to form the basis of diversity assessments a more comprehensive suite of characteristics would need to be evaluated. In future studies the resolution of phenotypic data available for classification and characterisation of the isolates could be improved by introducing a more comprehensive selection of phenotypic traits (e.g. Maximum growth temperature, comprehensive carbohydrate, organic acid utilisation and amino acid utilisation). Commercial phenotyping kits such as API 50CH, 50AO and 50AA strips (bioMérieux) have previously been successfully

applied for this purpose (Ferreira *et al.,* 2013; Somvanshi, *et al.,* 2006; Tóth and Lakatos, 2008; Hazir, *et al.,* 2004; Fischer-Le Saux *et al*., 1999). Additional specific characteristics that could prove useful in identifying and characterising these strains include lecithinase, DNase, annular haemolysis (sheep or horse blood agar) and utilisation of L-fucose, DL-glycerate, L(-) histidine and DL-lactate (Ferreira *et al.,* 2013; Boemare and Akhurst, 2006; Boemare and Akhurst, 2005).

Genomic fingerprinting proved to be more successful than phenotypic characteristics for the purpose of differentiating between *Photorhabdus* isolates. 16S rRNA gene PCR-RFLP was useful in differentiating Isolates 91, 165 and 178 from the remainder, but was unable to offer evidence of further diversity. Additional restriction endonuclease enzymes may contribute to greater resolution. However, in light of data obtained from 16S rRNA gene sequence analysis, this particular gene region is unlikely to uncover any significant diversity amongst these isolates using 16S PCR-RFLP. To date, little research has been published for RAPD-PCR fingerprinting on *Photorhabdus* spp.. However, the four RAPD primers used in this study consistently distinguished Isolate 178 and the two reference strains. Isolates 165 and 91 were also found to have unique fingerprint patterns in three of the primers. Within the RAPD fingerprint groupings generated, some levels of genomic diversity between *Photorhabdus* isolates were observed. Hence, it is possible that this technique could be useful for isolate grouping and differentiation. However, careful choice of primers, optimisation of PCR run parameters and a broad selection of type strains would be needed in order to determine the degree to which this technique would be effective for examining *Photorhabdus* spp. diversity.

Analysis of the 16S rRNA gene fragment sequences proved highly useful in establishing isolate identities and degrees of relatedness between isolates. Analysis of this gene sequence showed isolates to be highly similar, barring Isolate 91. BLAST analysis of the partial gene sequences confirmed Isolates 165 (*Pseudomonas* sp.) and 178 (*Xenorhabdus* sp.) not to be members of the genus *Photorhabdus*. The remaining isolates demonstrated highest similarity to *P. luminescens* subsp. *laumondii.* These isolates formed two distinct groups with Isolate 91 distinct from the single cluster of Isolates 151, 161, 173 and 180. Neither cluster grouped strongly with any South African *Photorhabdus* strains previously described. When phylogenetic relationships were inferred Isolate 91 was grouped relatively close to *P. luminescens* subsp.

noenieputensis, however the 16S rRNA partial gene sequence similarity between these two strains is only 98.4%. Isolates 151, 161, 173 and 180 formed a distinct cluster separate from established subspecies of *P. luminescens*.

Applying additional DNA-based approaches to the two distinct groupings observed in this study may result in the description of new *P. luminescens* subspecies, or refine the relationship of these South African *Photorhabdus* strains to those previously described. Alternative targets for gene sequencing that have been used previously for *Photorhabdus* spp. include: *rec*A, *gyr*B, *dna*N, *glt*X and *inf*B (Ferreira *et al*., 2013; An and Grewal, 2011; Tailliez, *et al.*, 2010; Akhurst *et al*., 2004). Moreover, the description of new species and subspecies of *Photorhabdus* has often applied such high-resolution approaches as DNA-DNA hybridisation (Ferreira *et al.,* 2014; An and Grewal, 2011; Akhurst *et al.,* 2004; Fischer-Le Saux *et al*., 1999; Grimont *et al.,* 1984). Higher resolution DNA-based approaches applied in conjunction with supporting phenotypic data have been used for the description of *Photorhabdus* sp. and subspecies (Ferreira *et al.,* 2014).

Whole-cell MALDI-TOF-MS analysis of isolates using the Bruker Biotyper system returned similar identifications of isolates to that of the BLAST search of the 16S rRNA partial gene sequence data. Analysis of mass spectra derived from MALDI-TOF-MS achieved clear differentiation of the type strains, Isolates 165 (*Pseudomonas* sp.) and 178 (*Xenorhabdus* sp.), as well as Isolate 91. This further supports the close relatedness between the isolates of this sample set. However, mismatches of the reference strains to their counterparts in the BDAL database highlight the issue of the inter-laboratory reproducibility using this method. The MALDI Biotyper system may not currently achieve results as dependable as those attainable with gene sequencing, but offers great potential as a high throughput technique for screening large numbers of *Photorhabdus* spp. isolates.

Initial evaluation of antimicrobial activity using bioassays determined that all of the *Photorhabdus* isolates demonstrated antibacterial activity, but no significant antifungal activity. Comparison of antibacterial compounds extracted from the cell pellet and cell-free

supernatant respectively showed marked differences in bioactivity. The presences of higher levels of activity from extracts of the cell pellet, especially in older broth culture, suggest this is an interesting target for future analyses. Hydroxystilbenes are reported to be lost from culture over time and indeed activity in the broth supernatant was noted to decrease significantly with longer incubation times in this study (Forst and Nealson, 1996). Hence if 3,5-dihydroxy-4-isopropyistilbene (ST) is determined to be the antimicrobial compound responsible for activity in the cell pellet extractions it is interesting that activity is retained over the extended incubation time. Alternatively should ST not be the compound responsible, the presence of an additional active compound that does not rapidly degrade would also be a significant result.

Greater diversity of *Photorhabdus* antimicrobial compounds have been described when extracted from insect cadavers as opposed to artificial culture media. Hence, for these South African strains there is the possibility of more antimicrobial compounds being characterised by this approach. When trehalose utilisation was assessed during phenotypic testing certain isolates produced a red pigment. Given that trehalose is an insect-derived sugar, it would be of interest to include this carbohydrate in antibiotic production media to ascertain whether this is an anthraquinone pigment. Additionally, a greater range of compounds may be expressed in the presence of trehalose. In addition to optimisation of medium constituents, extractions of greater volumes of *Photorhabdus* growth media may allow the detection of antimicrobial compounds produced at low concentrations.

Extract analysis using TLC revealed similar profiles for Isolates 91, 161 and 173, with some interesting differences between extraction techniques. Additionally, the most active spot is similar to the R_f value previously described for ST (Hu *et al.,* 1997). TLC also offered evidence of an unknown active compound(s) which was apparently not extracted using the lyophilised broth extraction technique. Direct extraction of this band from the TLC plate could be used in conjunction with mass spectroscopy in future studies for compound identification purposes.

Both antimicrobial compound extraction techniques evaluated were found to be suitable for extraction and concentration of active metabolites. However the ethyl acetate extraction

proved less efficient and more materials-intensive in comparison with the extraction of lyophilised broth. The disadvantage of lyophilised broth extraction is the elevated concentration of contaminants necessitating the inclusion of a clean-up step. Clean-up using Sep-Pak C18 syringe columns produced a cleaner extract in comparison with the raw ethyl acetate extract. However, this method carries the risk of loss of compounds of interest during the clean-up process. There is room for improvement in this step to utilise more compound-targeted separation protocols. For the purposes of this study, it is clear that the compound primarily responsible for observed antibacterial activity was purified to a reasonable degree using the Sep-Pak C18 approach. It is interesting that UPLC-ESI-TOF-MS analysis of extracts determined the active compound to be identical between Isolates 91, 161 and 173. The active compound was putatively identified as 3,5-dihydroxy-4-isopropyistilbene (ST), an antimicrobial compound previously described from *Photorhabdus* spp. The appearance of a common compound is interesting considering the selection of isolates based on genetic and phenotypic diversity, and is a further indication of low diversity within the subset of isolates.

In conclusion, the *Photorhabdus* spp. isolates within the subset studied were found to be closely related. Two groupings could be distinguished after using the data obtained from DNA fingerprinting analysis, 16S rRNA gene fragment analyses and whole-cell MALDI-TOF-MS. Additional DNA-based approaches are necessary to fully elucidate the degree of relatedness amongst these strains and to those previously described. Aspects of this data suggest these isolates may be categorised as novel *P. luminescens* subspecies, however further genetic and phenotypic characterisation is required for confirmation. Assessments of antimicrobial compound diversity also revealed these isolates to synthesise highly similar compounds; however this was not an exhaustive assessment of the full range of compounds produced as there are indications of additional compounds which were not identified in this research. In addition, while differences in compound production have been reported between *in vitro* and *in vivo* studies, differences have not been reported between cell-free broth supernatant and cell pellet extractions in previous studies. This may prove a promising avenue for further study of *Photorhabdus* compounds, produced both by these isolates as well as strains previously studied.

REFERENCES

Abu Hatab, M., Stuart, R.J. and Gaugler, R. (1998). Antibiotic resistance and protease production by *Photorhabdus luminescens* and *Xenorhabdus poinarii* bacteria symbiotic with entomopathogenic nematodes: variation among species and strains. *Soil Biology and Biochemistry*, 30, 1955–1961.

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

Alm, R., Johansson, P., Hjernø, K., Emanuelsson, C., Ringnér, M. and Häkkinen, J. (2006). Detection and identification of protein isoforms using cluster analysis of MALDI-MS mass spectra. *Journal of Proteome Research*, 5, 785–792.

An, R. and Grewal, P.S. (2011). *Photorhabdus luminescens* subsp. *kleinii* subsp. nov. (Enterobacteriales: Enterobacteriaceae). *Current Microbiology*, 62, 539–543.

Anke, H., Kolthoum, I., Zähner, H. and Laatsch, H. (1980). Metabolic products of microorganisms. 185. The anthraquinones of the *Aspergillus glaucus* group. I. Occurrence, isolation, identification and antimicrobial activity. *Archives of Microbiology*, 126, 223–230.

Arrebola, M.L., Ringbom, T. and Verpoorte, R. (1999). Anthraquinones from *Isoplexis isabelliana* cell suspension cultures. *Phytochemistry*, 52, 1283–1286.

Atlas, R.M. (2010). *Handbook of Microbiological Media*. 655, 1572, 1869. Washington: CRC Press.

Baghdiguian, S., Boyer-Giglio, M.-H., Thaler, J.-O., Bonnot, G. and Boemare, N.E. (1993). Bacteriocinogenesis cells of *Xenorhabdus nematophilus* and *Photorhabdus luminescens*: Enterobacteriaceae associated with entomopathogenic nematodes. *Biology of the Cell*, 79, 177–185.

Banerjee, S. (1984). Solubility of organic mixtures in water. *Environmental Science and Technology*, 18, 587–591.

Banerjee, S. and Mazumbar, S. (2012). Electrospray ionization mass spectrometry: a technique to access the information beyond the molecular weight of the analyte. *International Journal of Analytical Chemistry,* 1–40.

Betina, V. (1994). Bioactive secondary metabolites of microorganisms. In: *Progress in Industrial Microbiology*, 30, 16–50. Edited by Bushnell, M.E. Amsterdam, New York, Bratislava, Slovac Republic: Elsevier; Ister Science Press.

Bochner, B.R. (2009). Global phenotypic characterization of bacteria. *FEMS Microbiology Reviews*, 33, 191–205.

Bode, H.B. (2009). Entomopathogenic bacteria as a source of secondary metabolites. *Current Opinion in Chemical Biology*, 13, 224–230.

Boemare, N.E. and Akhurst, R.J. (2006). The genera *Photorhabdus* and *Xenorhabdus*. In: *The Prokaryotes,* 6, 451–494. Edited by Dworkin, M., Falkow, S., Rosenburg, E., Schleifer, K-H., Stackebrandt, E. New York, NY: Springer.

Boemare, N.E. and Akhurst, R.J. (2005). The Proteobacteria Part B: the Gamma Proteobacteria. In: *Bergey's Manual of Systematic Bacteriology,* 2, 732–740. Edited by Garrity, G.M., Boone, D.R., Castenholz, R.W., Brenner, D.J., Krieg, N.R. and Staley, J.T. New York, NY: Springer.

Boemare, N.E., Akhurst, R.J. and Mourant, R.G. (1993). DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. *International Journal of Systematic Bacteriology*, 43, 249–255.

Boemare, N.E., Boyer-Giglio, M.-H., Thaler, J.-O., Akhurst, R.J. and Brehelin, M. (1992). Lysogeny and bacteriocinogeny in *Xenorhabdus nematophilus* and other *Xenorhabdus* spp.. *Applied and Environmental Microbiology*, 58, 3032–3037.

Bowen, D., Rocheleau., T.A., Blackburn, M., Andreev, O., Golubeva, E., Bhartia, R. and ffrench-Constant, R.H. (1998). Insecticidal toxins from the bacterium *Photorhabdus luminescens*. *Science*, 280, 2129–2132.

Brown, S.E., Cao, A.T., Dobson, P., Hines, E.R., Akhurst, R.J. and East, P.D. (2006). Txp40, a ubiquitous insecticidal toxin protein from *Xenorhabdus* and *Photorhabdus* bacteria. *Applied and Environmental Microbiology*, 72, 1653–1662.

Brunel, B., Givaudan, A., Lanois, A., Akhurst, R.J. and Boemare, N. (1997). Fast and accurate identification of *Xenorhabdus* and *Photorhabdus* species by restriction analysis of PCR-amplified 16S rRNA genes. *Applied and Environmental Microbiology*, 63, 574–580.

Burdock, T., Brooks, M., Ghaly, A. And Dave, D. (2011). Effect of assay conditions on the measurement of dehydrogenase activity of *Streptomyces venezuelae* using triphenyl tetrazolium chloride. *Advances in Bioscience and Biotechnology,* 2, 214–225.

Carbonnelle, E., Mesquita, C., Bille, E., Day, N., Dauphin, B., Beretti, J.-L., Ferroni, A., Gutmann, L. and Nassif, X. (2011). MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. *Clinical Biochemistry*, 44, 104–109.

Castillo, J.C., Shokal, U. and Eleftherianos, I. (2012). A novel method for infecting *Drosophila* adult flies with insect pathogenic nematodes. *Virulence*, 3, 339–347.

Chukwujekwu, J.C., Coombes, P.H., Mulholland, D.A. and van Staden, J. (2006). Emodin, an antibacterial anthraquinone from the roots of *Cassia occidentalis*. *South African Journal of Botany*, 72, 295–297.

Daborn, P.J., Waterfield, N., Blight, M.A. and ffrench-Constant, R.H. (2001). Measuring virulence factor expression by the pathogenic bacterium *Photorhabdus luminescens* in culture and during insect infection. *Journal of Bacteriology*, 183, 5834–5839.

Daborn, P.J., Waterfield, N., Silva, C.P., Au, C.P.Y., Sharma, S. and ffrench-Constant, R.H. (2002). A single *Photorhabdus* gene makes caterpillars floppy (mcf) allows *Escherichia coli* to persist within and kill insects. *Proceedings of the National Academy of Sciences USA*, 99, 10742–10747.

Daffonchio, D., Borin, S., Frova, G., Manachini, P. and Sorlini, C. (1998). PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis. International Journal of Systematics and Evolutionary Microbiology,* 48, 107–116.

Dare, D. (2006). Rapid bacterial characterization and identification by MALDI-TOF mass spectrometry. In: *Advanced Techniques in Diagnostic Microbiology*, 117–133. Edited by Tang, Y.-W. and Stratton, C. W. New York, NY: Springer.

Eleftherianos, I., Boundy, S., Joyce, S.A., Aslam, S., Marshall, J.W., Cox, R.J., Simpson, T.J., Clarke, D.J., ffrench-Constant, R.H. and Reynolds, S.E. (2007). An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. *Proceedings of the National Academy of Sciences USA*, 104, 2419–2424.

Eloff, J.N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica,* 64, 711–713.

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

Fenselau, C. and Demirev, P.A. (2001). Characterization of intact microorganisms by MALDI mass spectrometry. *Mass Spectrometry Reviews*, 20, 157–171.

Ferreira, T., Van Reenen, C., Pagès, S., Tailliez P., Malan, A.P. and Dicks, L.M.T. (2013). *Photorhabdus luminescens* subsp. *noenieputensis* subsp. nov., a symbiotic bacterium associated with a novel *Heterorhabditis* species related to *Heterorhabditis indica*. *International Journal of Systematic and Evolutionary Microbiology*, 63, 1853–1858.

Ferreira, T., Van Reenen, C.A., Endo, A., Tailliez, P., Pagès, S., Spröer, C., Malan, A.P. and Dicks, L.M.T. (2014). *Photorhabdus heterorhabditis* sp. nov., a symbiont of the entomopathogenic nematode *Heterorhabditis zealandica*. *International Journal of Systematic and Evolutionary Microbiology*, 64, 1540–1545.

ffrench-Constant, R.H. and Bowen, D.J. (2000). Novel insecticidal toxins from nematode-symbiotic bacteria. *Cellular and Molecular Life Sciences*, 57, 828–833.

ffrench-Constant, R.H., Dowling, A., and Waterfield, N.R. (2007a). Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. *Toxicon*, 49, 436–451.

ffrench-Constant, R.H., Eleftherianos, I. and Reynold, S.E. (2007). A nematode symbiont sheds light on invertebrate immunity. *Trends in Parasitology*, 23, 514–517.

Fischer-Le Saux, M., Mauleon, H., Constant, P., Brunel, B. and Boemare, N. (1998). PCR-ribotyping of *Xenorhabdus* and *Photorhabdus* isolates from the Caribbean region in relation to the taxonomy and geographic distribution of their nematode hosts. *Applied and Environmental Microbiology*, 64, 4246–4254.

Fischer-Le Saux, M., Viallard, V., Brunel, B., Normand, P and Boemare, N.E. (1999). Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. *International Journal of Systematic Bacteriology*, 49, 1645–1656.

Flamini R. (2003) Mass spectrometry in grape and wine chemistry. Part I: Polyphenols. *Mass Spectrometry Reviews*, 22, 218–250.

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

Freiwald, A. and Sauer, S. (2009). Phylogenetic classification and identification of bacteria by mass spectrometry. *Nature Protocols*, 4, 732–742.

Gerrard, J.G., Joyce, S.A., Clarke, D.J., ffrench-Constant, R.H., Nimmo, G.R., Looke, D.F.M., Feil, E.J., Pearce, L. and Waterfield, N.R. (2006). Nematode symbiont for *Photorhabdus asymbiotica. Emerging Infectious Diseases,* 12, 1562–1564.

Gerrard, J.G., Waterfield, N., Vohra, R. and ffrench-Constant, R. (2004). Human infection with *Photorhabdus asymbiotica:* an emerging bacterial pathogen. *Microbes and Infection*, 6, 229–237.

Gerritsen, L.J.M., De Raay, G. and Smits, P.H. (1992). Characterization of form variants of *Xenorhabdus luminescens*. *Applied and Environmental Microbiology*, 58, 1975–1979.

Gerritsen, L.J.M., Georgieva, J. and Wiegers, G.L. (2005). Oral toxicity of *Photorhabdus* toxins against thrips species. *Journal of Invertebrate Pathology*, 88, 207–211.

Gerritsen, L.J.M., Van Der Wolf, J.M., Van Vuurde, J.W.L., Ehlers, R.-U., Kramsomil-Osterfeld, K.C. and Smits, P.H. (1995). Polyclonal antisera to distinguish strains and form variants of *Photorhabdus* (*Xenorhabdus*) *luminescens*. *Applied and Environmental Microbiology*, 61, 284–289.

Goodrich-Blair, H. and Clarke, D.J. (2007). Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. *Molecular Microbiology*, 64, 260–268.

Grimont, P.A.D., Steigerwalt, A.G., Boemare, N., Hickman-Brenner, F.W., Deval, D., Grimont, F. and Brenner, D.J. (1984). Deoxyribonucleic acid relatedness and phenotypic study of the genus *Xenorhabdus*. *International Journal of Systematic Bacteriology*, 34, 378–388.

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95–98.

Hathway, D.E. (1962). The use of hydroxystilbene compounds as taxonomic tracers in the genus *Eucalyptus*. *Biochemical Journal*, 83, 80–84.

Hatting, J., Stock, S.P. and Hazir, S. (2009). Diversity and distribution of entomopathogenic nematodes (Steinernematidae, Heterorhabditidae) in South Africa. *Journal of Invertebrate Pathology*, 102, 120–128.

Hazir, S., Stackebrandt, E., Lang, E., Schumann, P., Ehlers, R-U. and Keskin, N. (2004). Two new subspecies of *Photorhabdus luminescens*, isolated from *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae): *Photorhabdus luminescens* subsp. *kayaii* subsp. nov. and *Photorhabdus luminescens* subsp. *thracensis* subsp. nov. *Systematic and Applied Microbiology*, 27, 36–42.

Hemwimon, S., Pavasant, P. and Shotipruk, A. (2007). Microwave-assisted extraction of antioxidative anthraquinones from roots of *Morinda citrifolia*. *Separation and Purification Technology*, 54, 44–50.

Holding, A. J. and Collee, J. G. (1971). Routine biochemical tests. In: *Methods in Microbiology*, 6, 3–32. Edited by Norriss, J. R. and Ribbons, D. W. London: Academic Press Inc.

Hu, K. and Webster, J.M. (1998). *In vitro* and *in vivo* characterization of a small-colony variant of the primary form of *Photorhabdus luminescens* MD (*Enterobacteriaceae*). *Applied and Environmental Microbiology*, 64, 3214–3219.

Hu, K. and Webster, J.M. (2000). Antibiotic production in relation to bacterial growth and nematode development in *Photorhabdus-Heterorhabditis* infected *Galleria mellonella* larvae. *FEMS Microbiology Letters*, 189, 219–223.

Hu, K., Li, J., Li, B. and Webster, J.M. (1997). Quantitative analysis of a bacteria-derived antibiotic in nematode-infected insects using HPLC-UV and TLC-UV methods. *Journal of Chromatography B: Biomedical Sciences and Applications*, 703, 177–183.

Hu, K., Li, J., Li, B. and Webster, J.M. (1999). Nematicidal metabolites produced by *Photorhabdus luminescens* (Enterobacteriaceae), bacterial symbiont of entomopathogenic nematodes. *Nematology*, 1, 457–469.

Hu, K., Li, J., Li, B., Webster, J.M. and Chen, G. (2006) A novel antimicrobial epoxide isolated from larval *Galleria mellonella* infected by the nematode symbiont, *Photorhabdus luminescens* (Enterobacteriaceae). *Bioorganic and Medicinal Chemistry*, 14, 4677–4681.

Hu, K., Li, J., Wang, W., Wu, H., Lin, H. and Webster, J.M. (1998). Comparison of metabolites produced *in vitro* and *in vivo* by *Photorhabdus luminescens*, a bacterial symbiont of the entomopathogenic nematode *Heterorhabditis megidis*. *Canadian Journal of Microbiology*, 44, 1072–1077.

Janda, J.M. and Abbott, S.L. (2002). Bacterial identification for publication: when is enough enough? *Journal of Clinical Microbiology*, 40, 1887–1891.

Jerkovic, V., Nguyen, F., Timmermans, A. and Collin, S. (2008) Comparison of procedures for resveratrol analysis in beer: Assessment of stilbenoids stability through wort fermentation and beer aging. *Journal of the Institute of Brewing*, 114, 143–149.

Jukes T.H. and Cantor C.R. (1969). Evolution of protein molecules. In: *Mammalian Protein Metabolism*, 21–132. Edited by Munro H. N., Academic Press, New York.

Kim, Y., Lee, C., Kim, H. and Lee, H. (2004). Anthraquinones isolated from *Cassia tora* (Leguminosae) seed show an antifungal property against phytopathogenic fungi. *Journal of Agricultural and Food Chemistry,* 52, 6096–6100.

Kowalska, T., Kaczmarski, K. and Prus, W. (2003). Theory and mechanism of thin-layer chromatography. In: *Handbook of Thin-layer Chromatography*, 62–105. Edited by J. Sherma and B. Fried, USA: Marcel-Dekker, Inc.

Krásný, L., Hynek, R. and Hochel, I. (2013). Identification of bacteria using mass spectrometry techniques. *International Journal of Mass Spectrometry*, 353, 67–79.

Krasomil-Osterfeld, K.C. (1995). Influence of osmolarity on phase shift in *Photorhabdus luminescens*. *Applied and Environmental Microbiology*, 61, 3748–3749.

Kumar, S.V., Mulla, S.R. and Suresh, C.K. (2011). Isolation and molecular characterization of symbiotic bacterial isolates associated with entomopathogenic nematodes in agro climatic zone 5 of Karnataka. *Journal of Phytology*, 3, 25–29.

Langfield, R.D., Scarano, F.J., Heitzman, M.E., Kodo, M., Hammond, G.B. and Neto, C.C. (2004) Use of a modified microplate bioassay method to investigate antibacterial activity in the Peruvian medicinal plant *Peperomia galioides*. *Journal of Ethnopharmacology*, 94, 279–281.

Lay, J.O. (2000). MALDI-TOF mass spectrometry and bacterial taxonomy. *Trends In Analytical Chemistry*, 19, 507–516.

Lay, J.O. (2001). MALDI-TOF mass spectrometry of bacteria. *Mass Spectrometry Reviews* 20, 172–194.

Li, J., Chen, G., Wu, H. and Webster, J.M. (1995). Identification of two pigments and a hydroxystilbene antibiotic from *Photorhabdus luminescens*. *Applied and Environmental Microbiology*, 61, 4329–4333.

Li, W., Raoult, D. and Fournier, P.-E. (2009). Bacterial strain typing in the genomic era. *FEMS Microbiology Reviews*, 33, 892–916.

Liu, J., Berry, R.E. and Blouin, M.S. (2001). Identification of symbiotic bacteria (*Photorhabdus* and *Xenorhabdus*) from the entomopathogenic nematodes *Heterorhabditis marelatus* and *Steinernema oregonense* based on 16S rDNA sequence. *Journal of Invertebrate Pathology*, 77, 87–91.

Ma, X., Chen, Y. and Hui, R. (1989). Analysis of anthraquinones in *Rheum franzenbachii* Münt (Rhubarb) by thin-layer chromatography. *Chromatographia*, 27, 465–466.

Mahar, A.N., Munir, M., Gowen, S.R. and Hague, N.G.M. (2005). Role of entomopathogenic bacteria, *Photorhabdus luminescens* and its toxic secretions against *Galleria mellonella* larvae. *Journal of Entomology,* 2, 69–76.

McDonald, P.D. (2001). A sample preparation primer and guide to solid phase extraction methods development. Adapted from: *Solid Phase Extraction: Applications Guide and Bibliography: A Resource For Sample Preparation Methods Development: Method Development Manual and Comprehensive Compendium Of Worldwide Waters Sep-Pak Cartridge: Literature References Since 1978*. 6. Edited by: McDonald, P. D. and Bouvier, E. S. P. (1995) Milford: Waters.

Mellmann, A., Cloud, J., Maier, T., Keckevoet, U., Ramminger, I., Iwen, P., Dunn, J., Hall, G., Wilson, D., LaSala, P., Kostrzewa, M. and Harmsen, D. (2008). Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. *Journal of Clinical Microbiology*, 46, 1946–1954.

Olive, M.D. and Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *Journal of Clinical Microbiology*, 37, 1661–1669.

Olsen, G.J. and Woese, C.R. (1993). Ribosomal RNA: a key to phylogeny. *The FASEB Journal,* 7, 113–123.

Orozco, R., Hill, T. and Stock, S.P. (2013). Characterization and phylogenetic relationships of *Photorhabdus luminescens* subsp. *sonorensis* (γ-Proteobacteria:Enterobacteriaceae), the bacterial symbiont of the entomopathogenic nematode *Heterorhabditis sonorensis* (Nematoda: Heterorhabditidae). *Current Microbiology*, 66, 30–39.

Park, H.B. and Crawford, J.M. (2015). Lumiquinone A, an α-aminomalonate-derived aminobenzoquinone from *Photorhabdus luminescens*. *Journal of Natural Products,* 78, 1437–1441.

Pawlus, A.D., Su, B., Keller, W.J. and Kinghorn, A.D. (2005). An anthraquinone with potent quinone reductase-inducing activity and other constituents of the fruits of *Morinda citrifolia* (Noni). *Journal of Natural Products*, 68, 1720–1722.

Peel, M.M., Alfredson, D.A., Gerrard, J.G., Davis, J.M., Robson, J.M., McDougall, R.J., Scullie, B.L. and Akhurst, R.J. (1999). Isolation, identification, and molecular characterization of strains of *Photorhabdus luminescens* from infected humans in Australia. *Journal of Clinical Microbiology*, 37, 3647–3653.

Piel, J. (2004). Metabolites from symbiotic bacteria. *Natural Product Reports*, 21, 519–538.

Rademaker, J.L.W., Aarts, H.J.M. and Vinuesa, P. (2005). Molecular typing of environmental isolates. In: *Molecular Microbial Ecology,* 97–135. Edited by Osborn, A.M., and Smith, C.J. New York, NY: Taylor and Francis.

Rainey, F.A., Ehlers, R.-U. and Stackebrandt, E. (1995). Inability of the polyphasic approach to systematics to determine the relatedness of the genera *Xenorhabdus* and *Photorhabdus*. *International Journal of Systematic Bacteriology*, 32, 395–398.

Richardson, W.H., Schmidt, T.M. and Nealson, K.H. (1988). Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. *Applied and Environmental Microbiology*, 54, 1602–1605.

Saenz-Aponte, A., Pulido, O.F. and Jaramillo, C. (2014). Isolation and characterization of bacterial symbiont *Photorhabdus luminescens* SL0708 (Enterobacteriales: Enterobacteriaceae). *African Journal of Microbiology Research*, 8, 3123–3130.

Salie, F., Eagles, P.F.K. and Lens, H.M.J. (1996). Preliminary antimicrobial screening of four South African Asteraceae species. Journal of Ethnopharmacology, 52, 27–33.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406–425.

Sanders, T.H., McMichael, R.W. and Hendrix, K.W. (2000) Occurrence of resveratrol in edible peanuts. *Journal of Agricultural and Food Chemistry*, 48, 1243−1246.

Šedo, O., Sedláček, I. and Zdráhal, Z. (2011). Sample preparation methods for MALDI-MS profiling of bacteria. *Mass Spectrometry Reviews*, 30, 417–434.

Sharad, M. and Naved, S. (2005). Biosafety concerns on the use of *Photorhabdus luminescens* as biopesticide: experimental evidence of mortality in egg parasitoid *Trichogramma* spp.. *Current Science*, 89, 1268–1272.

Sherma, J. (2003) Basic TLC techniques, materials, and apparatus. In: *Handbook of Thin-layer Chromatography*, 1–61. Edited by J. Sherma and B. Fried, USA: Marcel-Dekker, Inc.

Singer, S. (2001) *Experiments in Applied Microbiology*. 33. Academic Press: San Diego.

Smigielski, A.J., Akhurst, R.J. and Boemare, N.E. (1994). Phase variation in *Xenorhabdus nematophilus* and *Photorhabdus luminescens*: differences in respiratory activity and membrane energization. *Applied and Environmental Microbiology*, 60, 120–125.

Somvanshi, V.S., Lang, E., Ganguly, S., Swiderski, J., Saxena, A.K. and Stackebrandt, E. (2006). A novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum* Ganguly and Singh, 2000. *Systematic and Applied Microbiology*, 29, 519–525.

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

Tamura, K., Nei, M. and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA)* 101, 11030–11035.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M. and Kumar S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739.

Thompson, N.S. (2003). Trehalose – The insect 'blood' sugar. In: *Advances in Insect Physiology*, 31, 205–285. Edited by Simpson, S. London, New York: Academic Press.

Tóth, T. and Lakatos, T. (2008). *Photorhabdus temperata* subsp. *cinerea* subsp. nov., isolated from *Heterorhabditis* nematodes. *International Journal of Systematic and Evolutionary Microbiology*, 58, 2579–2581.

Valentine, N., Wunschel, S., Wunschel, D., Petersen, C. and Wahl, K.L. (2005). Effect of culture conditions on microorganism identification by matrix-assisted laser desorption ionization mass spectrometry. *Applied and Environmental Microbiology*, 71, 58–64.

Van Belkum, A. (1994). DNA fingerprinting of medically important microorganisms by use of PCR. *Clinical Microbiology Reviews*, 7, 174–184.

Wafa, J., Abdelkefi-Mesrat, L., Tounsi, S., Jaoua, S. and Zouari, N. (2013). Potential of *Photorhabdus temperata* K122 bioinsecticide in protecting wheat flour against *Ephestia kuehniella*. *Journal of Stored Products Research*, 53, 61–66.

Wang, Z., Russon, L., Li, L., Roser, D.C. and Long, S.R. (1998). Investigation of spectral reproducibility in direct analysis of bacteria proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, 12, 456–464.

Waterfield, N., Kamita, S.G., Hammock, B.D. and ffrench-Constant, R. (2005). The *Photorhabdus* Pir toxins are similar to a developmentally regulated insect protein but show no juvenile hormone esterase activity. *FEMS Microbiology Letters*, 245, 47–52.

Welker, M., and Moore, E.R.N. (2011). Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. *Systematic and Applied Microbiology*, 34, 2–11.

Williams, J.S., Thomas, M. and Clarke, D.J. (2005). The gene stlA encodes a phenylalanine ammonia-lyase that is involved in the production of a stilbene antibiotic in *Photorhabdus luminescens* TT01. *Microbiology*, 151, 2543–2550.

Wunschel, S.C., Jarman, K.H., Petersen, C.E., Valentine, N.B., Wahl, K.L., Schauki, D., Jackman, J., Nelson, C.P. and White, E. (2005). Bacterial analysis by MALDI-TOF mass spectrometry: an inter-laboratory comparison. *Journal of the American Society for Mass Spectrometry*, 16, 456–462.

Yang, G. and Waterfield, N.R. (2013). The role of TcdB and TccC subunits in secretion of the *Photorhabdus* Tcd Toxin Complex. *PLOS Pathogens* 9, 1–13.

Zhang, Y., Gao, J., Zheng, H., Zhang, R. and Han, Y. (2011). The preparation of 3,5-dihydroxy-4-isopropylstilbene nanoemulsion and *in vitro* release. *International Journal of Nanomedicine*, 6, 649–657.

Zhu, L., Yu, S., Zeng, X., Fu, X. and Zhao, M. (2008). Preparative separation and purification of five anthraquinones from *Cassia tora* L. by high-speed counter-current chromatography. *Separation and Purification Technology*, 63, 665–669.

APPENDIX ONE

Additional UPLC-ESI-TOF-MS spectra from extracts of Isolates 91, 161 and 173 generated from several UPLC time intervals. Extracts comprised ethyl acetate extraction from cell-free broth supernatant and methanol extracts from lyophilised cell-free broth purified using a C18 protocol (Chapter 4). Also included are mass spectra resolved for reference standards of trans-stilbene and anthraquinone.

1.1. UPLC-ESI-TOF-MS mass spectra resolved at 2.9 minutes

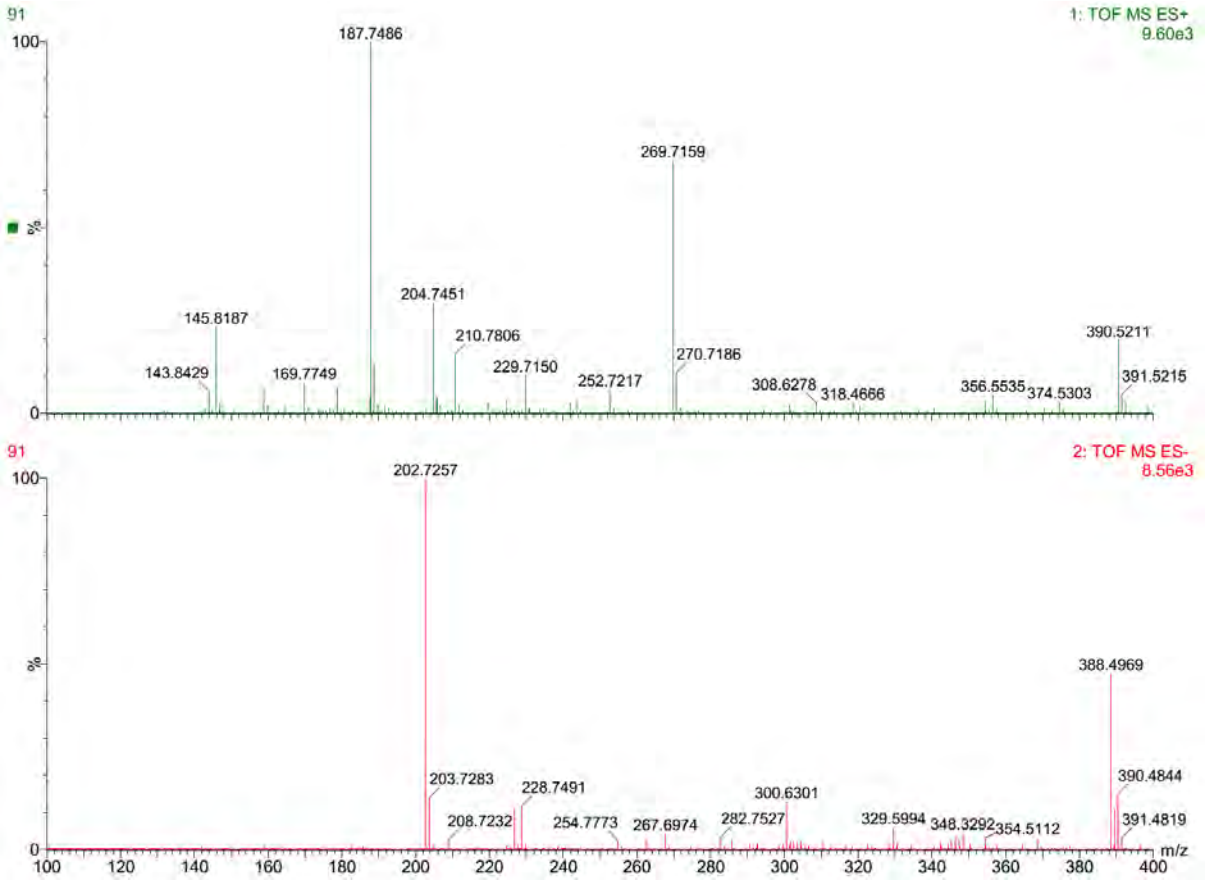


Figure A1.1.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 2.9 minutes from a methanol extract of C18-column cleaned lyophilised broth from Isolate 91.

1.2. UPLC-ESI-TOF-MS mass spectra resolved at 4.0 minutes

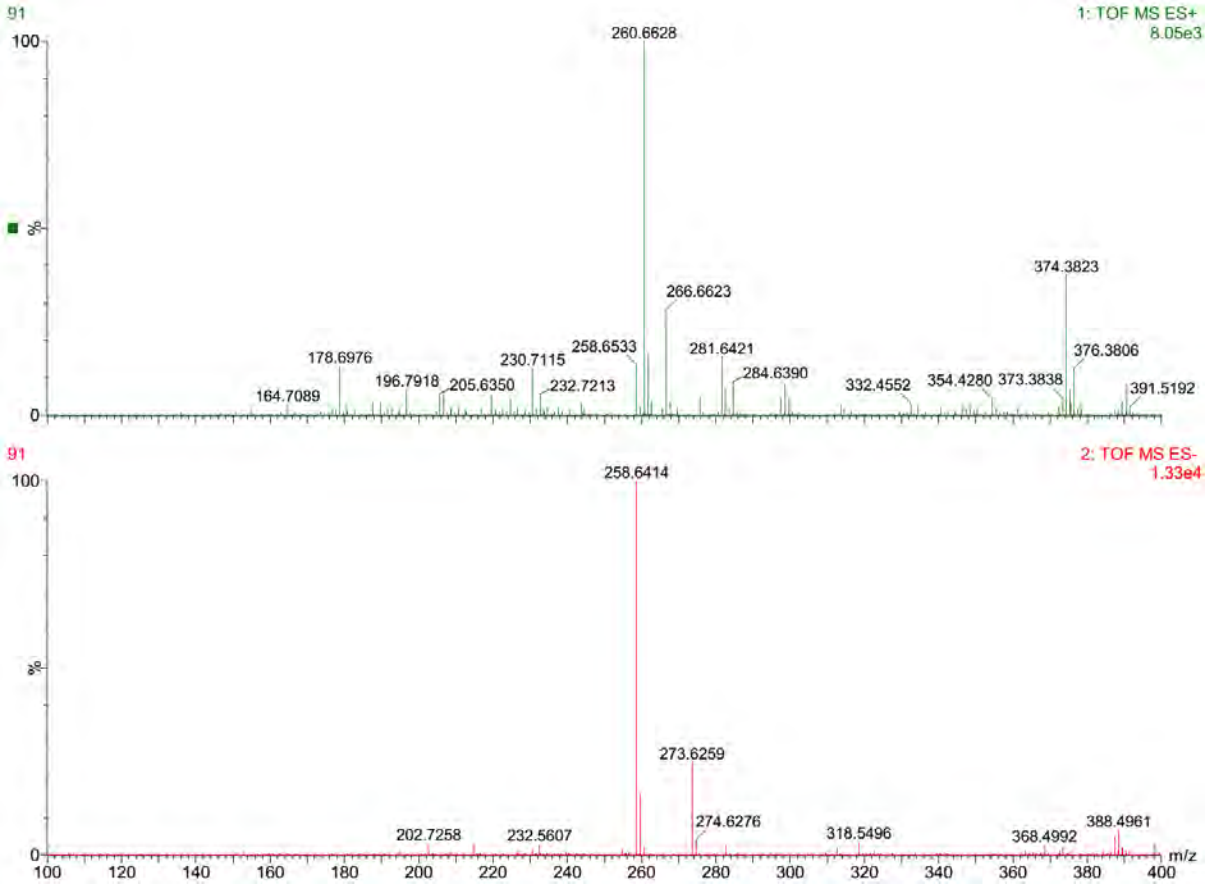


Figure A1.2.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 4.0 minutes from a methanol extract of C18-column cleaned lyophilised from Isolate 91.

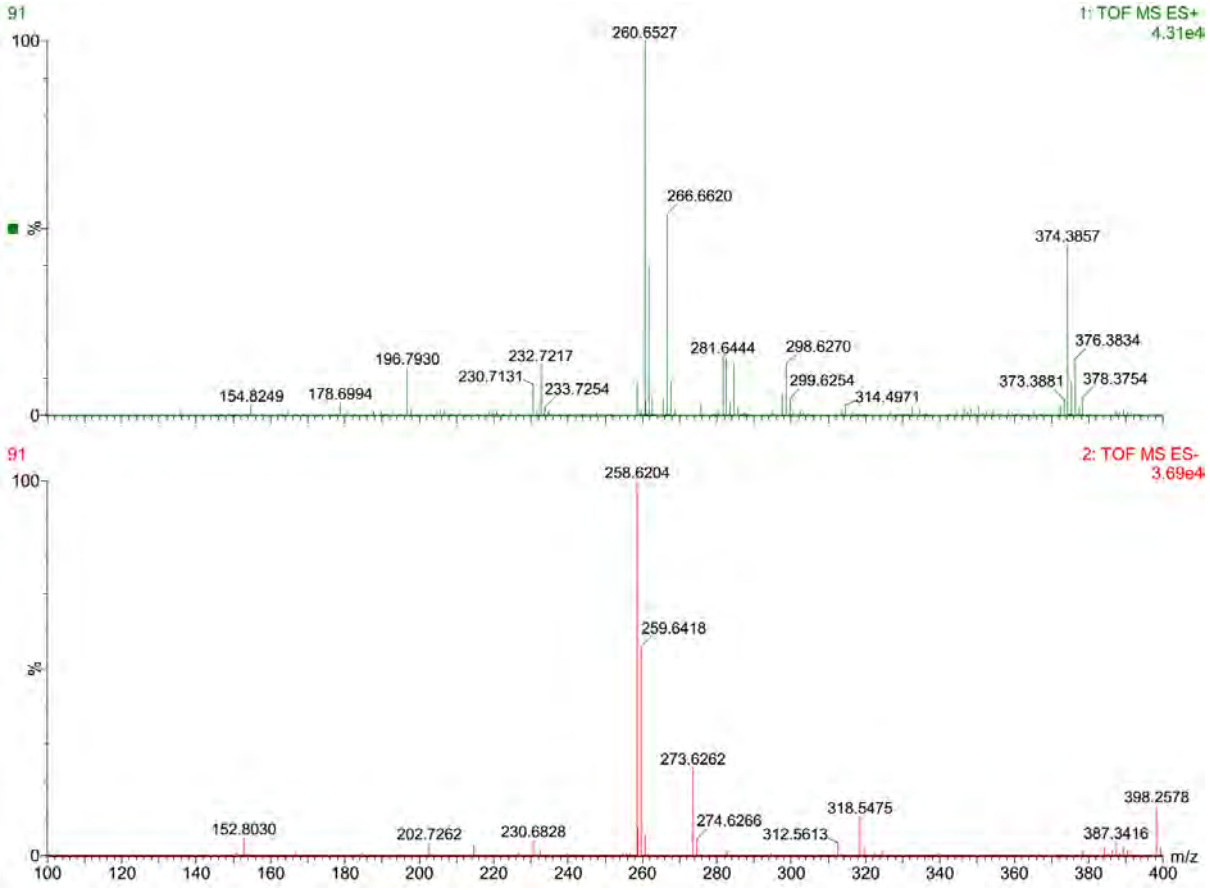


Figure A1.2.2. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 4.0 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 91.

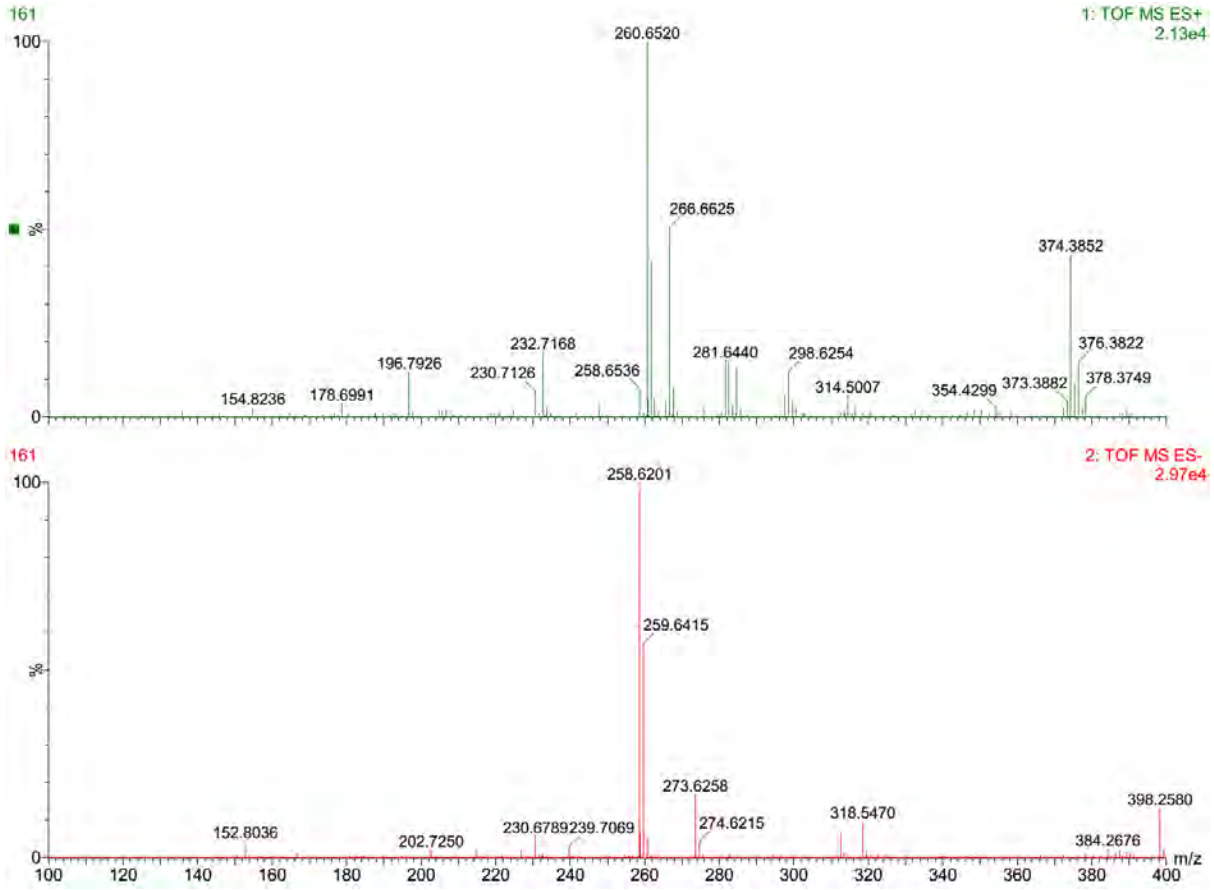


Figure A1.2.3. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 4.0 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 161.

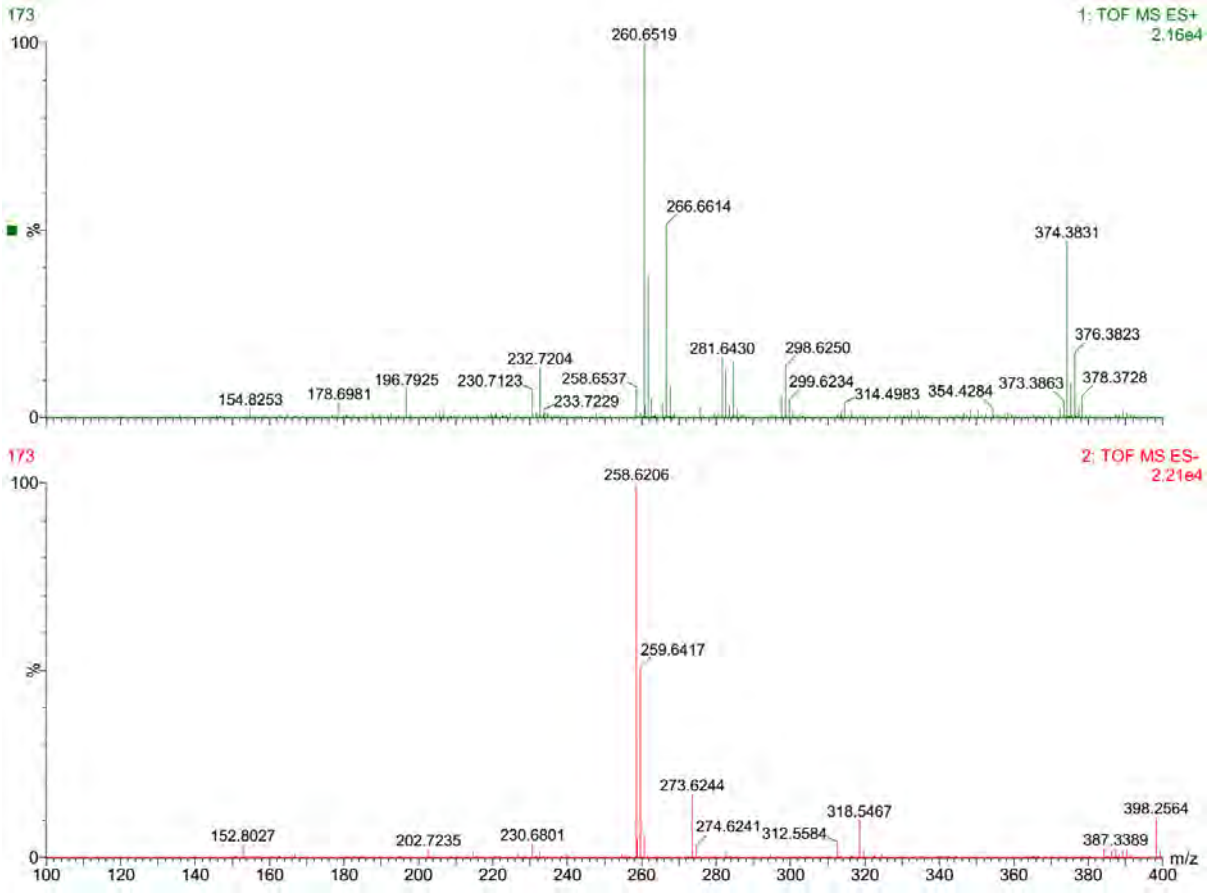


Figure A1.2.4. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 4.0 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 173.

1.3. UPLC-ESI-TOF-MS mass spectra resolved at 4.9 minutes

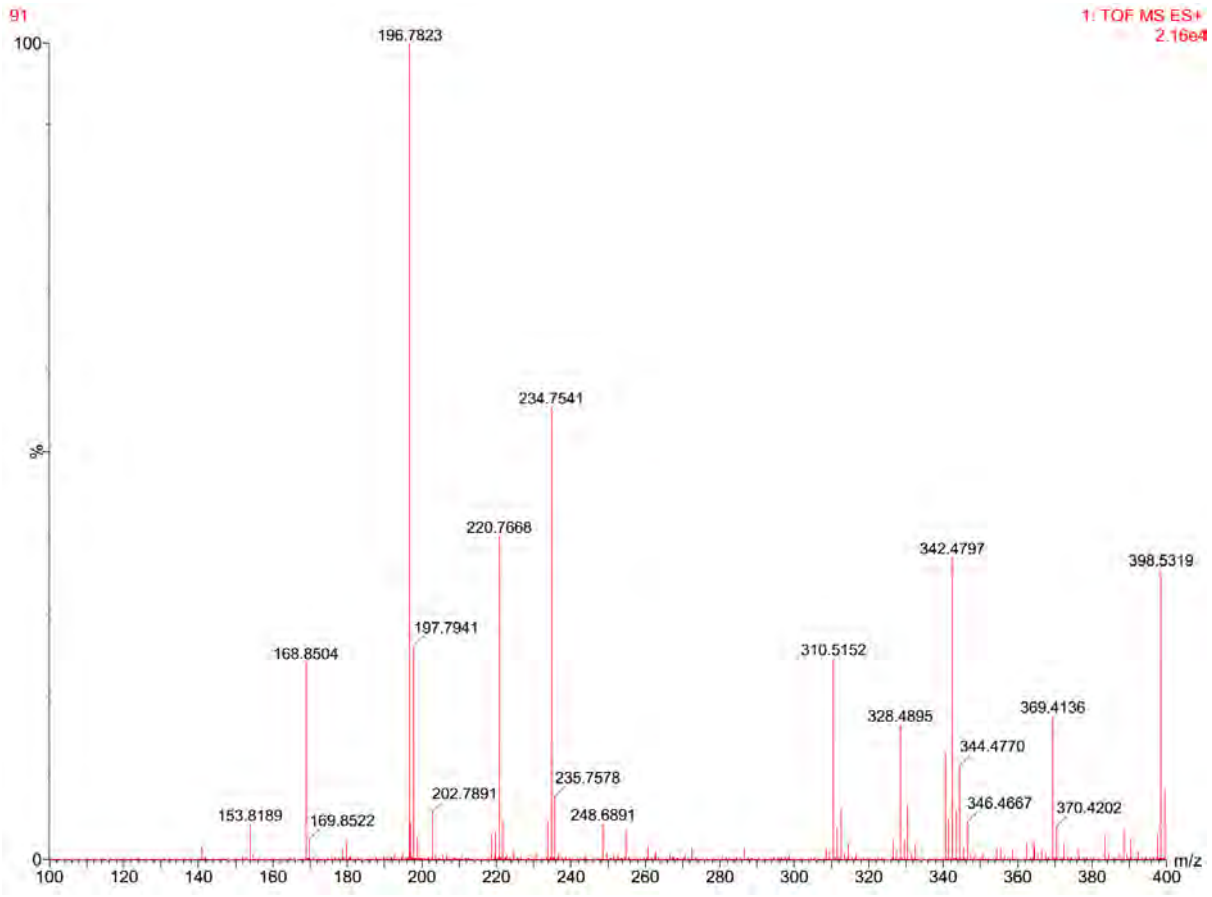


Figure A1.3.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 4.9 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 91.

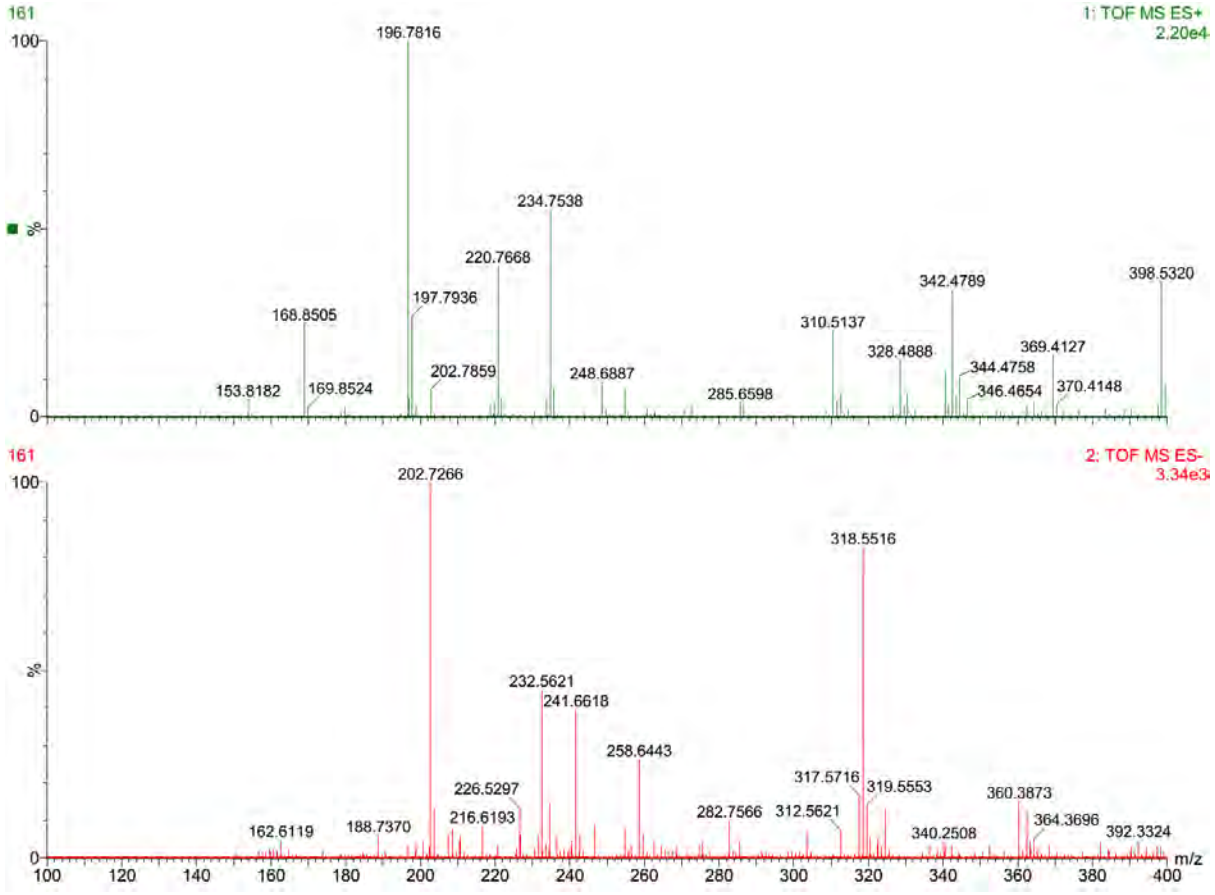


Figure A1.3.2. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 4.9 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 161.

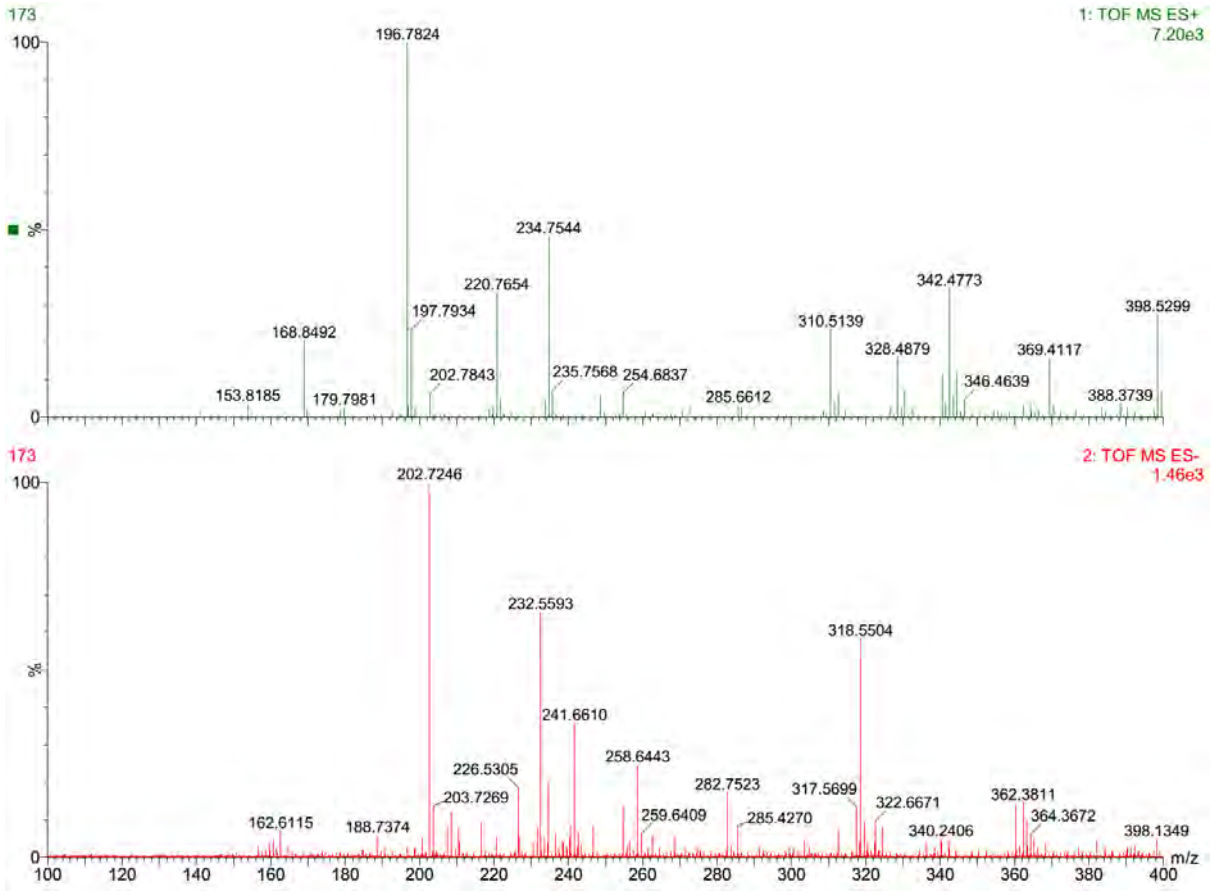


Figure A1.3.3. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 4.9 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 173.

1.4. UPLC-ESI-TOF-MS mass spectra resolved at 10.9 minutes

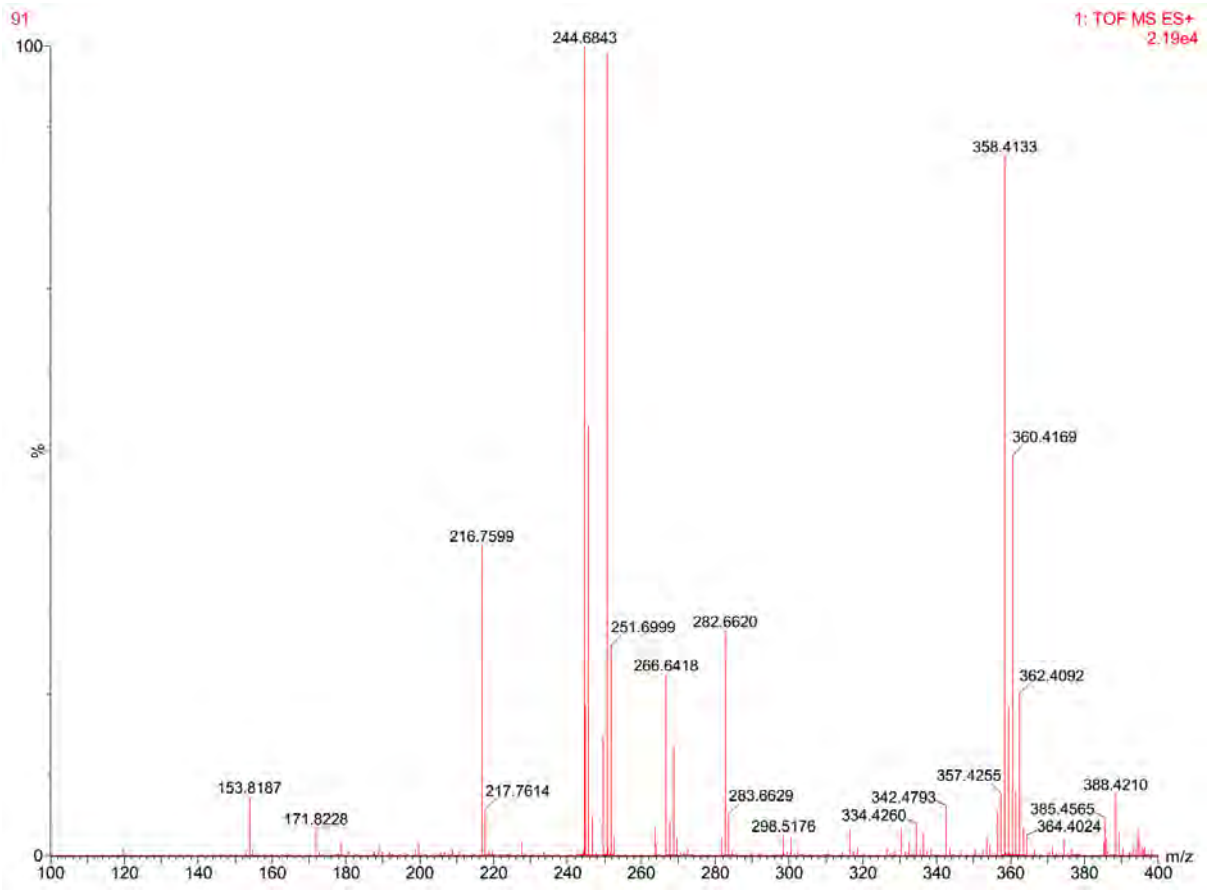


Figure A1.4.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 10.9 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 91.

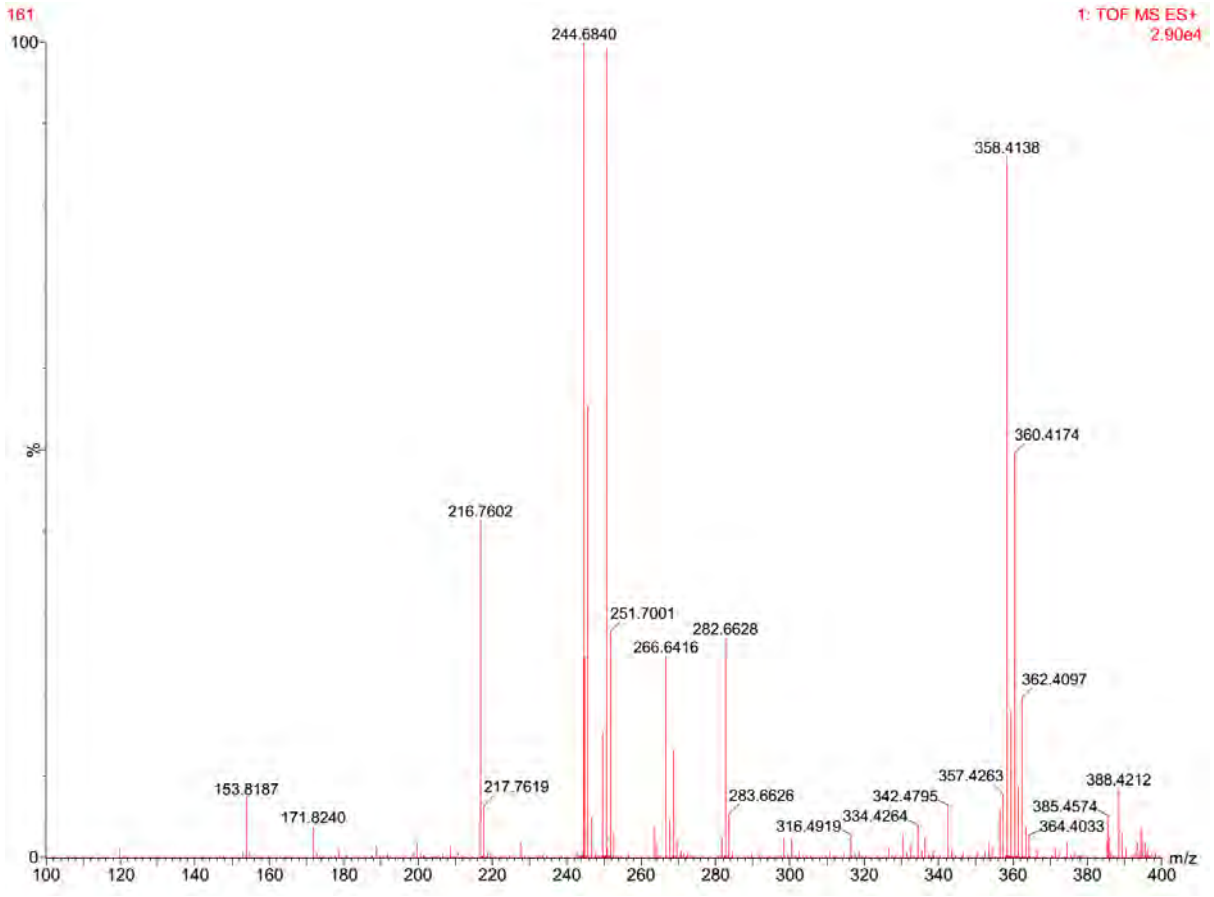


Figure A1.4.2. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 10.9 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 161.

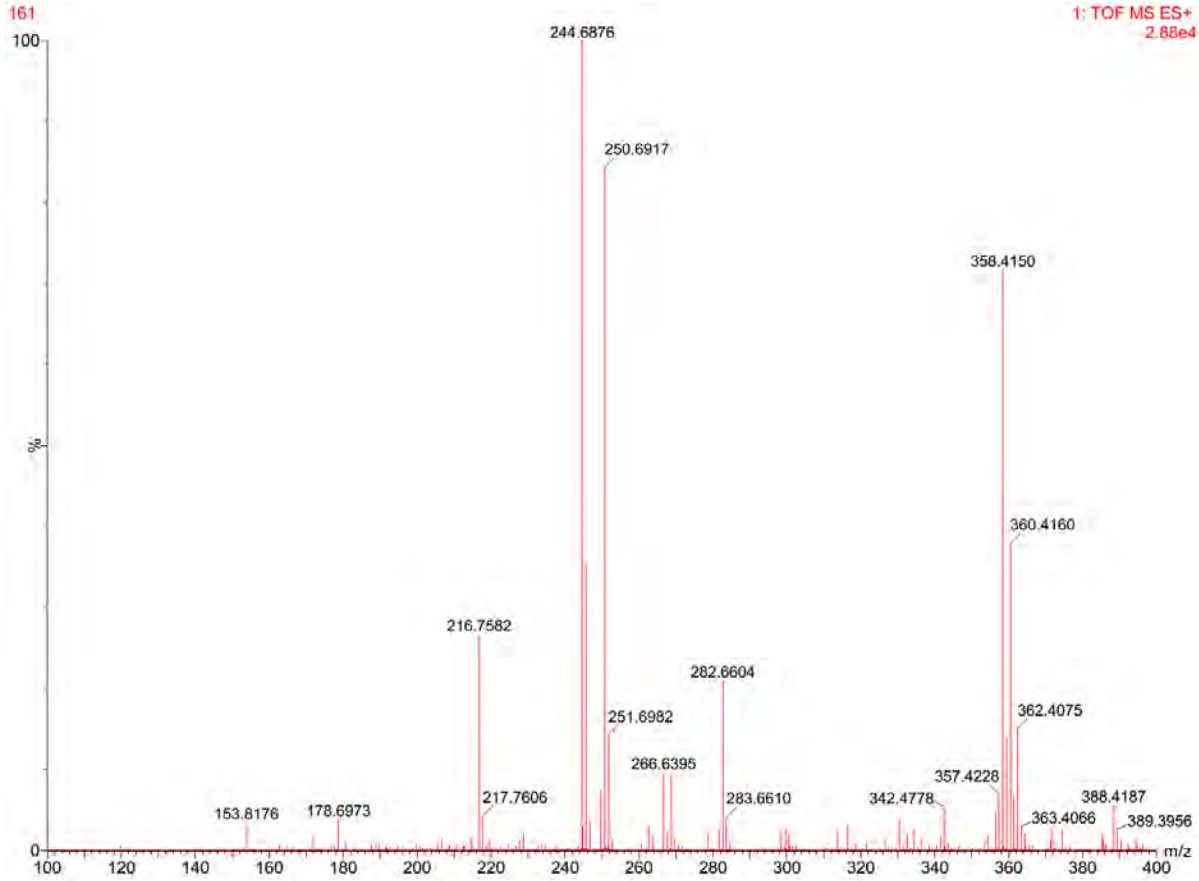


Figure A1.4.3. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 10.9 minutes from a methanol extract of C18-column cleaned lyophilised broth from Isolate 161.

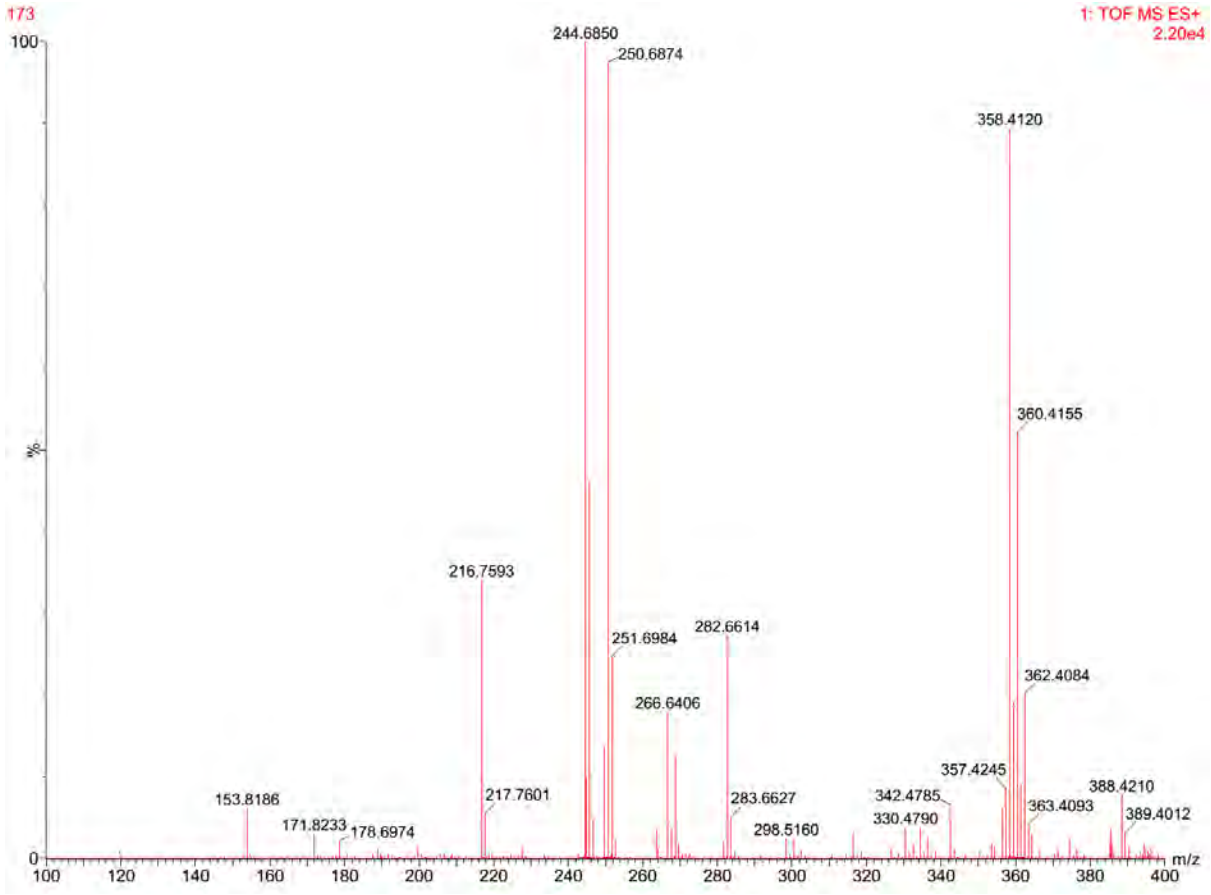


Figure A1.4.4. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 10.9 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 173.

1.5. UPLC-ESI-TOF-MS mass spectra resolved at 17.2 minutes

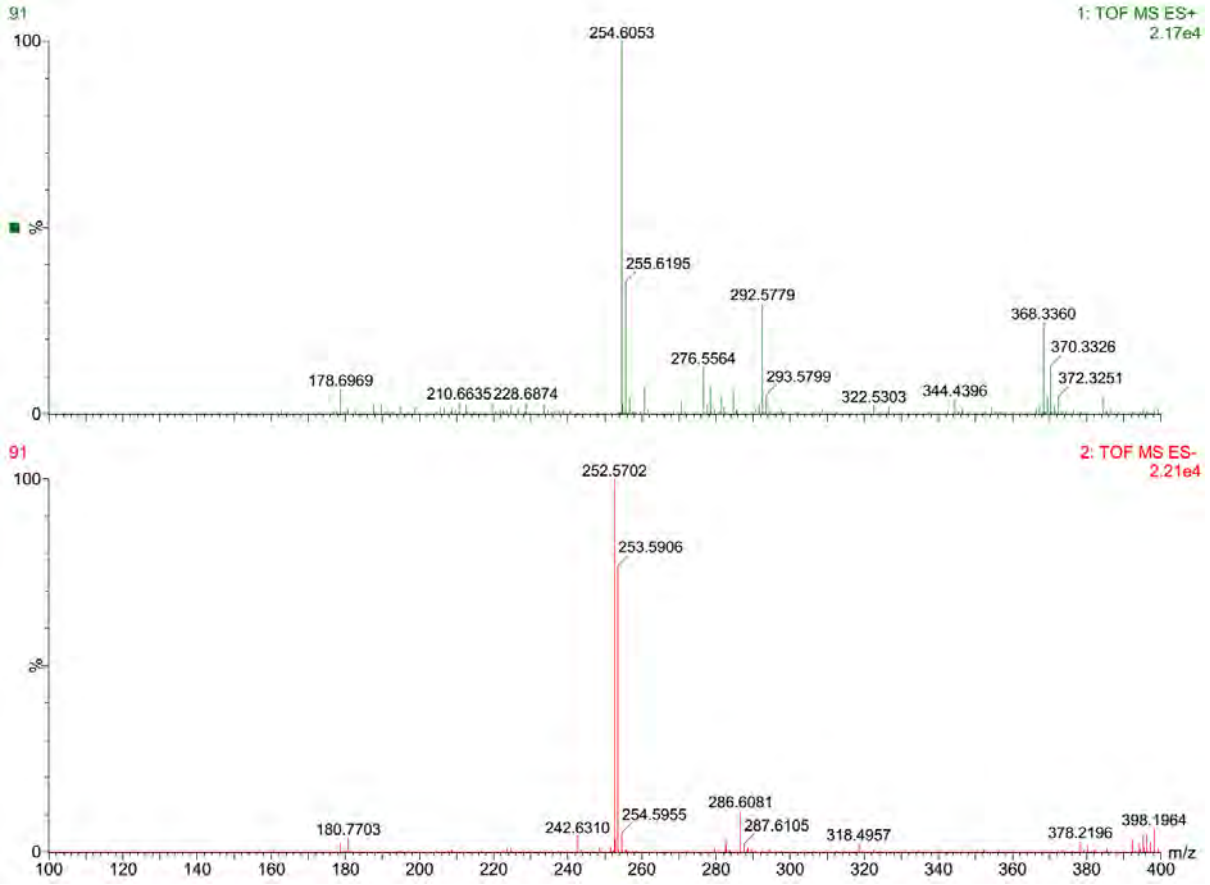


Figure A1.5.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 17.2 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 91.

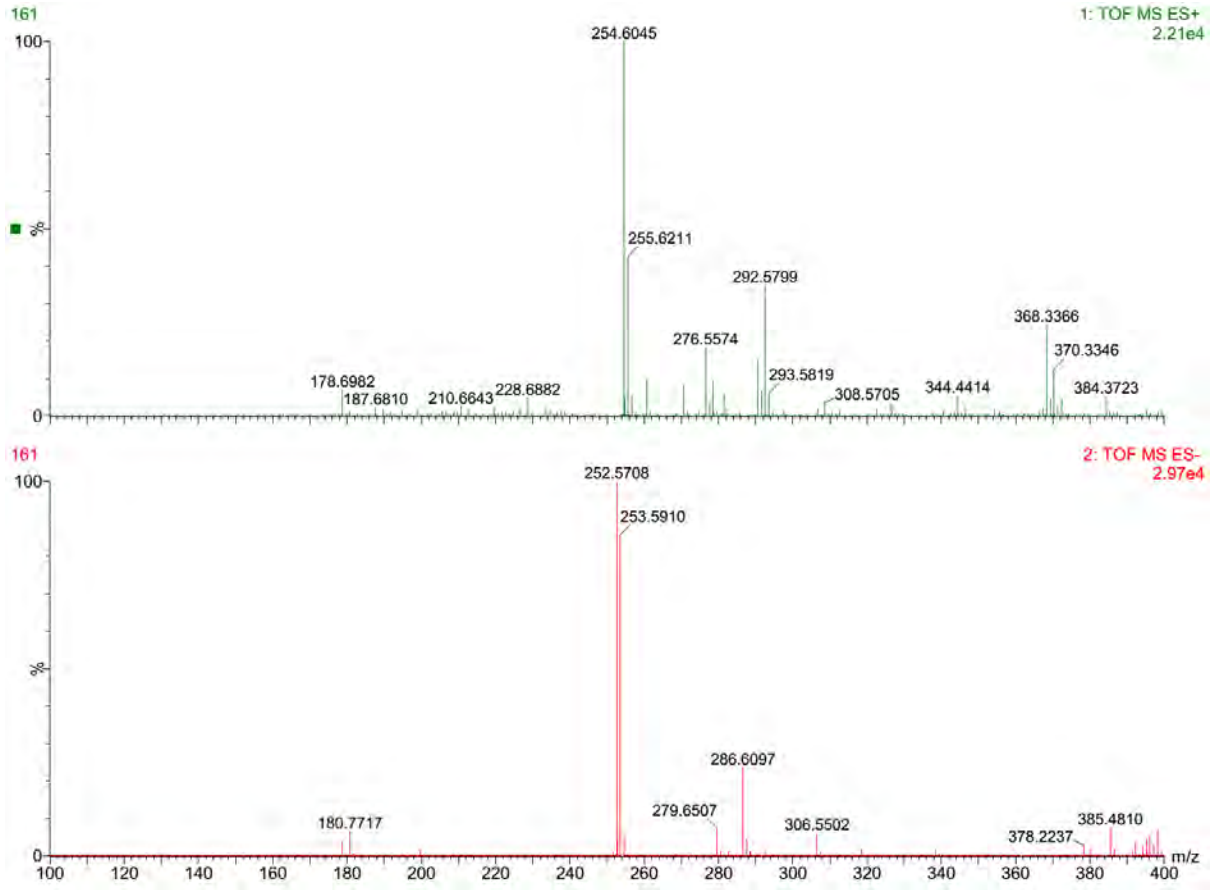


Figure A1.5.2. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 17.2 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 161.

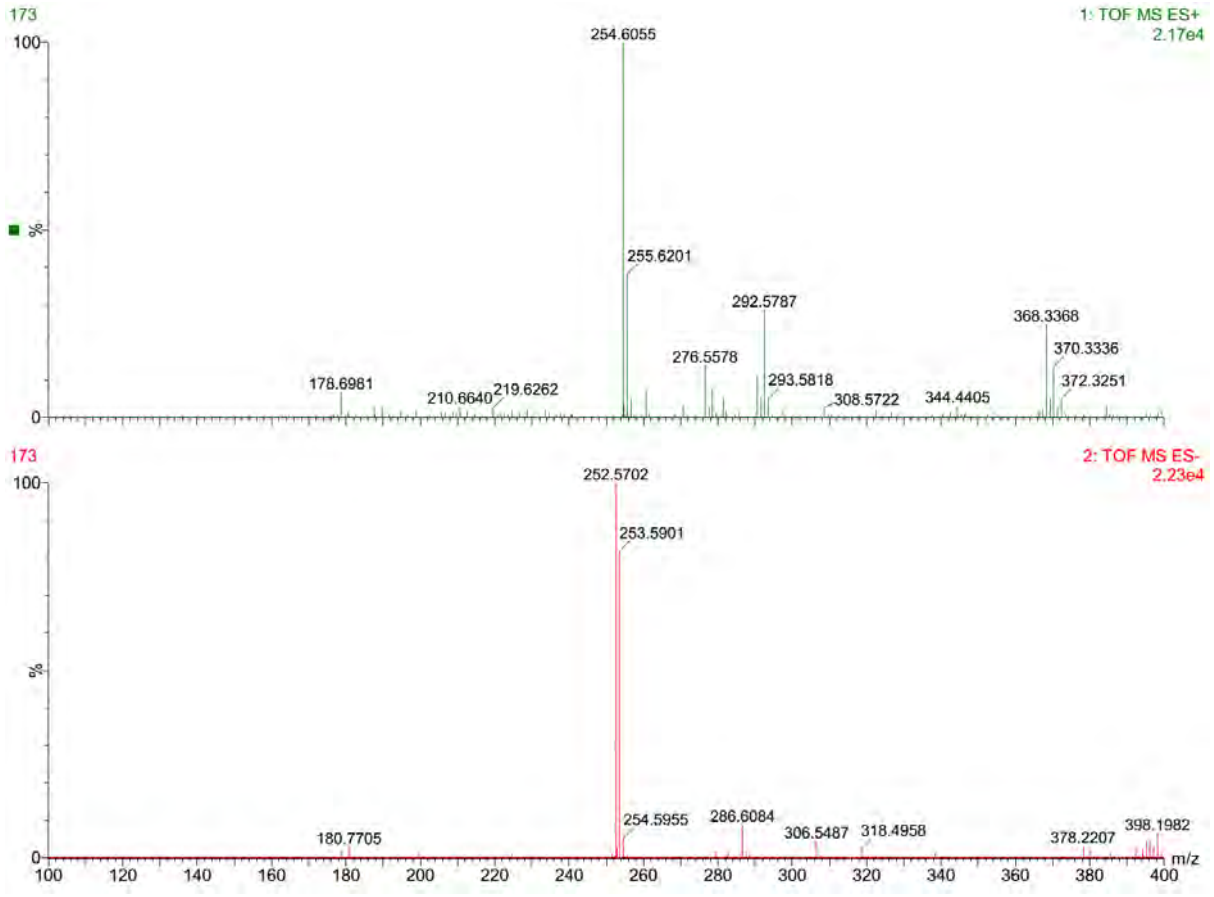


Figure A1.5.3. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 17.2 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 173.

1.6. UPLC-ESI-TOF-MS mass spectra resolved at 19.9 minutes

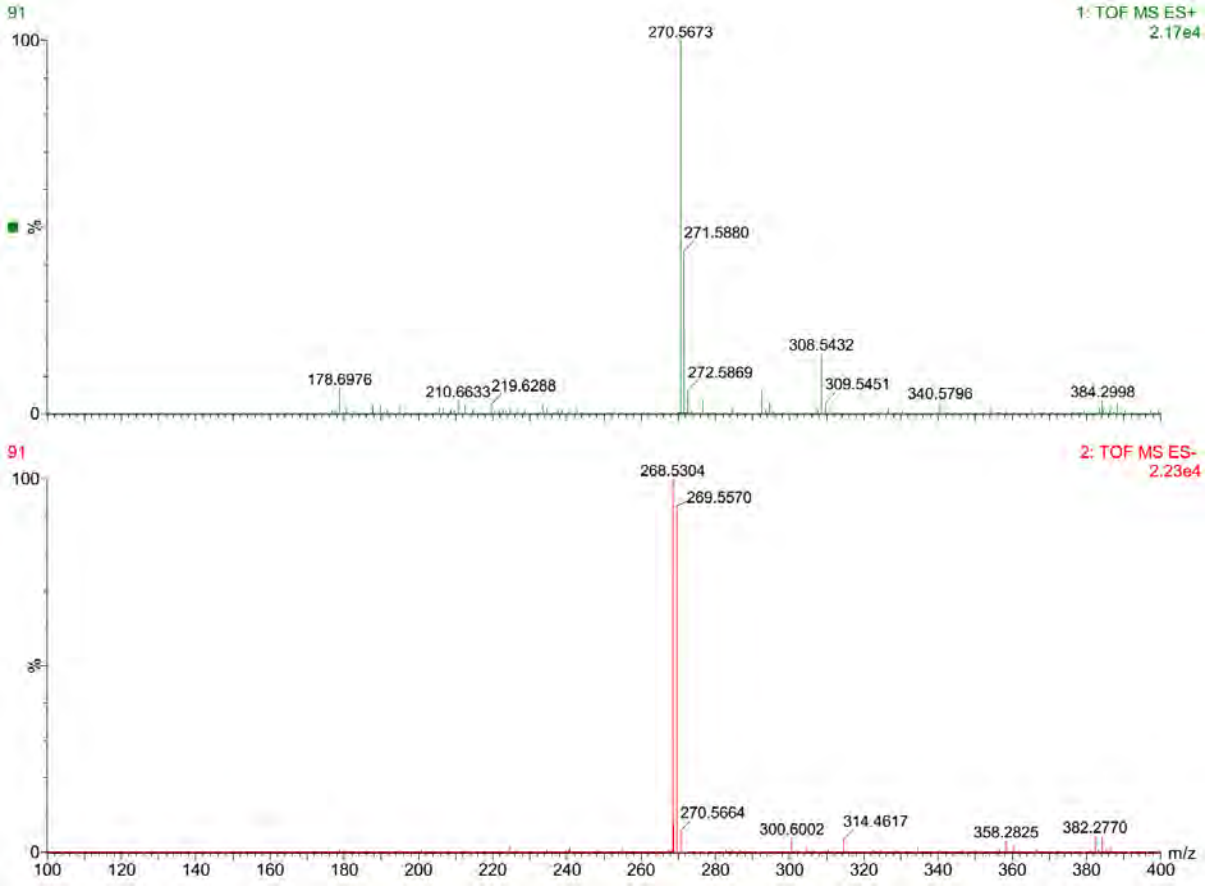


Figure A1.6.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 19.9 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 91.

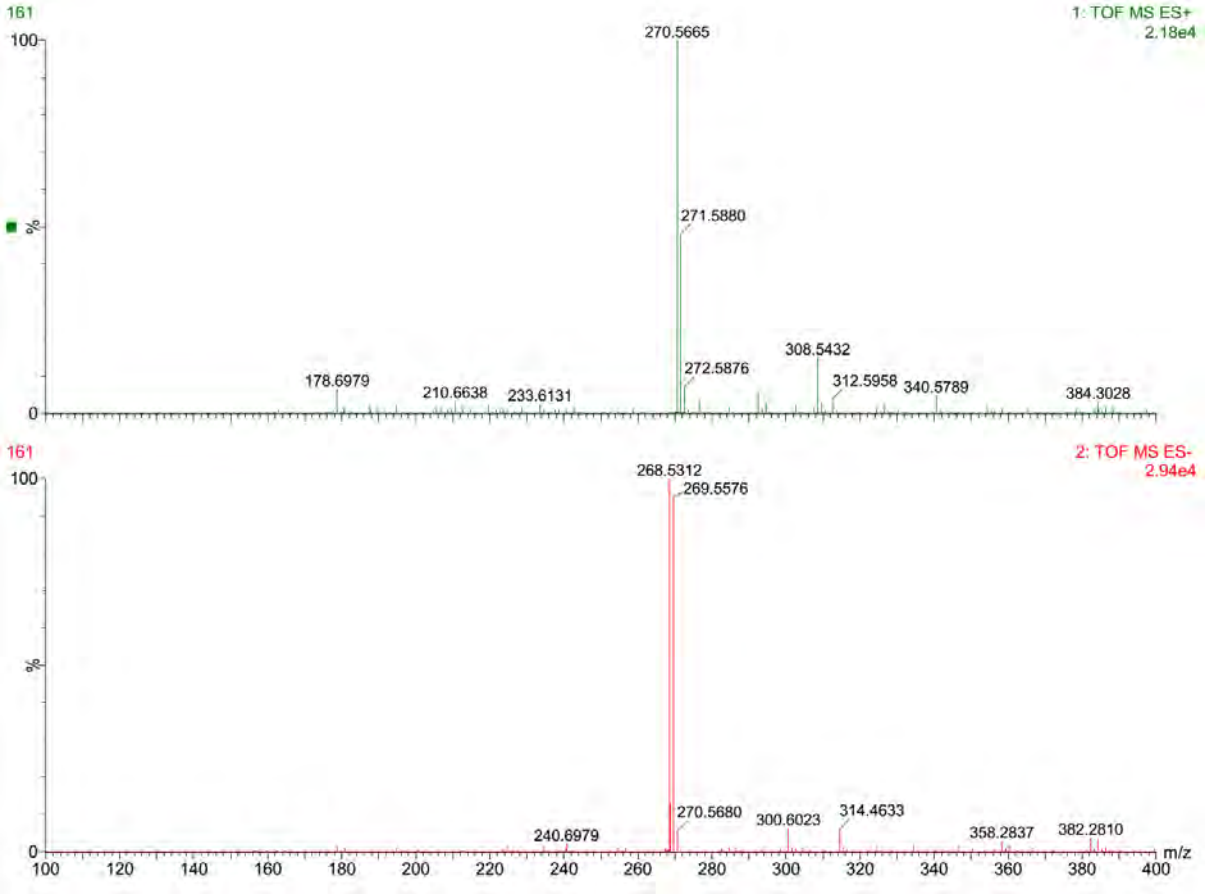


Figure A1.6.2. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 19.9 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 161.

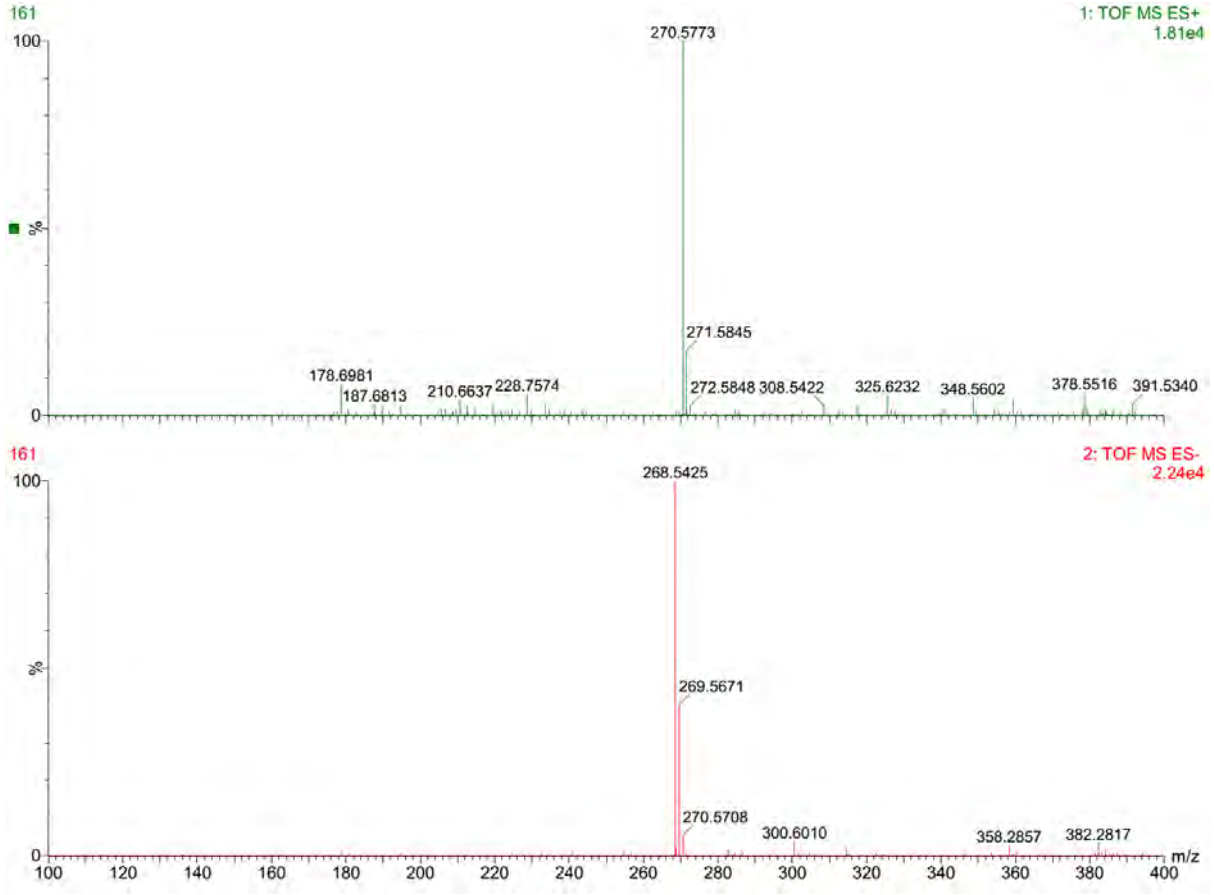


Figure A1.6.3. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 19.9 minutes from a methanol extract of C18-column cleaned lyophilised broth from Isolate 161.

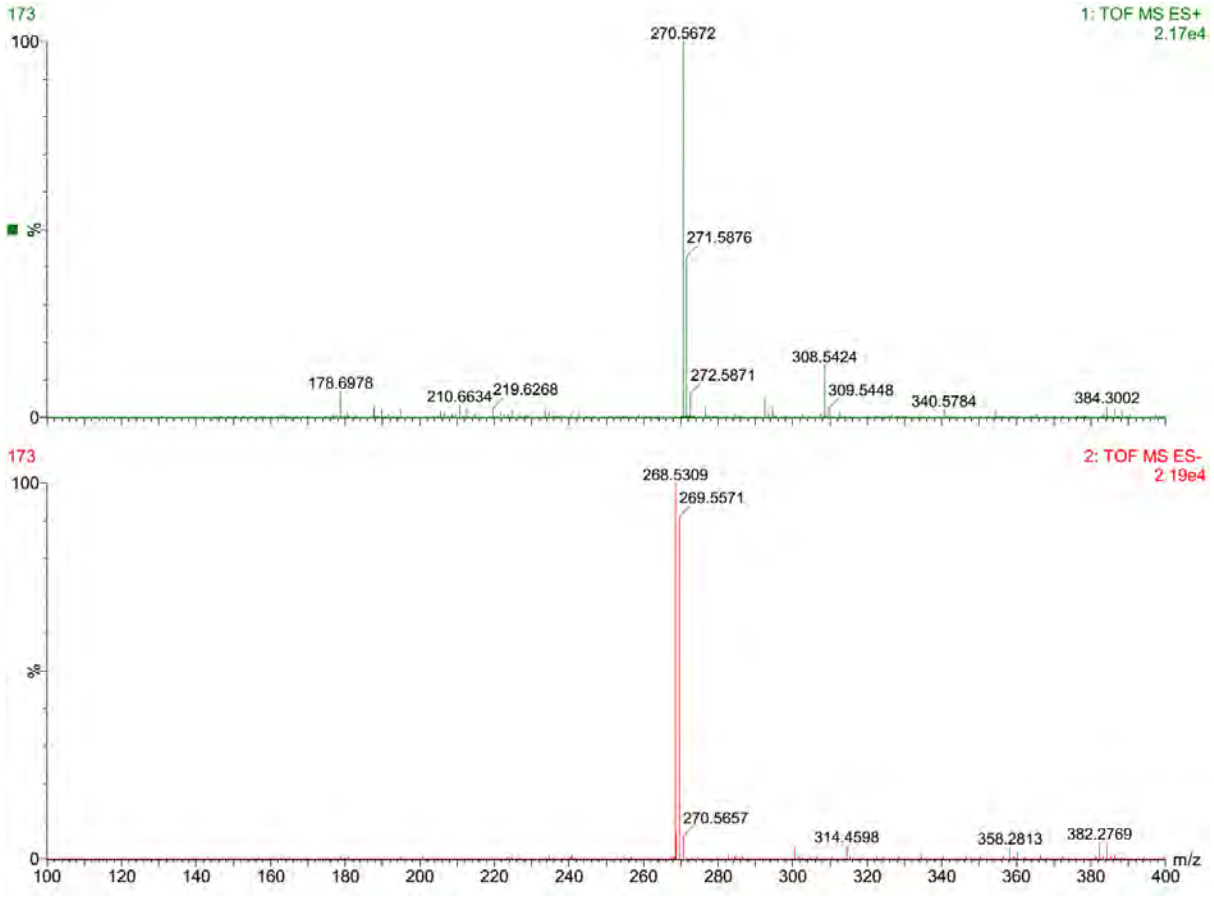


Figure A1.6.4. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 19.9 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 173.

1.7. UPLC-ESI-TOF-MS mass spectra resolved at 21.2 minutes

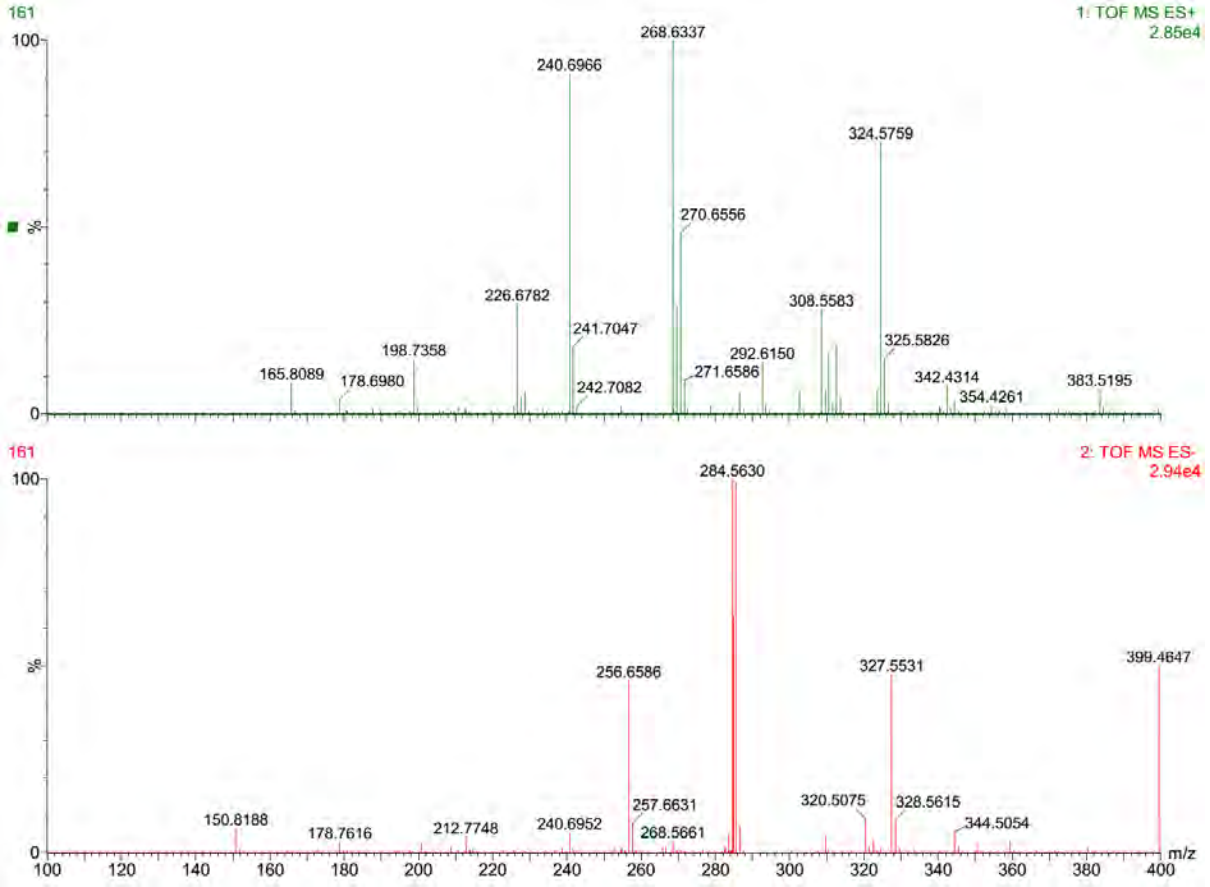


Figure A1.7.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 21.2 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 161.

1.8. UPLC-ESI-TOF-MS mass spectra resolved at 24.8 minutes

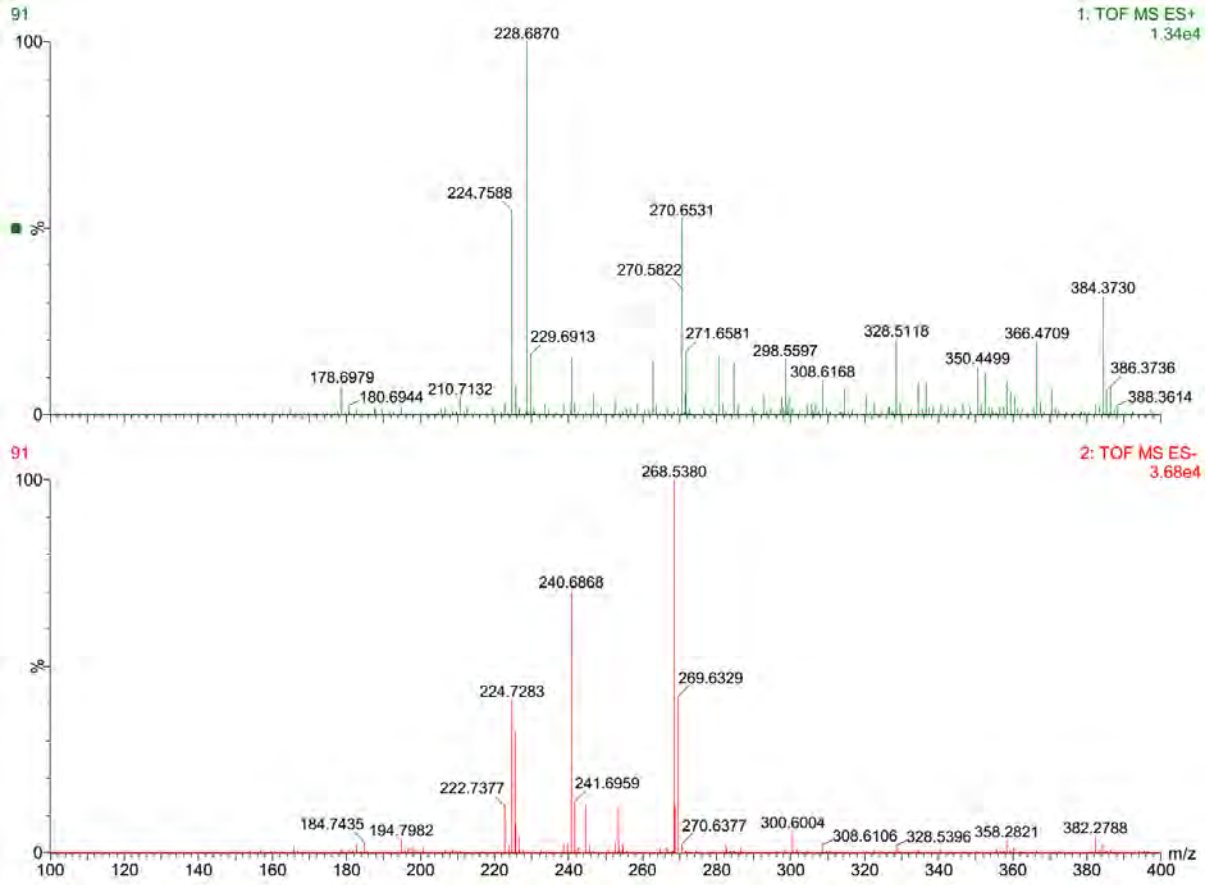


Figure A1.8.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 24.8 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 91.

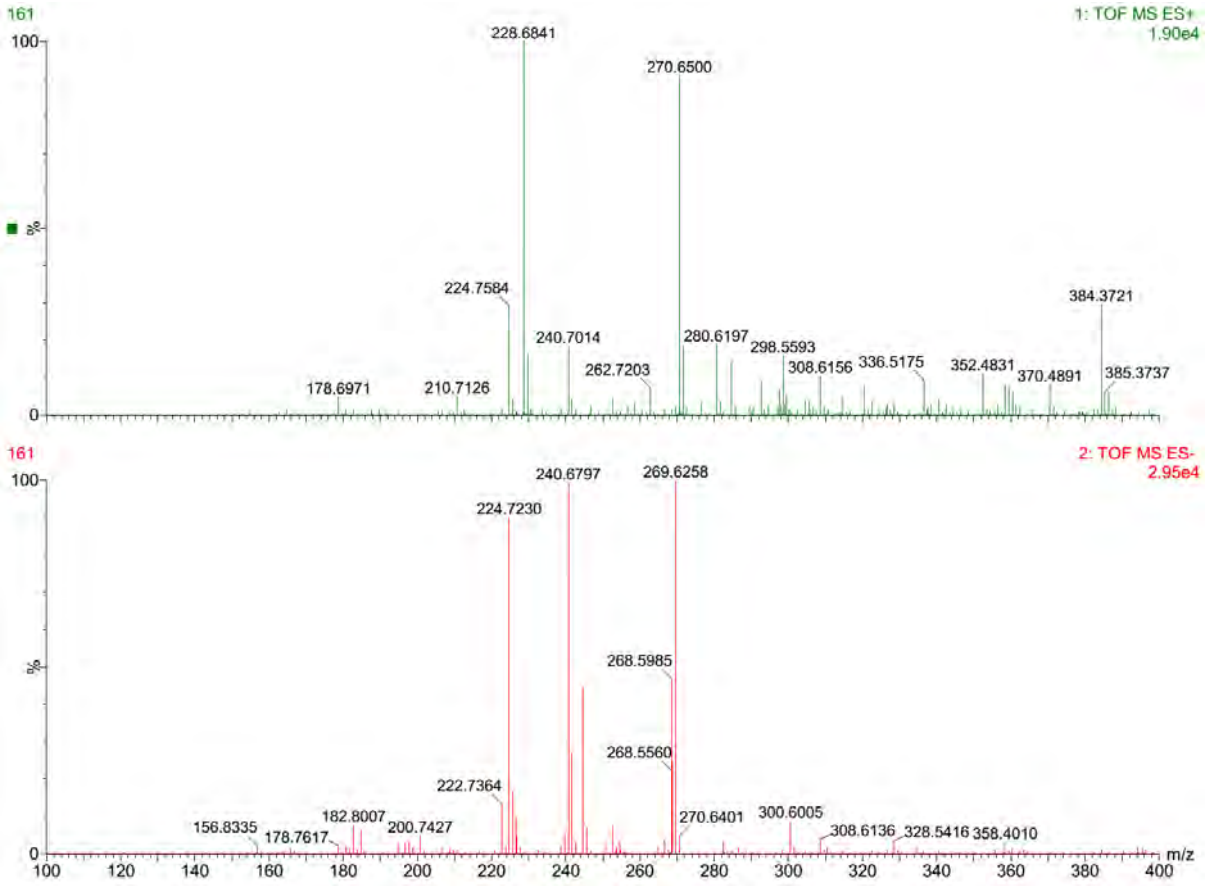


Figure A1.8.2. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 24.8 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 161.

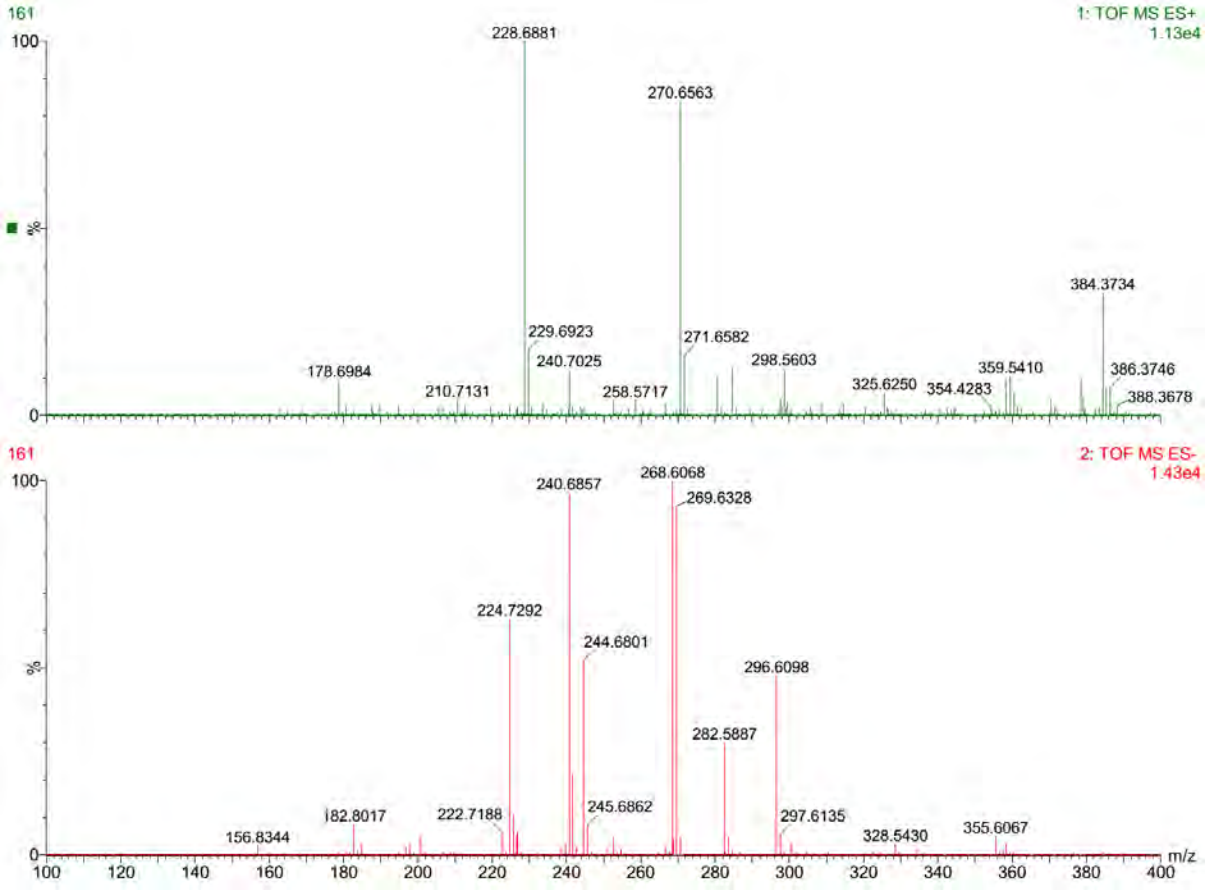


Figure A1.8.3. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 24.8 minutes from a methanol extract of C18-column cleaned lyophilised broth from Isolate 161.

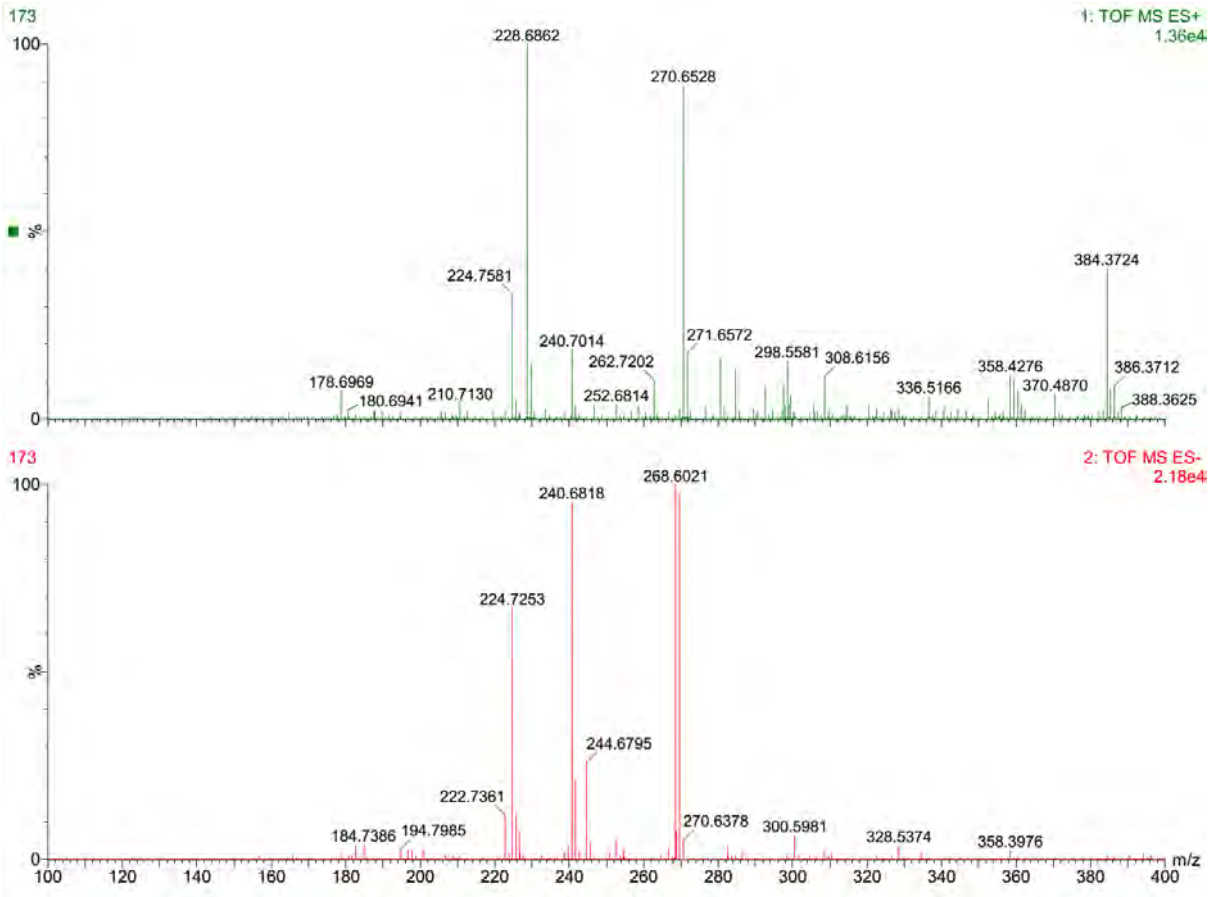


Figure A1.8.4. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 24.8 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 173.

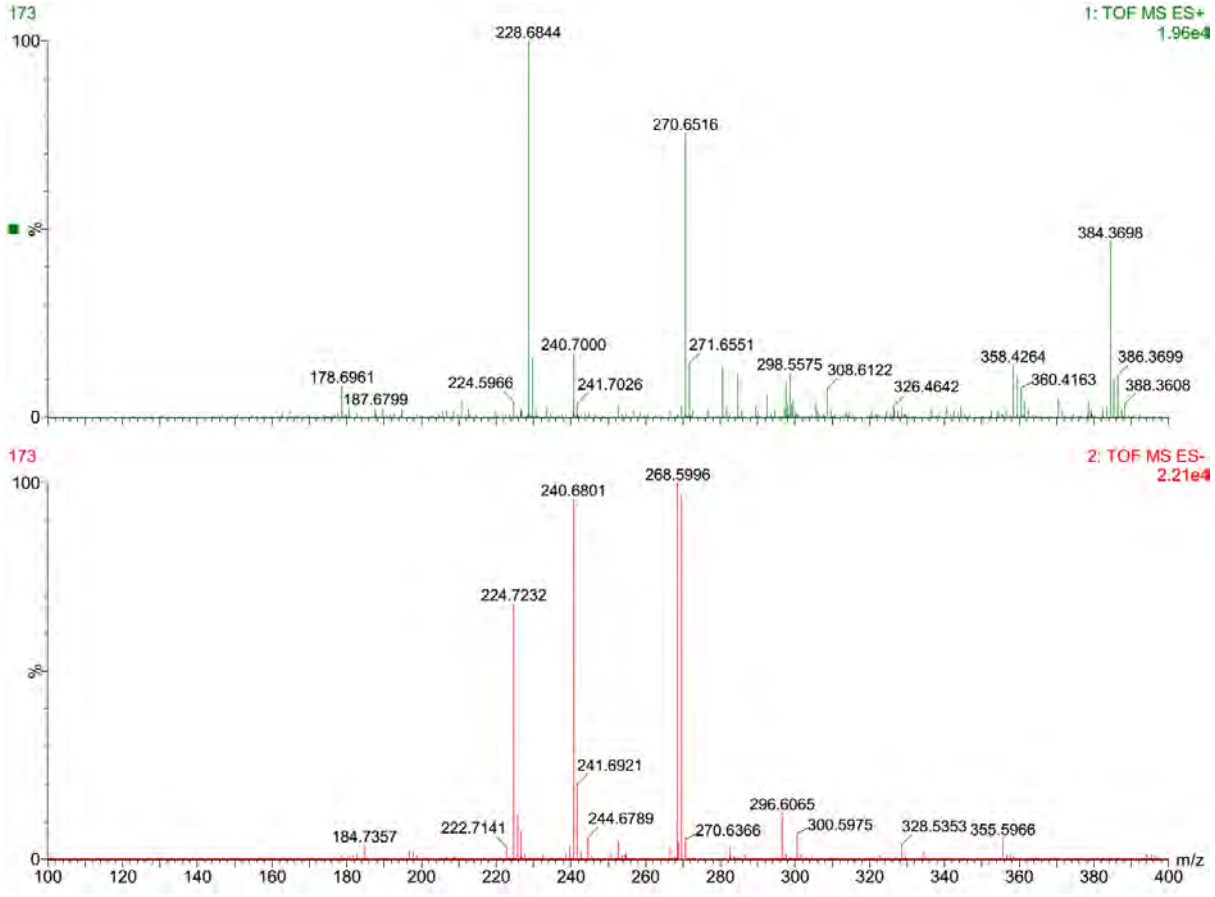


Figure A1.8.5. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 24.8 minutes from a methanol extract of C18-column cleaned lyophilised broth from Isolate 173.

1.9. UPLC-ESI-TOF-MS mass spectra resolved at 26.6 minutes

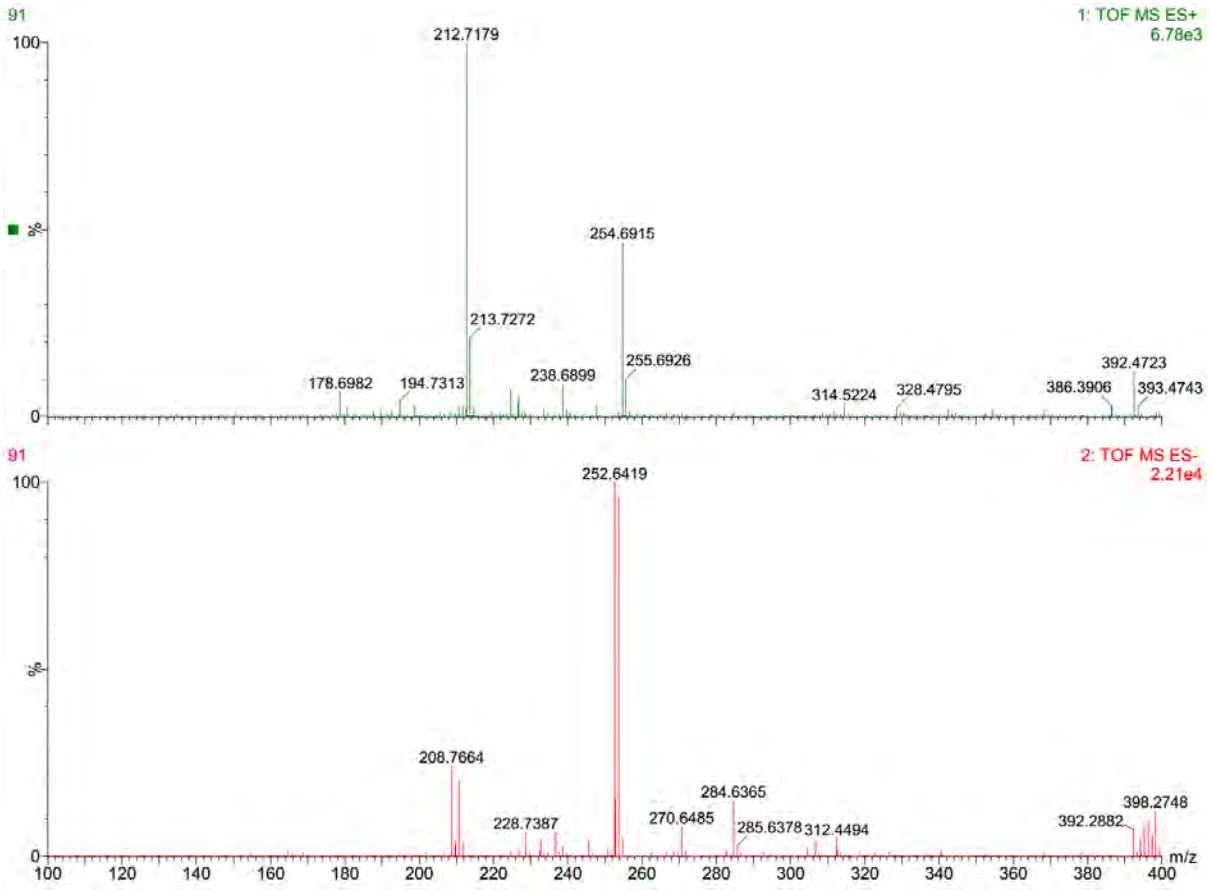


Figure A1.9.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 26.6 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 91.

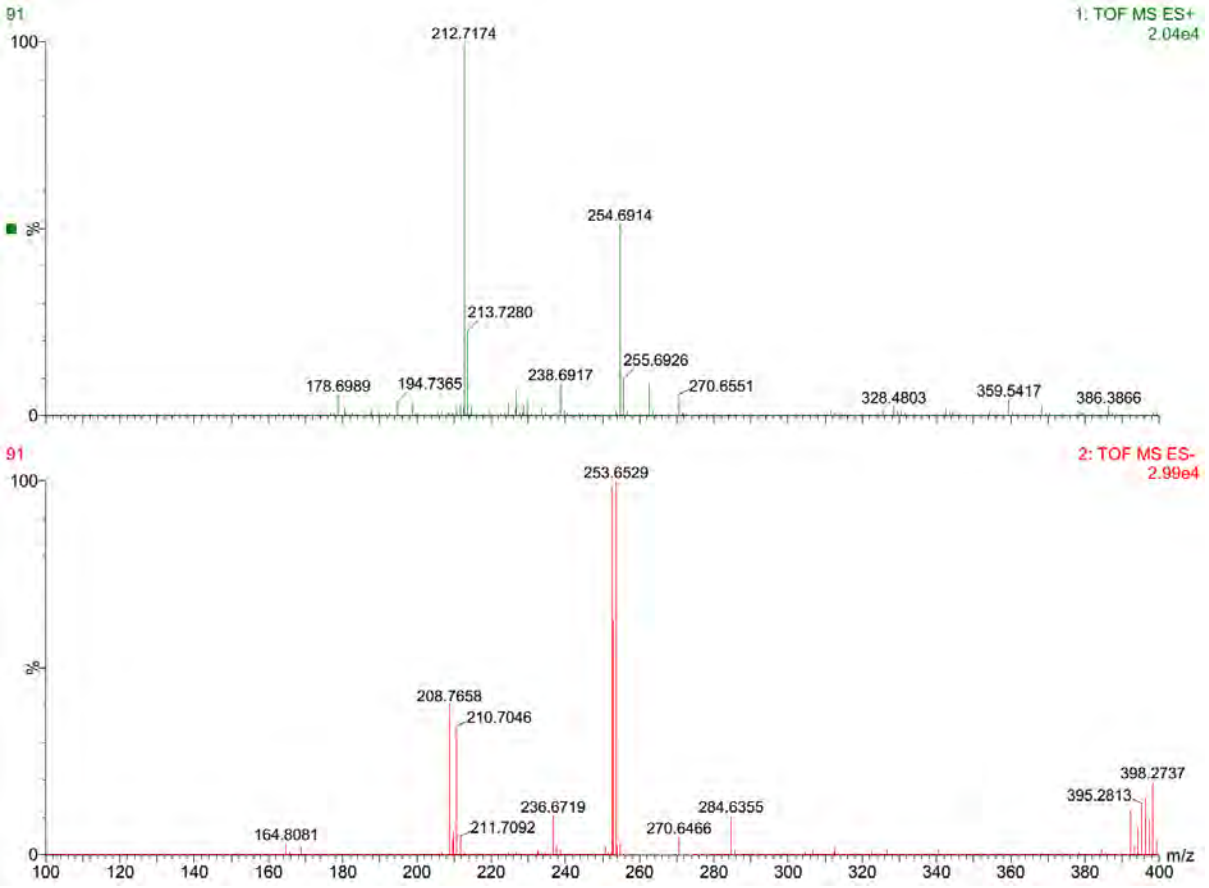


Figure A1.9.2. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 26.6 minutes from a methanol extract of C18-column cleaned lyophilised broth from Isolate 91.

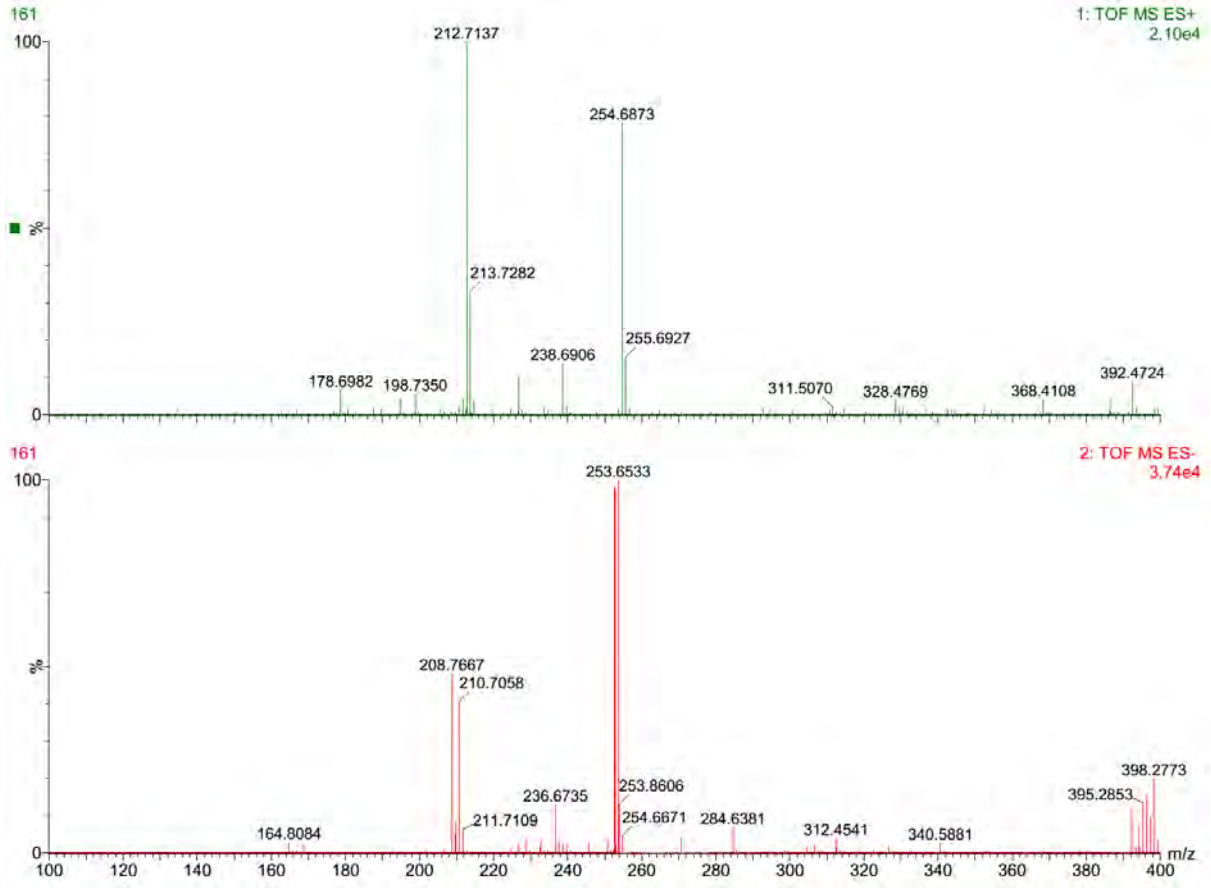


Figure A1.9.3. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 26.6 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 161.

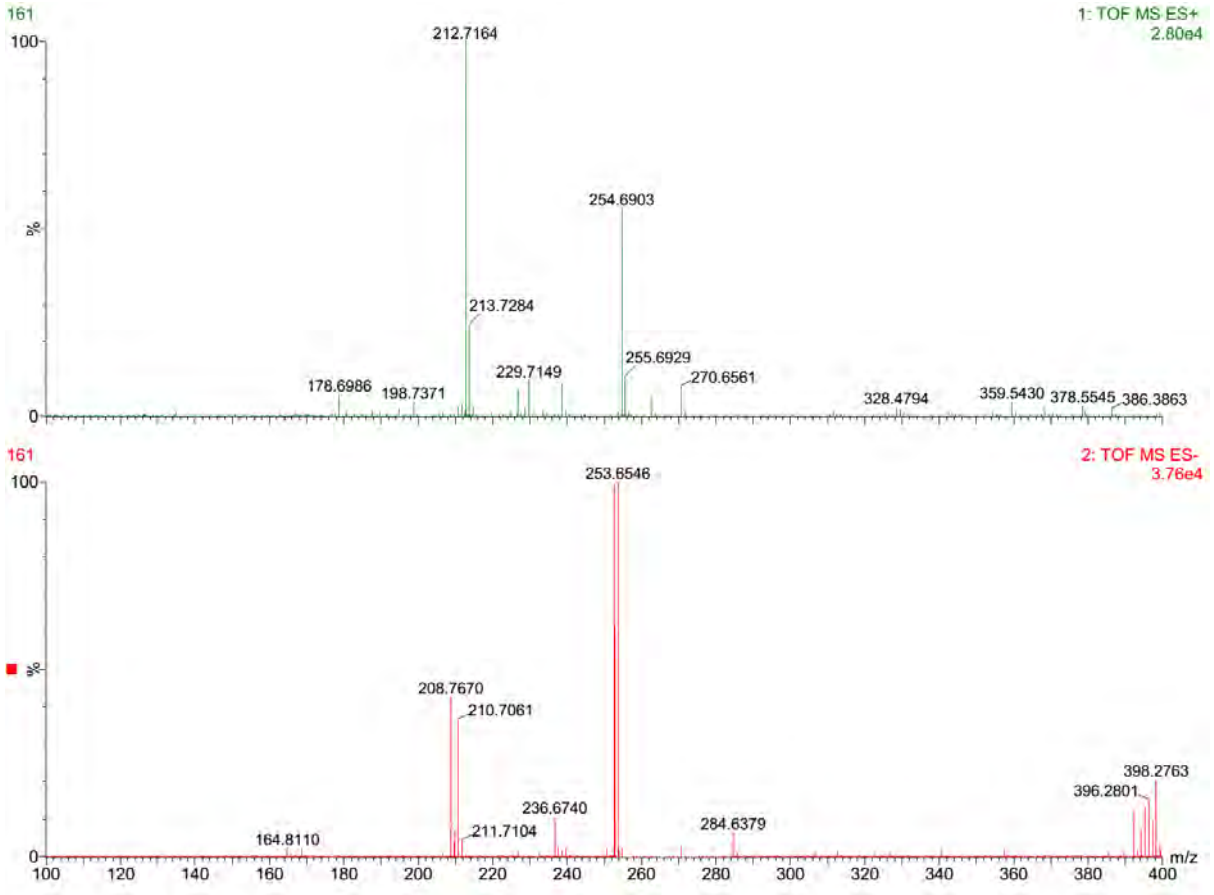


Figure A1.9.4. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 26.6 minutes from a methanol extract of C18-column cleaned lyophilised broth from Isolate 161.

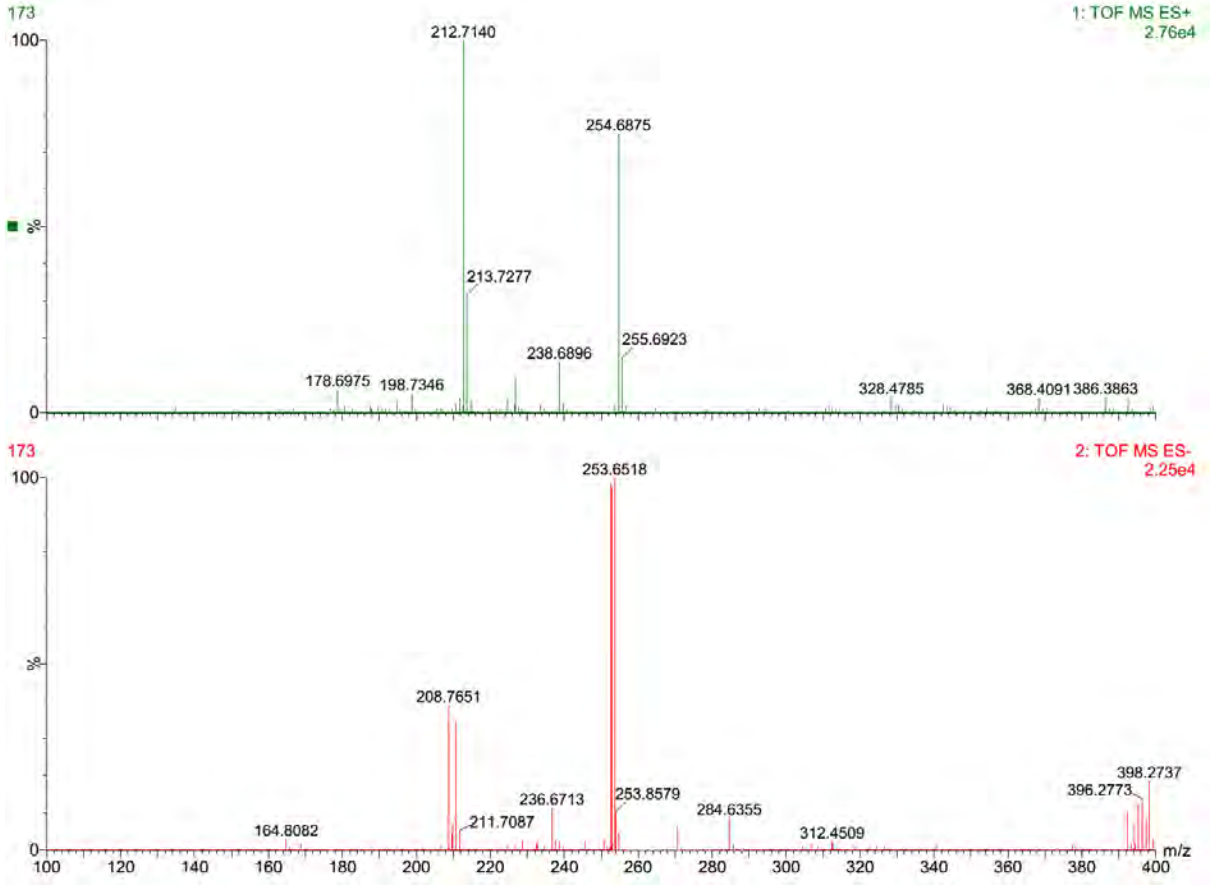


Figure A1.9.5. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 26.6 minutes from an ethyl acetate extract of cell-free culture broth from Isolate 173.

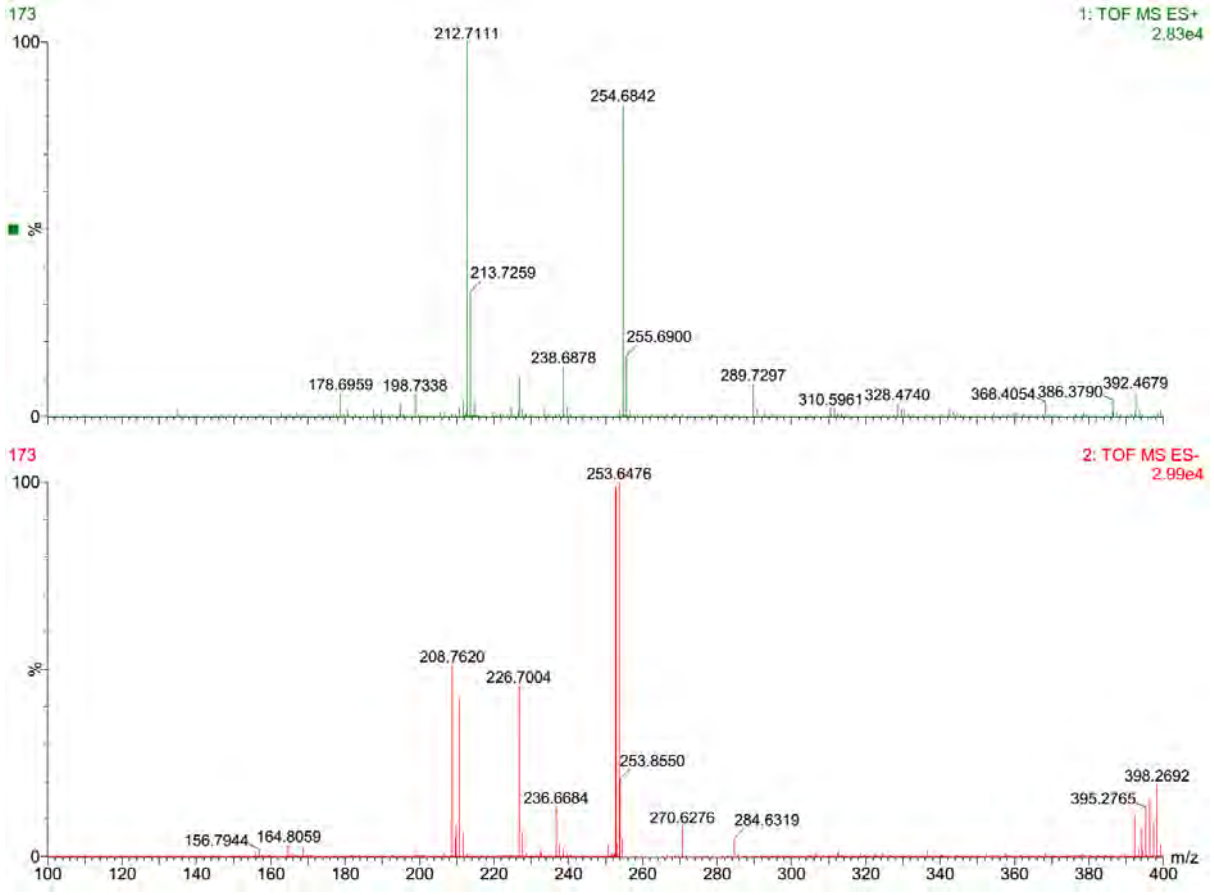


Figure A1.9.6. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 26.6 minutes from a methanol extract of C18-column cleaned lyophilised broth from Isolate 173.

1.10. UPLC-ESI-TOF-MS mass spectra resolved at 28.3 minutes

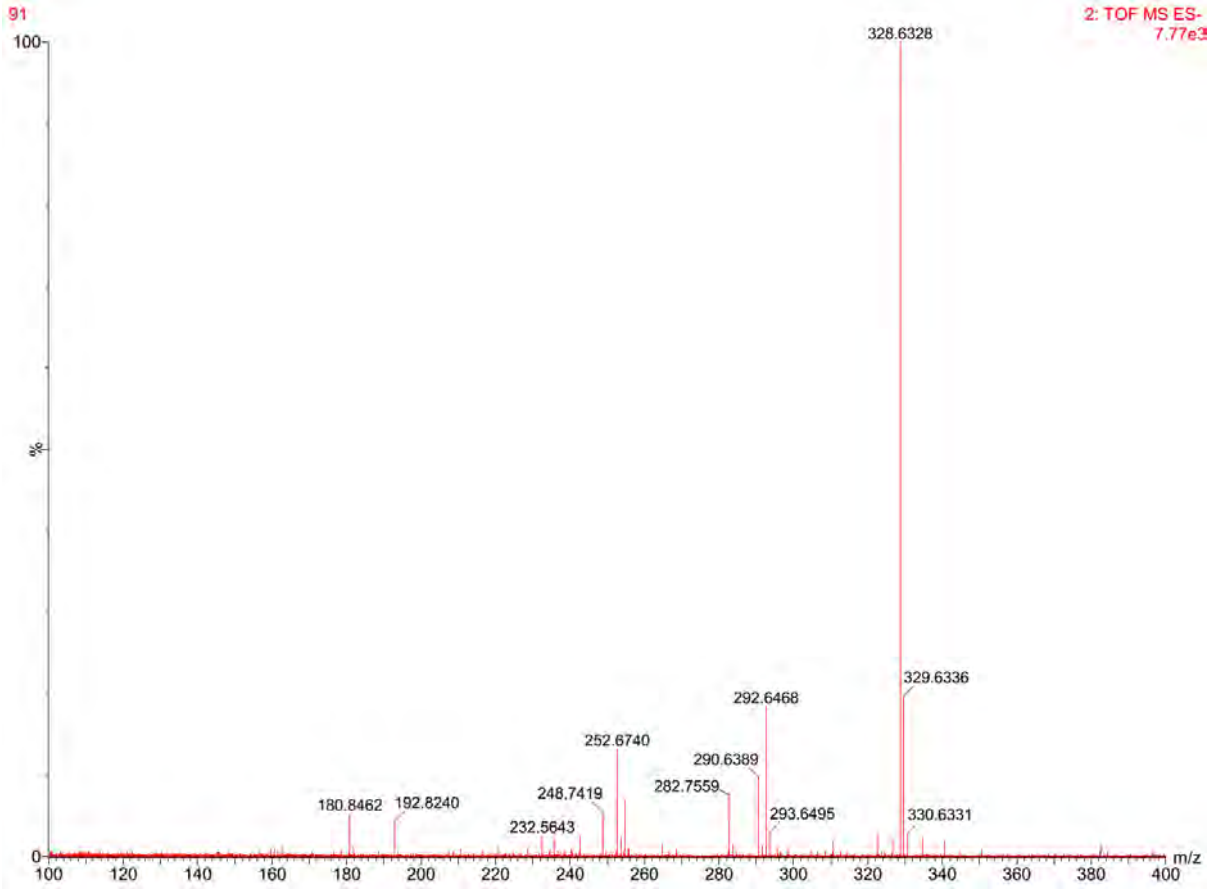


Figure A1.10.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 28.3 minutes from a methanol extract of C18-column cleaned lyophilised broth from Isolate 91.

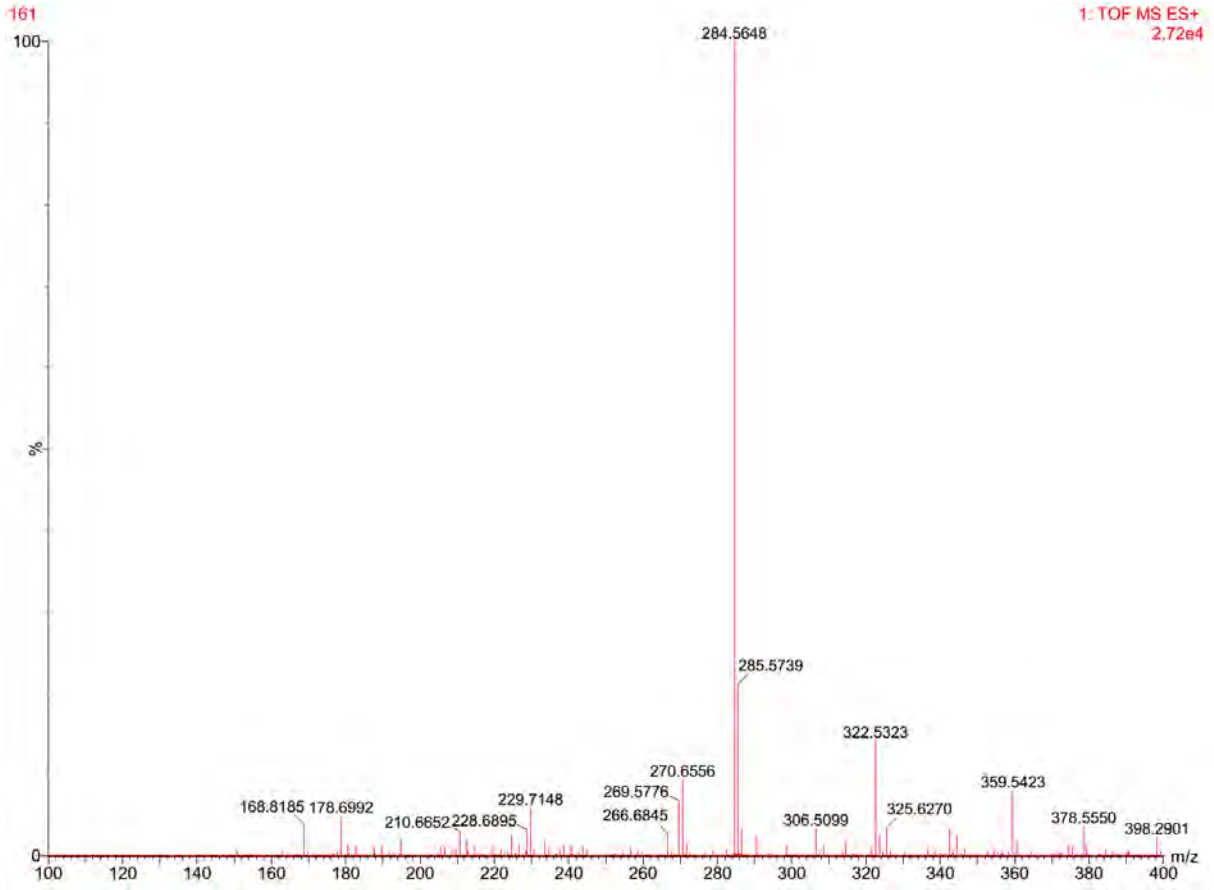


Figure A1.10.2. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 28.3 minutes from a methanol extract of C18-column cleaned lyophilised broth from Isolate 161.

1.11. UPLC-ESI-TOF-MS mass spectra resolved for the anthraquinone reference standard

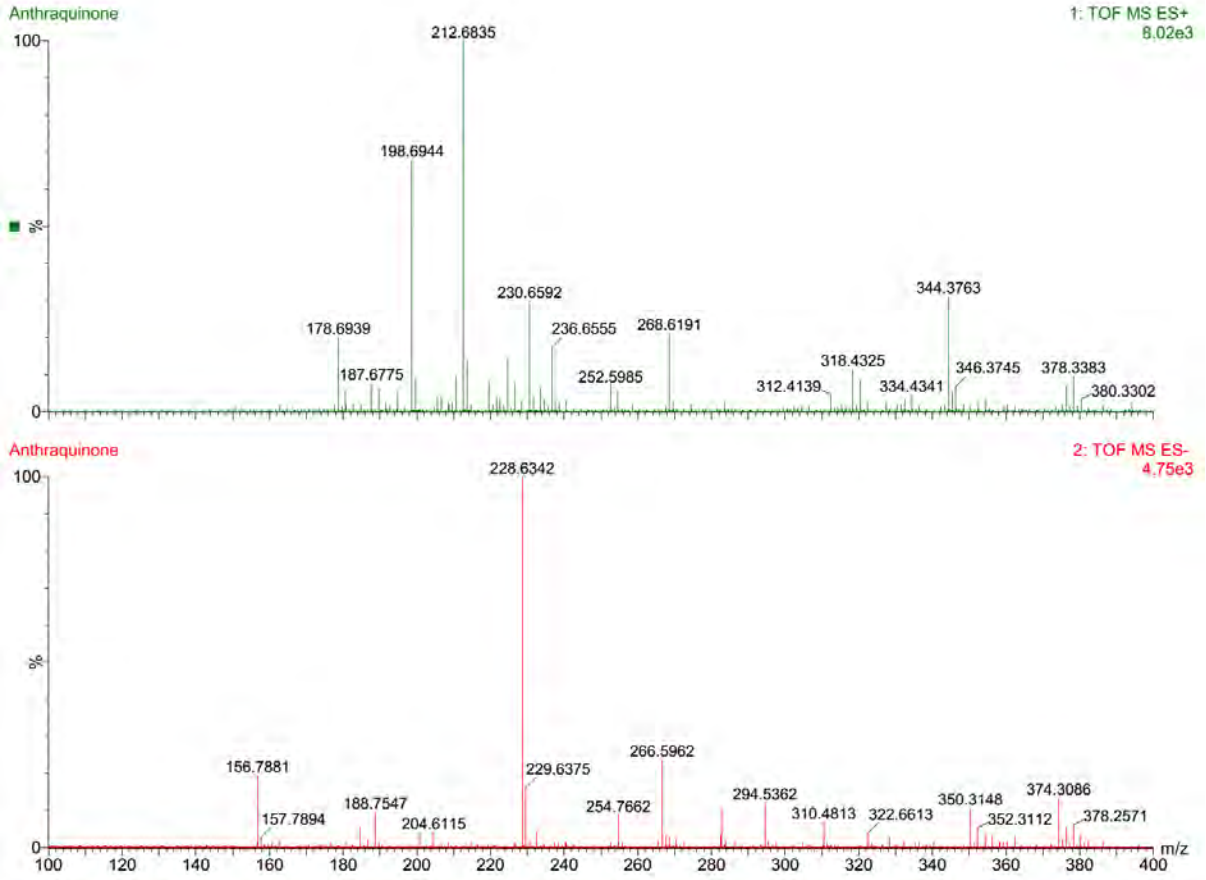


Figure A1.11.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 19.9 minutes from the anthraquinone reference standard.

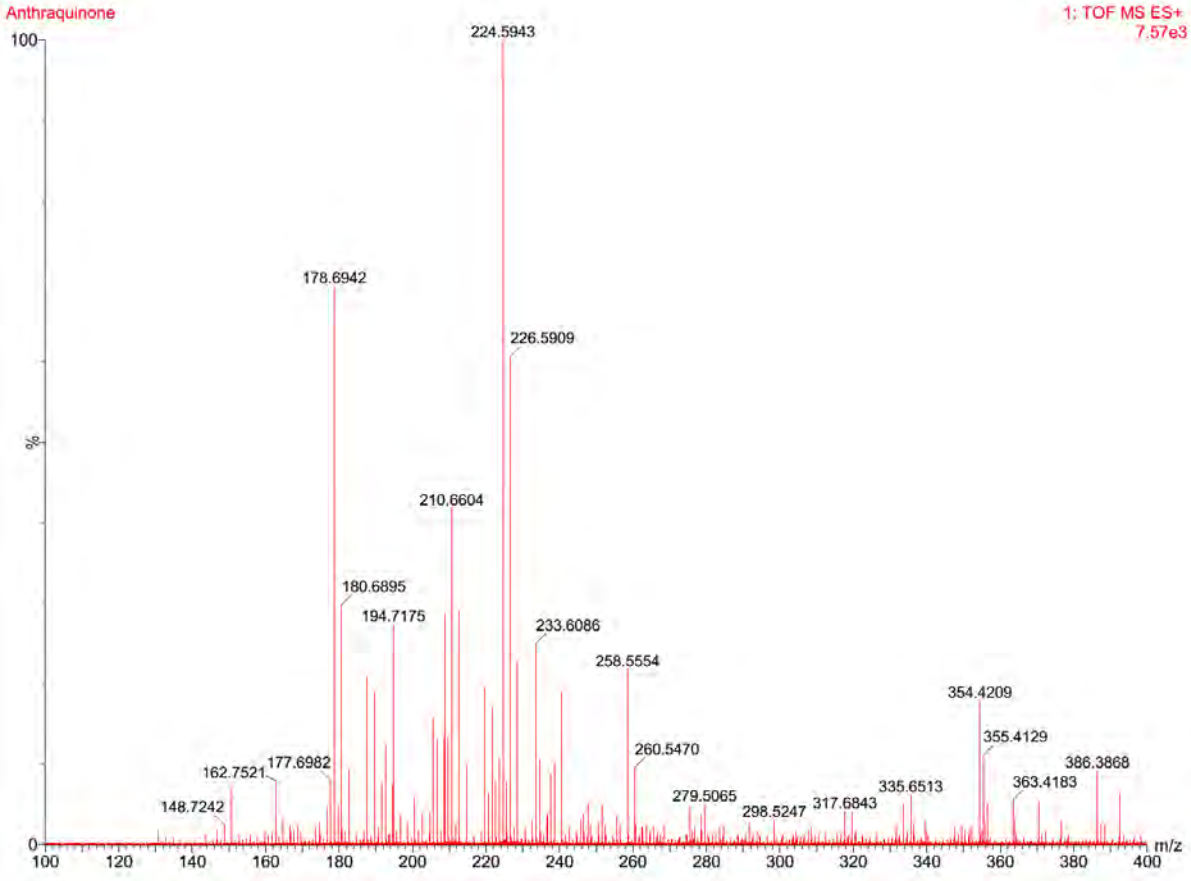


Figure A1.11.2. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 26.9 minutes from the anthraquinone reference standard.

1.12. UPLC-ESI-TOF-MS mass spectra resolved for the trans-stilbene reference standard

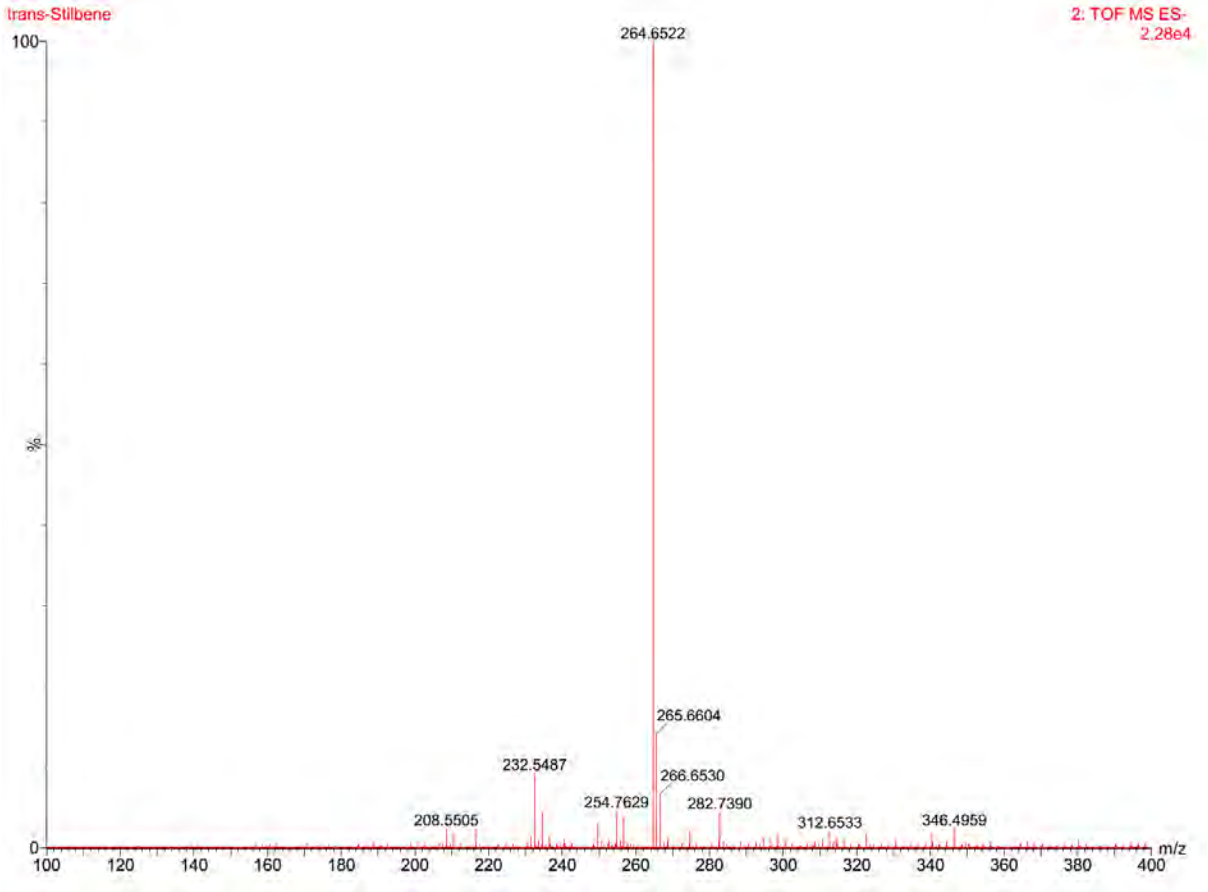


Figure A1.12.1. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 35.6 minutes from the trans-stilbene reference standard.

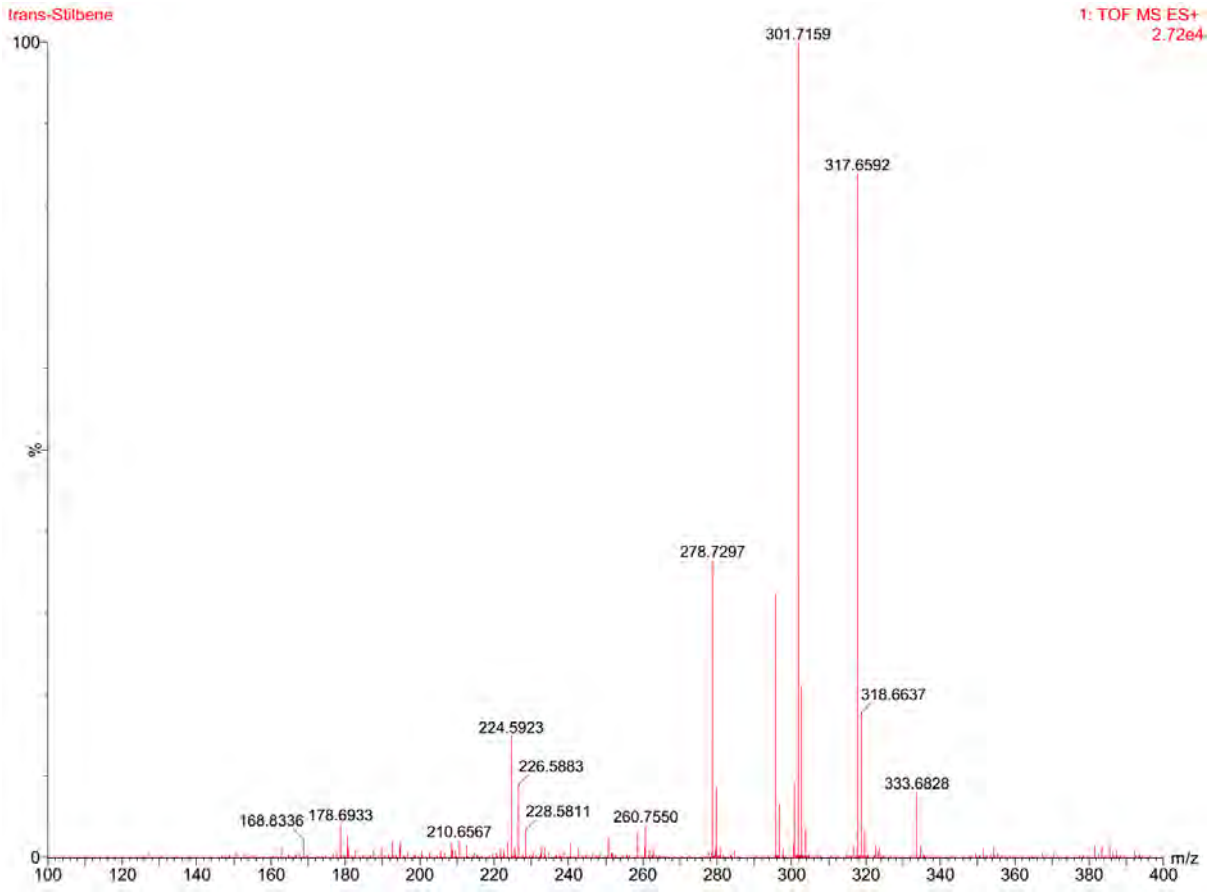


Figure A1.12.2. Mass spectra of a UPLC-ESI-TOF-MS peak resolved at 35.8 minutes from the trans-stilbene reference standard.