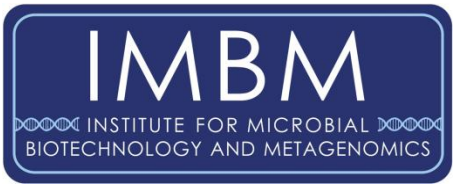


Biodiscovery of Novel Bioactivities from the South African Marine Environment

Reyghana Hoosen

UNIVERSITY of the
WESTERN CAPE

A thesis submitted in partial fulfilment of the requirements for the degree of

MAGISTER SCIENTIAE (M.Sc.)

Department of Biotechnology

University of the Western Cape

Bellville

Supervisor: Prof. Marla Trindade (Tuffin)

Date: 21st September 2016

Declaration

I, Reyghana Hoosen, hereby declare that "**Biodiscovery of Novel Bioactivities from the South African Marine Environment**" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Date: 21 September 2016

Signed: ……

http://etd.uwc.ac.za/

Abstract

There is currently a global health crisis taking place, which is the increasing occurrence of drug-resistant pathogens within clinical microorganisms. Drug-resistant pathogens are increasingly becoming more difficult to treat; whereas novel drug discovery has declined, therefore there is an urgency to develop novel drug candidates that are able to combat these infections.

The marine environment is an important source of biodiversity resulting from the various environmental niches that have formed due to a multitude of conditions such as, low temperatures, high pressure, various pH as well as salinity. These niches are potentially able to harbour diverse bacterial communities therefore making the marine environment a good source for the bioprospecting of novel microorganisms and antimicrobial compounds.

This study aimed to discover novel bioactive compounds from bacteria associated with two tunicate species, *Distaplia skoogi* and *Eudistoma sp.* 011RSASPN, that are endemic to the South African coast, to screen for antimicrobial activity as well as to identify the secondary metabolite genes responsible for the antimicrobial activity.

The methodology involved a culture-dependent strategy, where bacteria associated with the tunicate species were isolated and then subsequently screened for anti-microbial activity against a panel of indicator strains which included a multi-drug resistant *E. coli* strain. *Distaplia skoogi* yielded 157 morphologically distinct colonies, whereas 143 morphologically distinct colonies were isolated from *Eudistoma sp.* 011RSASPN. There were 33 isolates with antimicrobial activity associated with

http://etd.uwc.ac.za/

Distaplia skoogi, whereas, 45 isolates with antimicrobial activity were associated with *Eudistoma sp.* 011RSASPN.

Isolate PE12_11 displayed the most diverse activity profile and could potentially produce more than one antimicrobial compound. Isolate PE12_11 has 99% homology to *Paenibacillus polymyxa*. Several NRPS pathways were identified in PE12_11. The presence of *pmxA*, responsible for the production of polymyxin as well as the gramicidin synthase was detected by PCR amplification from PE12_11. Thus, it is expected that PE12_11 produces 2 well-known antibiotics.

This study highlights the South African coast as a diverse source of microbial natural products and should be exploited further for drug discovery.

Key words: marine biotechnology, tunicates, secondary metabolite production, drug-resistance, natural products, marine invertebrates, microorganisms, NRPS.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Acknowledgements

First and foremost, I would like to thank My Creator for guiding me and keeping me humble and focused during this journey as well as giving me the strength and ability to complete this study.

I would like to thank my parents for supporting me and affording me the opportunity to study at a Tertiary level.

I would like to thank my husband, Naasik Hendricks, for the constant support, encouragement, patience and understanding through this journey.

I would like to my supervisor Prof Marla Trindade for the opportunity to conduct this study in such an ideal environment and for guiding me through this study.

I would also like to thank Mr Lonnie Van Zyl, Dr Lucas Black and Dr William Mavengere for laboratory training and technical support.

The DST-NRF for providing the funding to allow me to fulfil this study.

IMBM for allowing me to complete my research in a world class laboratory.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Abbreviations

°C	Celsius
µF	Microfarad
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
µm	Micrometre
µM	Micromolar
ACP	Acyl carrier protein
ACM	Activated charcoal medium
A-domain	Adenylation domain
antiSMASH	Antibiotics and secondary metabolite analysis shell
AT	Acyltransferase
BLAST	Basic local alignment tool
Bp	Base pair
cDNA	Complementary DNA
C-domain	Condensation domain
CI	Chloroform isoamyl alcohol
Contig	Contiguous

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

dH_2O	Deionized water
DH-domain	Dehydratase domain
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ER	enoylreductase
g	Gravitational force
g/L	Grams per litre
GYM	Glucose yeast malt medium
HPLC	High-performance liquid chromatography
IMBM	Institute for Microbial Biotechnology and Metagenomics
Kb	Kilobase pairs
KCl	Potassium chloride
Km	Kilometres
KR-domain	Ketoreductase domain
KS-domain	Ketosynthase domain
Kv	Kilovolts
L	Litre
LB	Luria Bertani broth

UNIVERSITY of the

http://etd.uwc.ac.za/

M	Molar
Meq/ml	Milliequivalent per millilitre
mg/ml	Milligrams per millilitre
ml	Millilitre
ml/min	Millilitre per minute
mM	Millimolar
MRSA	Methicillin resistant Staphylococcus aureus
MS	Mass spectroscopy
MT	Methyltransferase
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NMR	Nuclear magnetic resonance
NP	Natural product
NRP(s)	Non-ribosomal peptide(s)
NRPS	Non-ribosomal peptide synthase
OD	Optical density
OSMAC	One strain many compounds
PCI	Phenol/Chloroform/Isoamyl alcohol
PCP	Peptidyl carrier protein

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

PCR	Polymerase chain reaction
PKS	Polyketide synthase
PK(s)	Polyketide(s)
PPTase	Phosphopantetheine transferase
RNA	Ribonucleic acid
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SCUBA	Self-contained underwater breathing apparatus
Secs	Seconds
TAE	Tris-Acetate-EDTA buffer
TE	Tris-EDTA buffer
TE	Thioesterase
TB	Tuberculosis
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Table of Contents

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

List of Figures

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

http://etd.uwc.ac.za/

List of Tables

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Chapter 1: Literature Review

1.1 Introduction

The marine environment is ubiquitous with organisms that produce potent bioactive compounds. Such natural products have mainly been mined from terrestrial sources; however, a shift in focus to marine-derived natural products has increased over the last few years. This is due to the rise in antibiotic resistant microorganisms, which has necessitated the development of alternative antibiotics (Sarker et al. 2007; Prabhu et al. 2011). Numerous natural products have been isolated from marine invertebrates such as sponges and ascidians. Sponges are the most predominant source of marine natural product research; however, ascidians have been classified as the second predominant source of novel natural products (Kennedy et al. 2011; Perez-Matos and Rosado, 2007). In 1993, the first marine compound that was used in anti-cancer human clinical trials was from the marine ascidian, *Tridemnum solidum*, a tunicate; it was a purified natural product; however, it was unsuccessful in further trials (Davidson, 1993).

UNIVERSITY of the
WESTERN CAPE

The need for novel antibiotics is rising each year as medically relevant bacteria are becoming resistant to known antibiotics. This places pressure on the pharmaceutical industry to develop new antibiotics to combat resistant bacteria (Wang et al. 2012). The marine environment is suitable as it has been shown to harbour an enormous diversity and abundance of organisms that have previously produced novel antibiotics (Perez-Matos and Rosado, 2007).

Pharmaceutical drugs are generally small molecules that are able to bind to specific molecular targets and are capable of activating or deactivating the targeted biological processes (McGuire et al. 2007). Many drugs are produced through organic

http://etd.uwc.ac.za/

synthesis, and a vast majority of them can be taken orally. However, biopharmaceuticals are generally large biological molecules, like proteins, that are developed to target regions that are unable to be accessed by the smaller molecular variations. Biopharmaceuticals are usually injected because of their large size, coupled with the difficulty of surviving the stomach, colon and liver (McGuire et al. 2007). Although chemical synthesis of novel drugs has been faster and cheaper than the biological approach of screening organisms and extracts for novel drug candidates, the synthetic chemical libraries that have been developed have not delivered novel drugs (Machado et al. 2015). It should also be noted that a high percentage of novel drugs that have entered the pharmaceutical market have been derived from natural products (Newman and Cragg, 2015).

The pharmaceutical industry has also relied heavily on the use of natural products from plants (Drewes, 2012). This has been a huge market for many years; however, certain drugs cannot be found in plants and therefore an alternate source had to be identified in order to produce novel drugs and drug therapies. The marine environment offers this alternative source (Drewes, 2012; Davies-Coleman, 2010).

Natural products derived from microorganisms and invertebrates from the marine environment have shown promising results to combat antibiotic resistance. Natural products have also been shown to be instrumental in the development of anti-bacterial, anti-fungal, anti-tumour and anti-cancer drug therapies (Wang et al. 2012; Arasu et al. 2013; Piel et al. 2004; Chait et al. 2010; Boopathy and Kathiresan, 2011).

This study, therefore, aims to discover novel bioactive compounds from the South African marine environment to be used in the pharmaceutical industry.

UNIVERSITY of the WESTERN CAPE
http://etd.uwc.ac.za/

1.2 The Marine environment as a novel source of natural products

Approximately 70% of the earth's surface is covered with water; with 98% of the total space that is available to life being in the ocean (Levinton, 2001). The marine environment is highly diverse; this diversity includes microorganisms as well as invertebrates and marine animals.

The average salinity in the ocean is 3.5% of sodium chloride; which is reduced in coastal waters that have an average salinity of 3.1% sodium chloride content. The mineral content of the ocean varies greatly and is not as simple as salinity (Speight and Henderson, 2010). There are 11 chemicals that are dominant in the marine environment, as well as a large number of trace elements that are pivotal for biological functions such as nitrogen and calcium. Nitrogen is important for the formation of amino acids, and calcium is one of the building blocks of exoskeletons for marine invertebrates. The above factors of salinity along with the nutrients within the ocean, make the marine environment a good source of novel bacteria, due to the nutrient diversity present in the oceans (Speight and Henderson, 2010). Due to the diverse nature of the marine environment itself, one can expect that the organisms that inhabit the marine environment would be very diverse. The different zones of the ocean contain different nutrients and minerals therefore the microorganisms that inhabit the various zones will have metabolites as well as functions that are conducive to its specific zone. Literature has indicated there is a high probability that the diversity as well as the potential for novel antibiotics and enzymes in the marine environment is promising (de Voogd and Clearly, 2008; Baker et al. 2010; Phelan et al. 2012).

International research has concentrated on soft bodied, sessile invertebrate phyla as a source for novel natural products. The rationale behind this is due to the fact that

UNIVERSITY of the WESTERN CAPE
http://etd.uwc.ac.za/

these phyla have been shown to produce an abundance of secondary metabolites for use in predator defence, antifouling, inhibition of overgrowth, protection from ultraviolet radiation as well as acting as mediators in the competition for settling space. These metabolites harbour pharmaceutical value (Pawlik, 1993; Williams et al. 1989).

1.3 Marine invertebrates as a source of natural products

According to the marine biota data it is recorded that there are 12914 species of marine flora and fauna, that translates to roughly 15% of all the coastal marine species known worldwide, with approximately 12% of these species being endemic to South African coastal marine areas (Branch et al. 1994), of which 2500 benthic invertebrate communities have been collected (Griffiths et al. 2010).

UNIVERSITY of the
WESTERN CAPE

It is estimated that marine species encompass approximately half of the total global biodiversity (De Vries and Beart, 1995) therefore it is reasonable to assume that the marine environment offers an enormous potential resource for novel compounds with possible pharmaceutical value. Marine invertebrates have evolved over millions of years in a constant environment and therefore have had time to divert their energy resources into the development of toxic secondary metabolites as an integral part of a chemical survival strategy (Jensen and Fenical, 1994). Due to the high diversity as well as species richness located in the marine environment, several animal phyla may be located in a small region and are able to coexist with each other (de Voogd and Clearly, 2008). In 1993, it was reported that there were 31 animal phyla found in marine habitats. Of these 31 phyla, 15 were found to be endemic to the ocean; another 3 phyla were added between 2008 and 2009. From these 15 phyla, 10 are

http://etd.uwc.ac.za/

benthic, 1 is pelagic as well as being present in the water column, 2 of these phyla are both pelagic and benthic and 2 are endoparasitic. The additional phyla indicate that many in marine habitats are yet to be identified and classified. In comparison, there are only 11 terrestrial phyla as compared to the 15 endemic phyla from marine habitats. This leads one to expect that the seabed contains the most diversity which can be attributed to the variety of habitats present (Slyusarev, 2008; Bourlat et al. 2008; Philippe et al. 2009). The relative abundance according to taxonomic group of marine species is as follows; 3% Macroalgae, 5% Chordates, 3% Viruses, 13% Protozoa, 66% Invertebrates and 10% other Algae (Speight and Henderson, 2010). The largest marine diversity is found in the tropics; however, the species richness tends to be aligned with the latitude within its specific region (Bourlat et al. 2008).

UNIVERSITY of the
WESTERN CAPE

1.3.1 Tunicates

Tunicates are marine invertebrates that belongs to the phylum Chordata and the subphylum Urochordata. They are sedentary animals that are widely distributed in the marine environment. Tunicates are the most primordial chordates and are inherently more similar to vertebrates than invertebrates (Burighel et al. 2008). There are three groups of tunicates namely; Ascidians, Thaliceans and Appendicularians/Larvaceans (Holland, 2007).

Tunicates are mainly composed of a substance that is similar to cellulose. Tunicates are also referred to as sea squirts since they are able to expel a stream of water when agitated (Wada et al. 1992; Karleskint, 2012). Tunicates are usually cylindrical and consist of two tubes that project from them (Figure 1.1). The incurrent siphon

http://etd.uwc.ac.za/

allows water and food to enter it and an excurrent siphon expels wastes and water. (Burighel et al. 2008).

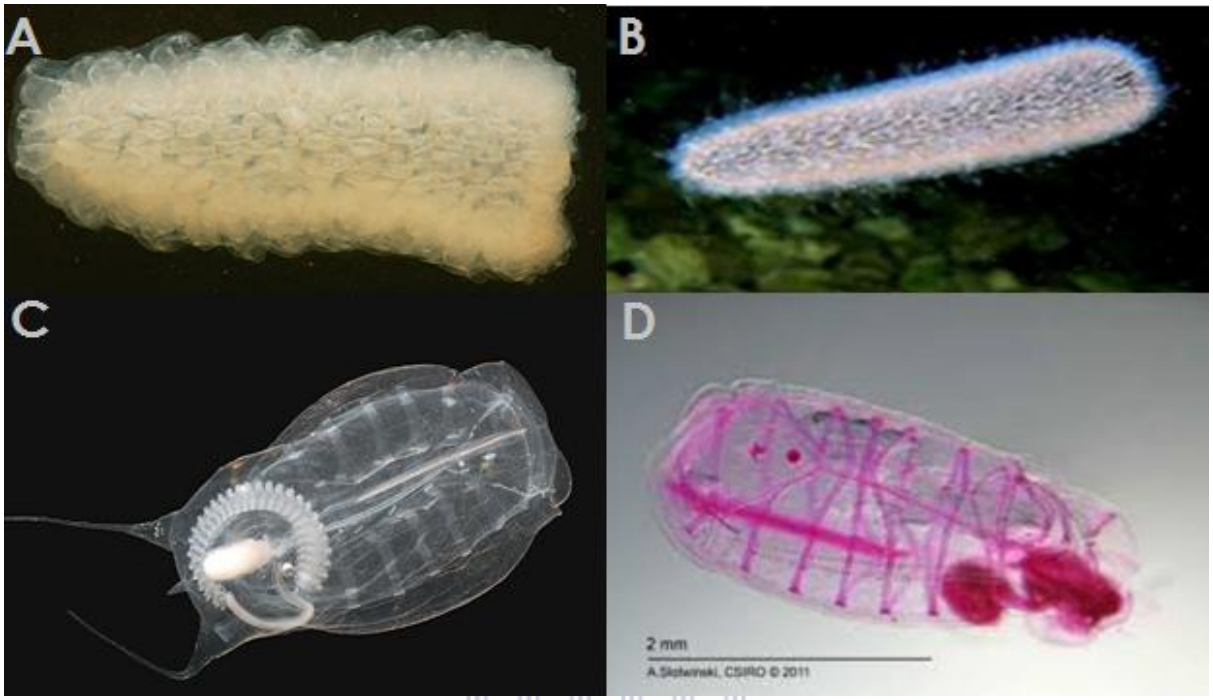


Figure 1.1: Tunicate species endemic to South Africa. A: *Pyrosoma* sp.; B: *Pyrosoma atlanticum*; C: *Thalia sp.* and D: *Thalia democratica*. Adapted from: http://www.biodiversityexplorer.org/mm/tunicates/pyrosoma.htm; http://www.sealifebase.org/identification/specieslist.php?famcode=1350&areacode; http://www.biodiversityexplorer.org/mm/tunicates/thalia.htm and www.imas.utas.edu.au respectively.

UNIVERSITY of the WESTERN CAPE

Thaliaceans are tunicates that are able to freely swim around and have their incurrent and excurrent siphons positioned opposite each other. They are able to pump water through their body as they swim, thereby extracting food as well as eliminating wastes. A number of these species are bioluminescent and their number increases rapidly when food is readily available (Karleskint, 2012; Aoki et al. 1989). These planktonic sea squirts consist of a muscular body that is surrounded by a jelly-like capsule. There are two orders endemic to South Africa namely; Salps and

http://etd.uwc.ac.za/

Doliolids (Branch et al. 1994). The three-tailed salp in the *Thalia* sp. (Figure 1.1 C&D) is found on the West coast leading up to Namibia, whereas the Fire Roller from the *Pyrosoma atlanticum* sp. (Figure 1.1B) is found all along the coast line. The Fire Roller is hollow and closed at one end and becomes luminescent when it is threatened or disturbed (Branch et al. 1994).

The survival of tunicates is dependent on the symbiotic relationship they have with their associated microorganisms since these aid in fulfilling both primary and secondary metabolic functions within the tunicate (Donia et al. 2011). The functional role of tunicates has been shown to be involved in nutrient cycling including nitrogen, oxygen and carbon (Kuhl et al. 2012). Tunicates are known to be abundant producers of secondary metabolites which are considered target compounds for pharmaceutical drugs for example anti-tumour, anti-cancer and antibiotics as well as cyclic peptides (Proksch et al. 2002, Adrian, 2007). It has been proposed that approximately 1080 natural products have been isolated from ascidians, with compounds from the Didemnidae family having contributed to 35% of these (Blunt et al. 2015 and Shenkar and Swalla, 2011).

UNIVERSITY of the
WESTERN CAPE

The major groups of metabolites that are produced from tunicates and their symbiotic microorganisms include alkaloids, peptides containing tyrosine and phenylalanine residues as well as cyclic peptides. Pharmaceutically they have produced antibacterial compounds that act against Gram-Positive organisms as well as antifungal, anticancer and antiviral compounds (Wang et al. 2012).

http://etd.uwc.ac.za/

1.4 Microbial symbionts as natural product producers

Marine invertebrates are known to contain both epibiotic as well as endobiotic microorganisms; this is due to the porous nature in the case of sponges and tunicates, therefore allowing the microorganisms to move in and out of the invertebrate freely. The symbiotic relationship that exists between tunicates and endosymbionts occurs through vertical transmission, ensuring that the offspring inherit the microorganisms that are required for their survival. This is believed to provide the tunicates with a competitive advantage from their early stage of development and is correlated with obligate symbioses (Lopez-legentil et al. 2011).

Endosymbionts are able to increase the integrity of their hosts as well as the hosts defence mechanisms. They produce extremely powerful secondary metabolites that are able to inhibit the physiological processes of their host's prey (Stachowicz et al. 2000; Donia et al. 2011, Schmidt et al. 2012). These natural products, often referred to as chemical weapons, have evolved into highly effective inhibitors and as such are sought after compounds in drug discovery studies.

UNIVERSITY of the
WESTERN CAPE

Interestingly, many natural products produced from invertebrates are either identical or structurally similar to the natural products produced by their associated microorganisms (Schmidt et al. 2012). Several natural products which have been identified from invertebrates have recently been shown to actually be produced by the associated symbionts (Phelan et al. 2012; Tsukimoto et al. 2011). Microorganisms live symbiotically with invertebrates therefore, the error in identifying the secondary metabolite from the invertebrate instead of the microorganisms is common (Donia et al. 2011, Schmidt et al. 2012).

http://etd.uwc.ac.za/

A typical example of this misconception was evident in the case of bryostatin, which is a protein kinase modulator that has been shown to have anti-cancer and anti-HIV bioactivity (Staveness et al. 2016). It is a type I polyketide, which was assumed to be produced by the marine bryozoan *Bugula neritina* instead of a symbiotic microorganism. A study by Hildebrand et al. 2004 in which a metagenomic approach was utilized, confirmed that the bryostatin was indeed produced by a microbial symbiont and not by the bryozoan (Davidson et al. 2001; Hildebrand et al. 2004; Trindade et al. 2015). A study by Steinert et al. 2015 has suggested that the marine ascidian-associated *Salinispora* and *Verrucosispora* bacteria may potentially be responsible for producing staurosporines that have previously been extracted from the ascidian *Eudistoma toealensis.* Staurosporines are characterised as potent protein kinase inhibitors. They are able to inhibit protein kinase A, protein kinase C, and protein kinase G (Karaman et al. 2008).

UNIVERSITY of the
WESTERN CAPE

Polytheonamides represent another example. They are classified as proteusins; they are able to perforate the bacterial membrane thus causing the leakage of components from the cell (Arnison, 2013). They were originally isolated in 1994 from the marine sponge *Theonella swinhoei* in the Fusetani and Matsunaga lab (Hamada et al. 1994), while the gene cluster of polytheonamides was discovered in 2012 by the Piel laboratory (Freeman et al. 2012). Due to the lack of introns in the gene cluster and the structural similarity to the bacterial-produced proteusins, it was proposed that it had a bacterial origin (Freeman et al. 2016). The bacterium responsible for producing polytheonamides has been identified as an uncultivated sponge symbiont generally referred to as ‘Entotheonella factor’. More recently it has been proposed that the ‘Entotheonella factor’ be called ‘*Candidatus* Entotheonella factor’ and ‘*Candidatus* Entotheonella gemina’ (Ueoka et al. 2015).

http://etd.uwc.ac.za/

Pseudovibrio species have been confirmed to be associated with marine invertebrates such as tunicates, corals and sponges (Harrington et al. 2014), entering the sponges through vertical transmission (Muscholl-Silberhorn et al. 2008). *Pseudovibrio sp.* D323 was found to be a symbiont from the red algal species *Delisea pulchra*. Tropodithietic acid, an antibacterial compound, was isolated from *Pseudovibrio sp.* D323 (Penesyan et al. 2010). A study by Harrington et al. 2014 has confirmed that *Pseudovibrio* sponge symbionts are responsible for producing this antibacterial compound that acts against fish pathogens.

These examples give confidence that marine organisms harbour diverse symbiotic microorganisms which represent a promising source of novel natural products.

UNIVERSITY of the
WESTERN CAPE

1.4.1 Structure and diversity of Microbial Derived Marine Natural Products

There are many different classes of marine natural products, such as polyketide, non-ribosomal peptides, terpenoids, alkaloids, etc. Approximately 250 bacterial secondary metabolites have been described from the marine environment since the year 2000. During that same period only 150 bacterial secondary metabolites were derived from terrestrial environments (Schmidt et al. 2012). The marine metabolites were isolated from marine invertebrates as well as their endosymbionts; and include *Cyanobacteria, Streptomycetes, Actinobacteria* and *Pseudomonas* (Kennedy et al. 2008). Secondary metabolites are biologically significant as well as being chemically interesting; and many are used as antibiotics, antifouling agents, immune-suppressive agents, and anticancer compounds (Perez-Matos and Rosado, 2007; Schmidt et al. 2012; Wang et al. 2012).

http://etd.uwc.ac.za/

Indolocarbazole alkaloids are a group of natural products that have unique structural features as well as therapeutic properties. The majority are characterized by an indolo[2,3-α]pyrrolo[3,4-c]carbazole core with a sugar attached to it. Studies revealed that indolocarbazoles display several biological activities; however, the main interest lies in the ones that produce antitumor and neuroprotective properties (Sanchez et al. 2005). The indolocarbazole core is formed by the decarboxylative synthesis of two units derived from tryptophan, while the sugar moiety comes from glucose (Pearce et al. 1988).

Alkaloids are a group of chemical compounds that occur naturally in the environment, and are mostly comprised of nitrogen atoms. They are produced by several organisms as well as microorganisms (Manske, 1965). They are pharmacologically relevant in that they have a very broad range of bioactivities which includes; antibacterial, anticancer and antimalarial to name a few (Kittakoop et al. 2014). Alkaloids are characterized by their structural diversity since there is no uniform classification (Cushnie et al. 2014).

UNIVERSITY of the
WESTERN CAPE

The terpenoids also known as isoprenoids are a large and diverse class of naturally occurring compounds that are similar to terpenes. They are a derivative of isoprene units with a five-carbon compound structure, and are assembled and adapted in many different ways. Most terpenoids are multi-cyclic compounds that vary from each other in functional groups as well as in their carbon skeletons. They are lipids that are found in all classes of living things, they constitute the largest group of natural products, with roughly 60% of known natural products belonging to the terpenoids class (Firn, 2010).

http://etd.uwc.ac.za/

1.4.2 Polyketides and Non-Ribosomal Peptides

The Polyketide (PK), Non-Ribosomal Peptide (NRP) as well as hybrids of these two classes represent classes of compounds which have been shown to have interesting biological activities (Fischbach and Walsh, 2006). PK and NRP are two large families of complex natural products built from simple amino acid monomers (NRP) or carboxylic acid monomers (PK) (Khosla et al. 2009).

There are substantial differences between these two families; however, they are both synthesized biologically under the control of large multifunctional proteins. NRPS and PKS have recurrent coordinated groups of active sites known as modules; each module is responsible for catalysis of one complete cycle of polyketide, polypeptide or non-ribosomal peptide chain elongation as well as its associated functional group modification. PK and NRP have medicinal as well as agrochemical properties. Examples of polyketide antibiotics include tetracycline and erythromycin; NRP antibiotics include cephalosporin, penicillin and vancomycin, which is a glycopeptide (Fischbach and Walsh, 2006).

UNIVERSITY of the
WESTERN CAPE

The number of polyketides and non-ribosomal peptides produced under normal culturing conditions are less than the number of respective pathways encoded. This has been identified due to extensive research that has taken place since the genomic revolution started. The discrepancy in the natural products acquired against the gene cluster analysis leads to the conclusion that the biosynthesis genes that encode for natural products are not constitutively expressed but rather the genes are tightly regulated (Jimenez et al. 2010). The biosynthetic pathways of NRP and PK have been shown to be conserved in marine and terrestrial systems; however, there are novel catalytic enzymes that are only located in the marine environment and

http://etd.uwc.ac.za/

these catalytic enzymes are accountable for the unique functional groups in marine natural products (Davidson et al. 2001).

1.4.2.1 Polyketide Synthases

Polyketide (PK) metabolites are very diverse in chemistry and function, containing antibiotic, antitumor and antifungal properties. Despite the huge diversity, research has shown that the biosynthesis of PKs occurs via the consecutive condensation of small carboxylic acids which is paralleled with the similar mechanism of fatty acid synthesis that occurs in bacteria as well as in both higher and lower eukaryotes (Figure 1.2). The genes that are encoded for a specific polyketide are usually organized in one operon/gene cluster in bacteria (Hopwood and Sherman, 1990). Polyketide synthases (PKS), the enzymes that make the polyketide compounds, perform three essential reactions namely, chain initiation, elongation and cyclization (Fujii et al. 2001); and are classified into 3 classes: Type I, Type II and Type III. The differences in the types of PKS are based on the mode of synthesis as well as the type of structural product that they produce (Jimenez et al. 2010).

http://etd.uwc.ac.za/

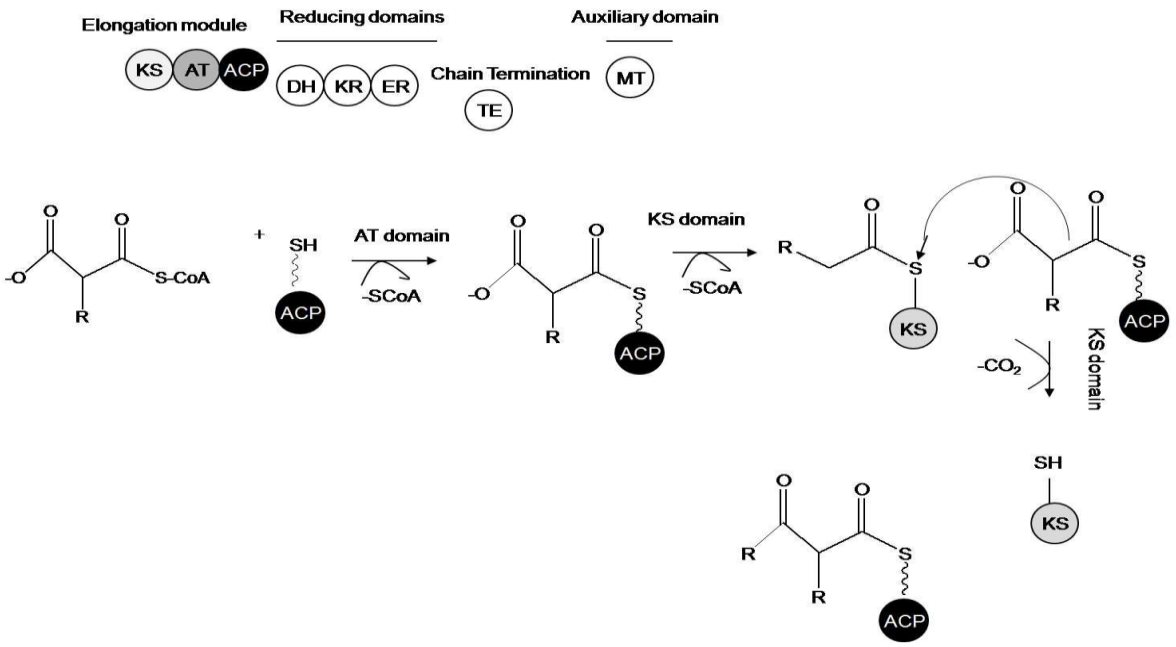


Figure 1.2: The organization and mechanisms of modular biosynthetic pathway systems that occur in polyketide biosynthesis (PKS). KS: ketosynthase, AT: acyltransferase, ACP: acyl carrier protein, DH: dehydratase, KR: ketoreductase, ER: enoylreductase, TE: thioesterase, MT: methyltransferase. (Adapted from, Schwarzer et al. 2003).

UNIVERSITY of the
WESTERN CAPE

Type I:

Bacterial type I PKs are compounds made by a multi-enzyme complex that are arranged into linear modules that are responsible for solitary chain elongation processes as well as post condensation modification of the resulting β-carbonyl. However, the fungal type I PKSs are comprised of a single giant protein that uses a module of a conserved set of domains to create the polyketide (Schwarzer et al. 2003).

http://etd.uwc.ac.za/

Type II:

Type II PKSs are also referred to as aromatic PKSs, they consist of uni-functional protein complexes that are identified by the use of a solitary set of distinct enzymes that are capable of constructing polyketide chains that are cyclised in order to create smaller molecules that consist of a system of aromatic rings (Jimenez et al. 2010).

Type III:

Bacterial PKS that belong to the enzyme family's stilbene synthase and chalcone synthase in plants have been characterized as Type III PKSs. This type differs from type I and II in that it consists of only one domain, one protein and only a single active site that is able to perform the three essential reactions of chain initiation, elongation as well as cyclization (Austin and Noel, 2003).

UNIVERSITY of the
WESTERN CAPE

1.4.2.2 Non-Ribosomal Peptides

Non-ribosomal peptides are a diverse family of natural products that have a vast range of biological activities as well as pharmacological properties. They are often toxins, pigments or siderophores. Non-ribosomal peptide antibiotics as well as immuno-suppressants are available commercially (Schwarzer et al. 2003; Marahiel et al. 1997).

Antibiotics such as penicillin and vancomycin are produced via NRPS. The occurrence of non-proteinogenic D-amino acids and their frequently cyclic nature is the distinguishing factor of NRPS synthesized compounds (Jimenez et al. 2010). The amino acids that form the peptide backbone generally undergo cyclization, this results in the formation of oxazolines and thiazolines; which can then be oxidized or

http://etd.uwc.ac.za/

reduced. Dehydration occasionally occurs on serines, resulting in the formation of dehydro-alanine. Non-ribosomal peptide synthase peptides are generally dimers or trimers comprised of identical sequences that are either chained, cyclized, or branched (Schwarzer et al. 2003; Marahiel et al. 1997).

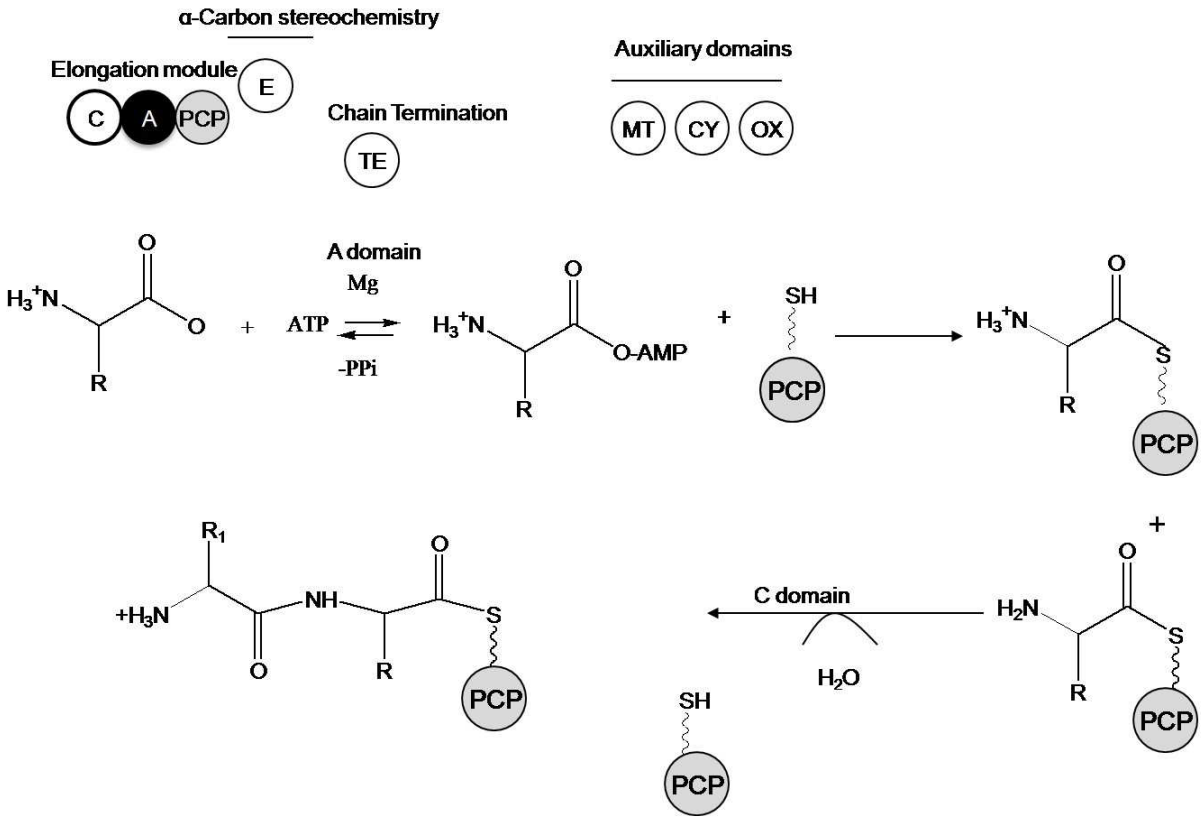


Figure 1.3: The organization and mechanisms of modular biosynthetic pathway systems that occur in non-ribosomal peptide synthase peptide synthesis (NRPS). C: condensation, A: adenylation, PCP: peptidyl carrier protein, E: epimerase, CY: cyclase, OX: oxidoreductase, TE: thioesterase, MT: methyltransferase. (Adapted from Schwarzer et al. 2003).

NRPS are similar to PKSs in that they are also comprised of multifunctional enzyme systems that are capable of building chains from specific building blocks that are

http://etd.uwc.ac.za/

individually selected. NRPS are arranged in modules that are each responsible for a single cycle of elongation with the incorporation of a solitary amino acid into the peptide chain (Walsh et al. 2001). Elongation modules each consist of three important domains that catalyse thiolation, condensation and adenylation (Figure 1.3) (Walsh et al. 2001). NRPs that are constructed on multi-modular enzymatic assembly lines, often attain biological activity by cyclization constraints that are introduced by tailoring enzymes. Tailoring enzymes are encoded by gene clusters in which the genes for coordinated regulation are clustered with the assembly line. NRP heterocyclizations to thiazoles and oxazoles can take place on the elongating framework of acyl-S enzyme intermediates (Fischbach and Walsh, 2006).

Post-assembly-line tailoring enzymes are generally responsible for the catalysis of oxidation, glycosylation, acylation, or alkylation (typically methylation) of promising PK and NRP frameworks (Walsh et al. 2001). Microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* have been genetically engineered for protein drug production, and are referred to as microbial biosynthetic factories. They have been engineered to have robust cellular systems through the efficient organization of the biosynthetic pathways. This ensures that the assembly-line protein mechanisms and enzymes that are necessary for chemical tailoring of NRP scaffolds as well as the diversion of primary metabolites to secondary metabolic monomers are collected into contiguous sets of operons. Tailoring enzymes are essentially responsible for the diversity of the natural products resulting in chemically interesting properties (Fischbach and Walsh, 2006).

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

1.5 One Strain Many Compounds (OSMAC) Approach

The OSMAC approach is a culturable approach used to describe the use of varying fermentation parameters in order to yield several different compounds from the same isolate. The effect that stress has on microorganisms is a well-known fact; however, stress in response to the production of secondary metabolite genes has been discovered to be one of the reasons that microorganisms produce antimicrobial compounds (Rateb et al. 2011; Kusari et al. 2012). The effect of the OSMAC approach varies from increasing the number of compounds that a single microorganism produces to the accumulation of previously unknown natural products (Rateb et al. 2011; Kusari et al. 2012). The OSMAC approach is based on the principle that “silent” secondary metabolites genes in microorganisms outnumber the genes that are actively expressed under conventional cultivation methods. By inducing a stress response many of these “silent”/inactive genes are “switched-on”/expressed (Craney et al. 2013).

UNIVERSITY of the
WESTERN CAPE

1.5.1 Fermentation Parameters of the OSMAC approach

Studies have shown that varying the culture conditions during the fermentation of microorganisms such as aeration, media composition, and the shape of the flask (which contributes to aeration) as well as temperature has led to the discovery of novel compounds in various actinomycetes, fungi and bacteria (Bader et al. 2010). Other parameters that have been used to test and prove the OSMAC approach include, varying nitrogen, carbon and phosphate sources, heat shock, salinity (in the case of marine microorganisms) as well as co-culturing with more than one strain of bacteria (Bode et al. 2002; Pettit, 2011, Greenbaum et al. 2001).

http://etd.uwc.ac.za/

1.6 Bottlenecks associated with drug discovery and overcoming them

There are many bottlenecks associated with novel drug discovery, these include but are not limited to, a supply and demand problem of marine invertebrates as well as redundancy of natural products, see also Figure 1.4 of which “Hit Identification” and Lead Optimization will be discussed due to the interest of this study.

The main difficulty associated with the supply and demand problem is that large quantities of biomass are required in order to produce a minute amount of the drug and/or natural product (Tsukimoto et al. 2011, Trindade et al. 2015). One of the ways in which the supply and demand problem can be eliminated through the use of microorganisms.

Microorganisms are generally easy to grow and can produce a large yield within a few hours. This is substantiated by the fact that many drug discovery studies have found that microbial symbionts associated with natural product producing invertebrates are responsible for the production of the bioactive compounds (Refer to section 1.4). Microbes can circumvent the supply problem in 2 ways; as a source of the natural products as well as by being engineered to express biosynthetic pathways from other organisms.

http://etd.uwc.ac.za/

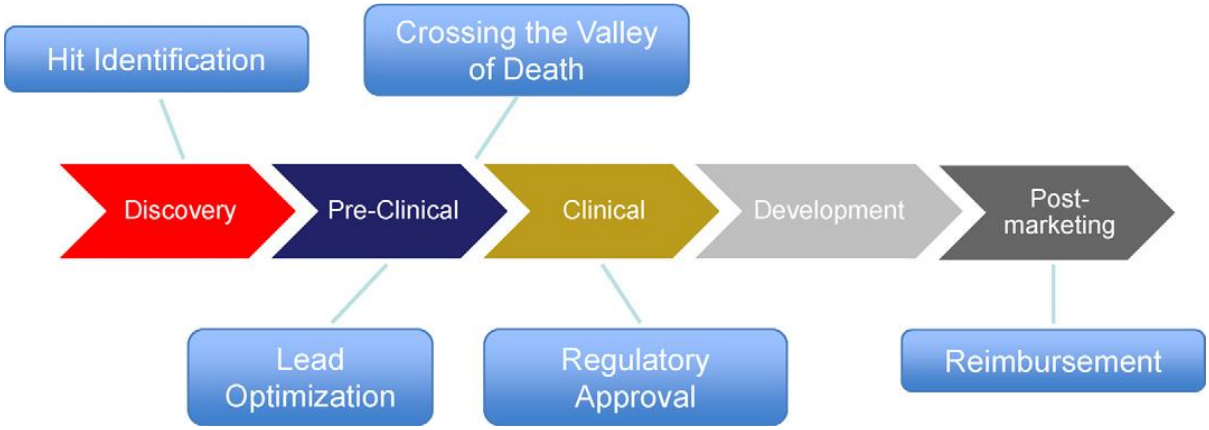


Figure 1.4: Identifying the bottlenecks of R&D in the value chain of pharmaceutical drug discovery platforms (Adapted from So et al. 2011).

UNIVERSITY of the
WESTERN CAPE

There are several bottlenecks associated with the R&D pipeline in natural drug discovery platforms (Figure 1.4). The first bottleneck one faces is the "hit identification," - this is challenging due to the high rate of redundancy that occurs in which common natural products as well as existing antimicrobials are rediscovered (Craney et al. 2013). This has previously been overcome by the use of combinatorial chemistry as well as the use of high throughput screening methods. So et al. (2011) reported that high-throughput screening for antibacterial drug candidates has had a considerably lower yield for antibacterial drug candidates compared to other therapeutic categories such as antifungal or anticancer drug candidates. Combinatorial chemistry has not yet been successful in that the compounds that were synthesised have lacked diversity and complexity, thereby not yielding many novel drug candidates (Wess et al. 2001; Zhang et al. 2005). An alternative method that has been employed to try overcome this bottleneck is the use of metagenomics in which a functional screen for secondary metabolite genes is performed and once

http://etd.uwc.ac.za/

successful, the natural products are expressed by a host organism such as *E. coli* (Penesyan et al. 2010). Metagenomics can contribute to novelty since it can access the genomic content of the 99% unculturable bacteria and in this way deliver novel products.

Lead optimization is the process in which one has to turn these leads into drugs that are able to enter preclinical trials; this is one of the stages in which a lot of attrition occurs. This can be overcome by ensuring that the drug is properly designed and that early drug supply and large-scale synthesis is carried out in order to determine the likelihood of the drug being successful (Wess et al. 2001; So et al. 2011).

Although there are many more bottlenecks associated with the natural drug discovery process these were selected based on their relevance to the study. The field of natural drug discovery and its platforms are ever growing and with the advances being made the bottlenecks we currently face could potentially be alleviated with the use of microorganisms.

UNIVERSITY of the
WESTERN CAPE

1.7 Marine Biotechnology in South Africa

The South African coastline is unique and diverse in that it is surrounded by two oceans, the Indian Ocean on the east and the Atlantic Ocean on the west. The South African coastline is 3650km long and consists of an exclusive economic zone that spans 1 million km^2 with an average depth of 5700m (Davies-Coleman, 2010). The warm Agulhas current flows from the east and the cold Benguela current from the west, joining at Cape Agulhas (Davies-Coleman, 2010). The diversity within the South African marine environment is due to the rare phenomenon where the mixing of waters from the warm Agulhas and cold Benguela currents occur; this in turn

http://etd.uwc.ac.za/

creates an environment that is unique to South Africa. Due to this phenomenon the coastal area that stretches from Cape Town to Port St. Johns is characterised with an abundant level of endemic marine species (Branch et al. 1994).

There are many species that are endemic to the South African coastline; making it a niche environment that could potentially harbour novel marine natural products. Secondary metabolites from the marine environment have been researched for the past 20 years in South Africa. In 2010 there were 46 bioactive compounds that had the potential to be manufactured as drugs providing they were successful in clinical trials (Davies-Coleman, 2010). There have been 8 natural products that have been derived from tunicates and soft corals as well as 7 from marine sponges (Davies-Coleman and Beukes, 2004). In 2012 it was reported that Tsitsikammamines were hypothesised to be produced by microbial symbionts, and it was also found that the compounds produced by the microbial symbionts were analogous to Tsitsikammamines produced by the endemic South African sponge *Tsitsikamma favus* (Dolusic et al. 2012; Walmsley et al. 2012). According to Davies-Coleman and Veale, (2015) there have been recent advances in marine drug discovery from the South African marine environment. Three marine invertebrates namely; *Cephalodiscus gilchristi*, a tube worm, *Lissoclinum sp*., an ascidian, as well as *Topsentia pachastrelloides*, a sponge, have demonstrated bioactivity as well as the production of anti-cancer secondary metabolites. The mandelalides and cephalostatins, which have been produced by the tube worm and ascidian, respectively, have been attracting international collaboration and interest within South African drug discovery platforms (Davies-Coleman and Veale, 2015).

South Africa is currently embarking on much international collaboration with regard to developing marine biotechnology in South Africa due to the vast potential and

UNIVERSITY of the WESTERN CAPE
http://etd.uwc.ac.za/

opportunities that are harboured within its marine environment. The South African marine environment has been relatively unexplored as a source of novel natural products as compared to the marine environments in Asia and Australia that have been vastly studied. Due to the South African marine environment being largely unexplored, and coupled to the evidence that suggests the high diversity within the endemic environment, it is a niche environment in that the biological diversity is filled with enormous potential for novel natural products.

http://etd.uwc.ac.za/

Chapter 2: Materials and Methods

2.1 Chemical and biological reagents

All chemicals and reagents used were supplied by Merck Chemical and Laboratory Supplies (Darmstadt, Germany), Sigma-Aldrich Chemical Company (Deissenhofen, Germany) and Kimix Chemical and Laboratory Supplies (South Africa) unless otherwise stated. The oligonucleotides for use in the polymerase chain reaction (PCR) were synthesized by Inqaba Biotech (Johannesburg, South Africa). The DNA molecular markers and DNA modifying enzymes (polymerases & restriction endonucleases) were purchased from Fermentas Life Sciences (Vilnius, Lithuania).

UNIVERSITY of the
WESTERN CAPE

2.2 Bacterial isolation

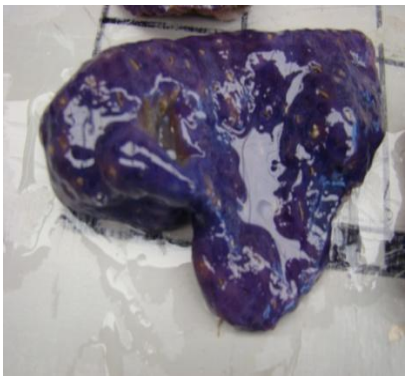
2.2.1 Sample Collection

Two tunicate samples were collected using SCUBA from two different sites off the Port Elizabeth Coast namely; Philips Reef, Algoa Bay (33:58.452S, 25:40.867E) and White Sands (34:00.406S, 25:43.117E) by Dr. S. Parker-Nance in January and April 2013 at a depth of 10-13 m and 23-25 m respectively.

Samples were stored on ice and shipped to Cape Town within 24 hours. The samples were processed by dissecting them into smaller pieces and stored in glycerol at -20°C until required.

http://etd.uwc.ac.za/

Table 2.1 Sample Classification and description of Tunicates

Sample Name	Sample Classification	Description	Image
PE06	Phylum: Chordata Subphylum: Tunicata Class: Ascidiacea Order: Aplousobranchia Family: Holozoidae Genus: *Distaplia* Species: *skoogi* (Hartmeyer, 1913)	Colonies are found in a variety of colour variants, blue (as per this collection), white and brown.	
PE12	Phylum: Chordata Subphylum: Tunicata Class: Ascidiacea Order: Aplousobranchia Family: Polycitoridae Genus: *Eudistoma* sp. A	This is a characteristic species and not very common. Larvae are required for a positive identification.	

UNIVERSITY of the
WESTERN CAPE

2.2.3 Bacterial isolation from Tunicate samples

Distaplia skoogi (PE6) and *Eudistoma sp. A* (PE12) samples were homogenised using a sterile mortar and pestle after which a 1:10 dilution was made using sterile (autoclaved) sea water collected from Algoa Bay. Serial dilutions (10 fold dilutions) were performed and 100 µl of each dilution were spread plated onto 16 different agar media (Appendix A). The plates were incubated at 15°C, for up to 6 weeks, in order to account for the slow growers. Bacterial colonies were picked and then re-streaked

http://etd.uwc.ac.za/

onto the respective media until pure cultures were obtained. Glycerol stocks were prepared for all isolates and stored in 96 well plates at -80°C.

2.3 Screening for antimicrobial activity from Tunicate isolates

2.3.1 Screening for antibacterial activity

All the isolates were cultured in a grid format, in which each isolate was spotted onto a Zobell agar plate on a grid, with each isolate having a 1 cm x 1 cm area. The plates were incubated at 37°C until all the isolates had grown (approximately 2 weeks), after which they were tested for antibacterial activity against 5 indicator strains. For anti-Gram-positive bacterial activity *Bacillus cereus* ATCC10702 and *Staphylococcus epidermidis* ATCC14990 were used as indicator strains, whereas *Pseudomonas putida* ATCC12633 was used to determine activity against Gram negative bacteria. *Escherichia coli* 1699 (Cubist, USA) (Appendix B) was also selected as an indicator strain since it has been genetically engineered for resistance against 52 known antibiotics. *Mycobacterium smegmatis* LR222 was selected due to it sharing the same genus with *Mycobacterium tuberculosis*, the strain known to cause TB.

UNIVERSITY of the
WESTERN CAPE

The indicator strains (*B. cereus, M. smegmatis, P. putida* and *S. epidermidis*) were inoculated in 10 ml Tryptic Soy Broth (TSB), whereas *E. coli* 1699 was inoculated into 10 ml LB and grown aerobically at 37°C overnight in a shaking incubator (150 RPM). A 1:100 dilution of each strain was made in 25 ml of TSB and LB 0.7% sloppy agar, respectively, and then overlaid onto the grids after which they were incubated at 30°C overnight. Antibiotic producers were identified by a zone of clearance surrounding the isolate (Janssen et al. 1986).

http://etd.uwc.ac.za/

This project forms part of a larger European Union (EU) and Seventh Framework funded project entitled PharmaSea, in which bio-medically relevant activities are screened for on various high-throughput platforms in order to identify and possibly produce novel antimicrobial compounds from marine bacteria. Therefore, certain screening results were generated by the consortium partners via these high throughput platforms, which were unable to be screened for at UWC, IMBM (Table 2.2).

2.3.2 Screening of tunicate isolates for antimicrobial and biomedical activity using the One Strain Many Compounds (OSMAC) approach

A secondary screening for antimicrobial activity of the isolates from *Distaplia skoogi* and *Eudistoma sp. A* (Section 2.2.3) was performed in the same grid format (Section 2.3.1) using the OSMAC approach, in which the isolates were screened on the following four media, ZBA, GYM, TSA and ACM (Appendix A) and incubated at 15°C for 2 weeks. This formed part of an ongoing study within the PharmaSea project.

The screening of relevant biomedical activity was performed by the following PharmaSea partners. Table 2.2 indicates the name of the PharmaSea partner as well as the assays that were performed on the selected bioactive extracts.

http://etd.uwc.ac.za/

Table 2.2: Screening of biomedically relevant bioactivity using high throughput drug discovery platforms performed by the following PharmaSea partners

PharmaSea Partner	Assay Performed
Fundación Medina	**Anti-Bacterial:** *E. coli*-MB2884, *S. aureus-MRSA*-MB5393, *P. aeruginosa* PA01, *P. aeruginosa* PA01_synergy with IMP. **Anti-Fungal:** *A. fumigatus* ATCC 46645 and *C. albicans* MY1055
Leuven	Neurological bioactivity
MarBio	Anti-Inflammatory and Toxicity

I was awarded a NRF mobility grant to go to the PharmaSea partner, Fundación Medina, in Granada, Spain, for a week's training on their high throughput drug discovery platform.

UNIVERSITY of the
WESTERN CAPE

2.4 16S rRNA identification of bioactive isolates, sequencing and phylogenetic analysis

2.4.1 16S rRNA gene amplification

In order to identify the organisms producing antibacterial activity, colony PCR of the 16S rRNA gene was conducted by polymerase chain reaction (PCR) using universal primers E9F 5'-GAGTTTGATCCTGGCTCAG-3' (Farrely et al. 1995) and U1510R 5'-GGTTACCTTGTTACGACTT-3' (Reysenbach et al. 1995). An amplicon of approximately 1.5 kb was expected. Each 50 µl PCR reaction contained a final concentration of 1 X reaction buffer, 2 mM $MgCl_2$, 150 µM of each dNTP, 0.5 µM of each primer, a single bacterial colony and 0.25 U of Dream*Taq* polymerase (Fermentas). The amplification was performed in an automated thermocycler

http://etd.uwc.ac.za/

(Multigene Thermal Cycler, Labnet). The following cycling conditions were used: an initial denaturation at 95˚C for 4 minutes, followed by 35 cycles at 95°C for 30 seconds, an annealing temperature of 55˚C for 30 seconds, extension at 72˚C for 1.5 minutes and a final extension step of 72˚C for 10 minutes. A negative control containing no bacterial colony was used in all PCR experiments. The amplicons were visualised on a 1% agarose gel prepared in 1 X TAE and electrophoresed for 1.5 hr at 80V.

2.4.2 DNA sequencing

PCR amplicons were purified using the Nucleospin PCR and Gel Band Purification Kit (Separations), according to the manufacturer's specifications. The sequencing reactions were performed by the DNA sequencing facility at the Stellenbosch Central Analytical Facility. The forward and the reverse sequencing primers were used in order to obtain the partial 16S rRNA gene sequence.

UNIVERSITY of the
WESTERN CAPE

2.4.3 16S rRNA analysis

The sequences were analysed using the software packages, Chromas LITE Version 2.1.1 (Technelysium Pty Ltd) and DNAMAN Version 4.1.3 (Lynnon Biosoft). The sequences were trimmed using Chromas LITE Version 2.1.1 and the sequences were aligned using DNAMAN Version 4.1.3. Sequence identification was conducted by comparison to the sequences in the GenBank database. The Basic Local Alignment Search Tool (BLASTn) programme (Altschul et al. 1996) was used in order to identify known species in the GenBank database as well as to determine the

http://etd.uwc.ac.za/

sequence similarity with known species, using software from the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/).

2.5 Bioinformatic analysis of gene clusters for *Paenibacillus sp.*

In order to determine the gene clusters, present in the genus *Paenibacillus*, bioinformatic analysis was performed. The following genomes for the *Paenibacillus* species were used for the analysis; these genome sequences were obtained from the NCBI database (Table 2.3).

Table 2.3: *Paenibacillus* species with their associated accession numbers

Accession Number	Species
NC_014622.1	*Paenibacillus polymyxa SC2*
NC_014483.1	*Paenibacillus polymyxa* E681
NC_017542.1	*Paenibacillus polymyxa* M1
CP006872.1	*Paenibacillus polymyxa strain CF05*
CP006941.2	*Paenibacillus polymyxa CR1*
CP009909.1	*Paenibacillus polymyxa SQR-21*
CP010268.1	*Paenibacillus polymyxa strain Sb3-1*
NC_012914.1	*Paenibacillus sp.* JDR 2
NC_016641.1	*Paenibacillus terrae* HPL-003
NC_017672.3	*Paenibacillus mucilaginosus* K02
NC_016935.1	*Paenibacillus mucilaginosus* 3016
NC_015690.1	*Paenibacillus mucilaginosus* KNP414
NC_013406.1	*Paenibacillus sp*. Y412MC10

The genomes were analysed using the Antibiotics and Secondary Metabolite Analysis SHell programme (antiSMASH) (Blin et al. 2013). antiSMASH is an online programme that identifies and annotates secondary metabolite biosynthetic gene clusters that are present in bacterial as well as fungal genomes (Blin et al. 2013).

http://etd.uwc.ac.za/

2.6 Antimicrobial extract preparation of PE12_11 and PE6_56

2.6.1 Antimicrobial extract preparation

Extracts of the 2 bacterial isolates PE12_11 and PE6_56 were prepared by inoculating the isolate into 100 ml of GYM and Zobell media, respectively, and incubating at room temperature shaken at approximately 130 RPM, until confluence was reached, after 7 days, in order to induce stress. Once the culture had grown, an equal volume of acetone was added to each flask and incubated at room temperature for 1 hr and shaken at approximately 130 RPM. Thereafter, each culture was rotor evaporated at 45°C until the acetone/culture volume had reduced by half.

A column was prepared using Sepa SP207ss resin beads (Sigma-Aldrich) and Amberlite Mb-150 (Sigma-Aldrich) beads respectively. The beads were packed in a column with a bed volume of 10 ml. The beads were conditioned by passing 50 ml of acetone through the column, thereafter, 50 ml of methanol and 100 ml of distilled water were sequentially passed through the column. The extract was then added to the column and the flow-through fraction was collected, and the bound material was eluted with 50 ml of acetone and the eluent was collected. Distilled water (50 ml) was then passed through the column in order to remove residual extract. The collected fractions were rotor evaporated at 45°C until dry.

UNIVERSITY of the
WESTERN CAPE

2.6.2 High Pressure Liquid Chromatography (HPLC) of antimicrobial extracts

The eluted extract was prepared for HPLC by adding 3 ml of distilled water to the antimicrobial extract, thereafter the extract was sonicated for 3 x 20 secs, after which the sample was filtered using a 0.22 µm filter. The samples were injected (3 x 200 µl) into the HPLC column (Dionex Ultimate 3000) and fractionated on a Rezex RHM –

http://etd.uwc.ac.za/

C18 column with the following dimensions, 300 x 7.80 mm. The samples were run at a flow rate of 0.600 ml/min at 33 bar for 1 hr at a temperature of 45°C. An elution gradient of 2-98% acetonitrile was used to elute bound samples. Fractions (1.2 ml) were collected. The acetonitrile was evaporated from the fractions using a rotor evaporator at 60°C.

2.6.3 Well Diffusion Antibiotic Assay of HPLC Fractions

Luria Bertani Agar (LBA) plates were prepared and wells were made in the agar plate using a p20 tip under aseptic conditions, thereafter; the well diffusion assay was performed using 4 indicator strains namely; *E. coli* 1699, *B. cereus, S. epidermidis and P. putida*. The indicator strains (100 µl) were spread plated onto the LBA plates, respectively, and allowed to dry for 10 minutes, thereafter, 5 µl of each fraction was dispensed into each well respectively and the plates were incubated at 30°C overnight. Antibiotic activity of the HPLC fraction was indicated by a clearance zone around the fraction/well.

UNIVERSITY of the
WESTERN CAPE

2.7 PCR screening for biosynthetic gene clusters in PE12_ 11

2.7.1 Genomic DNA extraction of PE12_ 11

A large scale genomic DNA (gDNA) extraction was performed on isolate PE12_ 11 according to Wilson, (1987). This was performed in order to determine if the gDNA was able to be amplified since good quality, pure DNA was required for screening for secondary metabolite gene clusters. The DNA was electrophoresed on a 1% agarose gel prepared in 1 X TAE for 1.5 hr at 100V. The concentration of the gDNA

http://etd.uwc.ac.za/

was determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA).

2.7.2 PCR Screening for a NRPS Gene Cluster

In order to identify the gene cluster(s) responsible for producing antimicrobial activity in PE12_11, amplification of a NRPS gene, which was selected based on the bioinformatics analyses, was performed using degenerate primers that targeted the A domain of NRPS, the following primers were used; MTF2 5'-CCNCGDATYTTNACYT-3' (Vizcaino et al. 2005) and MTR 5'-GCNGGYGGYGCNTAYGTNCC-3' (Vizcaino et al. 2005). An amplicon of 900bp-1kB was expected. The amplification was performed in an automated thermocycler (Multigene Thermal Cycler, Labnet). Reaction conditions are displayed in Table 2.4.

UNIVERSITY of the
WESTERN CAPE

Table 2.4: Reaction conditions for amplification of a NRPS gene cluster

Reagent	20 µl Reaction (ul)	50 µl Reaction (ul)	Final Concentration
Nuclease-free water	10.4	30.5	
5 X Phusion Buffer	4	10	1 X
10 mM dNTPs	0.4	1	200 uM
10 uM Forward Primer	1	2.5	0.5 uM
10 uM Reverse Primer	1	2.5	0.5 uM
Template DNA	100 ng	100 ng	<250 ng
Phusion DNA Taq Polymerase	0.2	0.5	1.0 units/50 ul reaction

The following cycling conditions were used: an initial denaturation at 98°C for 30 seconds, followed by 35 cycles at 98°C for 10 seconds, an annealing temperature of 57°C for 30 seconds, elongation at 72°C for 1 minute and a final extension step of

http://etd.uwc.ac.za/

72°C for 10 minutes. The amplicons were visualised on a 1% agarose gel prepared in 1 X TAE and electrophoresed for 1.5 hr at 80V.

2.7.3 Cloning of NRPS amplicons

2.7.3.1 Preparation of electro-competent cells

A colony of *E. coli* EPI300 was inoculated in 10 ml LB and incubated at 37°C overnight on a shaker at 250 RPM. A conical flask containing 400 ml of LB was inoculated with 4 ml of the overnight culture and incubated at 37°C on a shaker. The cells were grown until an OD600 between 0.35-0.4 was reached. Thereafter, the cells were immediately placed on ice to cool, shaking occasionally to ensure uniform cooling. Cells were harvested at 7000 × g for 20 minutes at 4°C and the supernatant was discarded. Cells were re-suspended in 200 ml of ice cold de-mineralised water and harvested at 7000 × g for 10 minutes at 4°C. The supernatant was discarded and the cells were re-suspended in 40 ml ice cold distilled water. The cells were harvested by centrifugation at 7000 × g for 5 minutes at 4°C. This washing step was repeated 5 times. After the final wash, the supernatant was discarded and the cell pellet re-suspended in 1 ml ice cold 10% glycerol. Aliquots of the cell suspension (70 µl) were transferred to chilled 1.5 ml micro-centrifuge tubes on ice. The electro-competent cells were snap frozen with liquid nitrogen and stored at -80°C.

UNIVERSITY of the
WESTERN CAPE

2.7.3.2 Preparation of the pUC18 cloning vector

The cloning vector (pUC18) was prepared for ligation by digestion with 1U of fast-digest SmaI endonuclease per µg of vector DNA in a 20 µl reaction according to manufacturer's instructions (Thermo scientific). The digested vector DNA was

http://etd.uwc.ac.za/

resolved in agarose gel (0.8% [w/v]) and the linearized vector was purified using the NucleoSpin® gel and PCR clean-up kit (Macherey-Nagel) according to the manufacturer's instructions.

2.7.3.3 Ligation of PCR products

The linearized pUC18 vector was ligated with insert DNA through blunt-end ligation, in the ratio 1:5 (vector to insert). The reaction mixture consisted of 1 U of T4 DNA ligase, 1 X T4 DNA ligase buffer and nuclease free water to yield a final volume of 20 µl. The ligation reaction was incubated at 16°C, overnight. The blunt-ended inserts were created using T4 DNA polymerase as per manufacturer's recommendations (Fermentas Life Sciences Ltd).

UNIVERSITY of the
WESTERN CAPE

2.7.3.4 Transformation of the ligated products

Electro-competent *E. coli* EPI300 cells were transformed directly with the ligation mixture. An aliquot of electro-competent cells (70 µl) was thawed for approximately 1 minute on ice and 5 µl of the ligation mix was subsequently added to the thawed cells. The mixture was kept on ice for 60 seconds and transferred to a chilled 0.1 cm sterile electroporation cuvette (Bio-Rad). The cells were electroporated using the Bio-Rad MicroPulser™ (USA) with the following parameters; 1 pulse of 1.8 Kv, 25 µF and 200 Ohms (Ω). Immediately after electroporation, 1 ml of LB was added to the cells and the mixture was transferred to a sterile 2 ml micro-centrifuge tube and incubated at 37°C for 1 hr. The electroporated cells (100 µl) were spread onto LB plates containing ampicillin at a final concentration of 100 µg/ml and 80 µg/ml X-gal and incubated overnight at 37°C.

http://etd.uwc.ac.za/

2.7.3.5 Screening of transformants

The clones were selected based on blue-white selection, where the white colonies contained the insert and the blue colonies did not. Plasmid DNA was extracted using the alkaline lysis method as follows: A single colony was inoculated into 10 ml of LB supplemented with ampicillin at a final concentration of 100 µg/ml and grown overnight at 37˚C with shaking at 250 RPM. Thereafter, 2 ml of the overnight culture was transferred to a sterile micro-centrifuge tube and the cells were harvested at 16,000g for 30 seconds. The supernatant was discarded and the cell pellet was re-suspended in 100 µl of solution 1 (50 mM glucose, 10 mM EDTA, and 25 mM Tris (pH 8.0)) and immediately placed on ice for 5 minutes. Thereafter, 200 µl of solution 2 (0.2 M NaOH and 1% SDS) was added and thoroughly mixed by inverting the tube and immediately placed on ice for 5 minutes. Solution 3 (3 M KOAc (pH 6.0)) at a volume of 150 µl was added, briefly vortexed at maximum speed and immediately placed on ice for 5 minutes. Cell debris and chromosomal DNA was centrifuged for 3 minutes at 16,000 x g at room temperature. The supernatant was transferred to a sterile micro-centrifuge tube and the plasmid DNA was precipitated by adding 800 µl of 95% ethanol and incubated at room temperature for 2 minutes. Plasmid DNA was obtained by centrifugation at 16,000 g for 1 minute at room temperature and subsequently washed with 1 ml of 70% ethanol, thereafter the plasmid DNA was air-dried and re-suspended in 50 µl of TE buffer (pH 8).

PCR analysis was conducted to evaluate whether the transformants had the expected insert sizes, using touchdown PCR and M13 vector primers (Norrander et al. 1983). The PCR reaction conditions are indicated in Table 2.5.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Table 2.5: Reaction conditions for amplification of the clones with M13 vector primers

Reagent	20 µl Reaction (ul)	50 µl Reaction (ul)	Final Concentration
Nuclease-free water	10.4	30.5	
5 X Phusion Buffer	4	10	1 X
10 mM dNTPs	0.4	1	200 uM
10 uM Forward Primer (5'CCCACTCACGACGTTGTAAAACG3')	1	2.5	0.5 uM
10 uM Reverse Primer (5'AGCGGATAACAATTTCACACAGG3')	1	2.5	0.5 uM
Template DNA	100 ng	100 ng	<250 ng
Phusion DNA Taq Polymerase	0.2	0.5	1.0 units/50 ul reaction

UNIVERSITY of the WESTERN CAPE

All reactions were carried out in an automated thermal cycler (T100 thermal cycler, BioRad). The following cycling conditions were used: initial denaturation at 94°C for 2 minutes, followed by 10 cycles at 94°C for 30 seconds, an annealing temperature of 65°C (-1°C/cycle) for 30 seconds, and elongation at 72°C for 50 seconds. This is followed by 25 cycles at 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 50 seconds. The amplicons were visualised on a 1% agarose gel prepared in 1 X TAE and electrophoresed for 1.5 hr at 80V.

To ensure that the plasmid DNA was suitable for sequencing, plasmid extraction was carried out using the Qiaprep® MiniPrep Kit (Qiagen) according to the manufacturer's specifications.

2.7.4 Sequence analysis of the NRPS amplicons

Cloned inserts of the NRPS, A-Domain, were sequenced at the University of Stellenbosch Central Analytical Sequencing Facility (ABI PRISM 377 automated

http://etd.uwc.ac.za/

DNA sequencer) in both the forward and reverse direction (MTF 5'–CCNCGDATYTTNACYTG-3' and MTR 5'- GCNGGYGGYGCNTAYGTNCC-3') (Vizcaino et al. 2005). The chromatograms were manually edited using Chromas Lite version 2.1 software package (Technelysium Pty Ltd, 2012). The curated nucleotide sequences (forward and reverse) were assembled using DNAMAN version 4.13 software package (Lynnon Biosoft) to construct a consensus sequence.

http://etd.uwc.ac.za/

Chapter 3: Results and Discussion of Isolation of Tunicate symbionts and Screening for Novel Antimicrobials

3.1 Introduction

The marine environment harbours a rich diversity found in not only marine invertebrates but also in the microorganisms that are associated with marine invertebrates (Kennedy et al. 2008). Marine invertebrates have been shown to be a novel source of natural products in which many medically relevant compounds have been isolated. Compounds that have been isolated from marine invertebrates have been shown to be chemically similar if not identical to compounds isolated from microorganisms (Velho-Pereira and Furtado, 2012). For years it was understood that the compounds were produced by the invertebrates, however, this hypothesis has been discredited with recent studies showing that it is in fact the microorganisms that are producing the compounds (Wilson et al. 2014). Due to this discovery, one of the major bottlenecks of natural product discovery has been eliminated, since microorganisms can easily be grown in large scale especially with the use of heterologous expression systems (Harvey et al. 2015).

UNIVERSITY of the
WESTERN CAPE

In this chapter, the isolation and screening of microbial symbionts from two tunicate species from the South African marine environment is described. The following screening methods will be discussed; antimicrobial overlay assays, screening for bio-medically relevant activity as well as the OSMAC approach for the induction of antibacterial activity.

http://etd.uwc.ac.za/

3.2 Isolation of Tunicate symbionts and Screening for Novel Antimicrobials

3.2.1 Isolation of bacterial symbionts from *Distaplia skoogi* (PE6) and *Eudistoma sp. A* (PE12)

3.2.1.1 Sample Collection

Figure 3.1: Tunicate samples from the coast of Algoa Bay. A: *Distaplia skoogi*, B: *Eudistoma sp.* 011RSASPN.

UNIVERSITY of the
WESTERN CAPE

Distaplia skoogi (Figure 3.1A) is taxonomically identified as belonging to the phylum Chordata and the subphylum Tunicata. It belongs to the Ascidiacea class in the order Aplousobranchia and the Holozoidae family. Halogenated oxindoles and indoles have previously been extracted from *Distaplia skoogi* that was harvested off the coast of Algoa Bay, and which have been found to be moderately cytotoxic anticancer agents (Bromley et al. 2013).

Eudistoma sp. 011RSASPN (Figure 3.1B) is taxonomically identified as belonging to the phylum Chordata with its subphylum being Tunicata. It belongs to the Ascidiacea class in the order Aplousobranchia and the Polycitoridae family. The tunicate *Eudistoma sp.* 011RSASPN is described as a novel candidate species as no similar taxonomic classification has previously been observed (Parker-Nance, 2013). ß-carbolines which belong to the group of indole alkaloids have previously been

http://etd.uwc.ac.za/

extracted from the *Eudistoma* species and are known to have antibacterial and antifungal properties (Schupp et al. 2003).

3.2.2 Bacterial isolation from *Distaplia skoogi* (PE6) and *Eudistoma sp.* 011RSASPN (PE12)

A total of 157 and 143 morphologically distinct colonies were isolated from *Distaplia skoogi* and *Eudistoma sp.* 011RSASPN samples respectively, using a range of different isolation agar based media. The following media were selected in order to ensure that maximum diversity of isolates as well as compounds were isolated from the Tunicate samples.

General Media

Zobell: Isolation of a wide range of Gram-negative marine microorganisms

UNIVERSITY of the
WESTERN CAPE

Zobell with Crystal Violet: Inhibits the growth of Gram-positive microorganisms

Seawater Agar: Isolation of slow growing microorganisms, due to the low nutrient content

Activated Charcoal Medium: An anaerobic medium used for the isolation of microorganisms that use pyruvate as a primary carbon source

Sponge Extract Medium: Isolation of bacteria that requires nutrients found in the host sponge

Reasoners 2 agar (R2A): Used to isolate bacteria from potable water, targeting slow growing bacterial species

http://etd.uwc.ac.za/

pH Media

Medium A Agar: Isolation of alkaliphilic as well as halo-tolerant microorganisms

Trypticase Soya Agar pH 4.5: Isolation of Gram-positive microorganisms that are acidophilic at a pH of 4.5

Firmicute Media

Trypticase Soya Agar: Isolation of Gram-positive bacteria

Shivji Nutrient Agar: Isolation of Firmicute bacteria, such as *Bacillus sp.*

Actinobacteria Media

Modified 172F: Isolation of Actinobacteria. The high levels of calcium ions present in this media specifically selects for actinobacteria

Oatmeal Agar: Standard Actinobacteria isolation medium

UNIVERSITY of the
WESTERN CAPE

Actinomyces Isolation Medium: Standard medium for isolation of Actinomyces

GYM Streptomyces Medium: Standard Streptomyces isolation medium

Cyanobacteria Media

Pringsheim's Cyanobacteria Specific Medium: Standard isolation medium for cyanobacteria

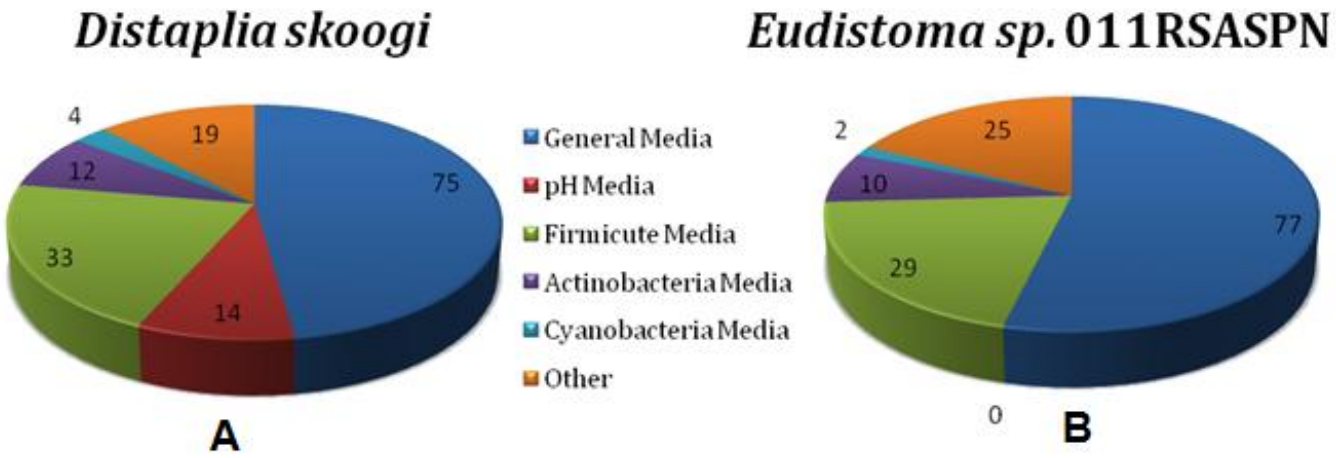
Ashby's Nitrogen Free Medium: Isolation of nitrogen fixation bacteria in the presence of light

Blue Green 11 Medium: For the enrichment of photosynthetic bacteria. Isolates

http://etd.uwc.ac.za/

cyanobacteria capable of using inorganic salts as carbon and nitrogen sources

Other Media
Planctomycetes Medium: Isolation of Plantomycetes



UNIVERSITY of the
WESTERN CAPE

Figure 3.2: Pie chart representing the media related differences in the number of isolates obtained from the tunicates *Distaplia skoogi* (PE06) (A) and *Eudistoma sp.* 011RSASPN (PE12) (B).

It is evident from Figure 3.2A that the majority of the isolates from *Distaplia skoogi* were cultured on general media. This is expected since these media are used in order to culture a wide range of microorganisms able to grow on various carbon and nitrogen sources. A number of isolates were isolated on “pH media”, chosen in order to isolate acidophilic as well as alkaliphilic microorganisms, due to the pH range that was covered. This could be a possible reason that 8.9% of the isolates were cultured on this class of media. Figure 3.2A indicates that 21% of the isolates were cultured on Firmicute media, this class of media is used for the isolation of bacteria such as

http://etd.uwc.ac.za/

Bacillus sp. and *Clostridia*. TSA is a general medium that provides bacteria with nitrogen as well as amino acids and is used to isolate Gram-positive microorganisms.

The majority of the isolates for *Eudistoma sp.* 011RSASPN were cultured on general media and Firmicute media which was expected (Figure 3.2B). There were more isolates cultured on general media from *Eudistoma sp.* 011RSASPN than there was for *Distaplia skoogi*. No isolates were cultured on “pH specific” media from *Eudistoma sp*. 011RSASPN.

Based on the percentages of isolates cultured on the different media types, it is evident that there is no distinct difference between the culturable bacteria from the *Distaplia skoogi* and *Eudistoma sp*. 011RSASPN tunicates with the exception of the "pH medium". According to Tianero et al. (2015) the ascidian microbiome is extremely diverse and the diversity is not correlated to geographical location. They found that the ascidian microbiomes as well as their metabolomes contained species-specific and location-specific components. Bacteria that were location-specific were found in low abundance which represented strains that were widespread. Based on the study by Tianero et al. (2015) it was expected that the microbiome associated with each tunicate would differ slightly since they were harvested from different areas within Algoa Bay and the tunicates were from different families, therefore, the isolates cultured from these tunicates respectively would be present in various quantities as well as diversity, (Nystrom and Folke, 2001). Previous studies based on the microbiome of tunicates revealed that the majority of microorganisms isolated from tunicates belong to the phylum *Proteobacteria* with *Alphaproteobacteria* being the dominant class (Reisenfeld et al. 2008; López-Legentil et al. 2015; Steinert et al. 2015). These results (from this study) do not

http://etd.uwc.ac.za/

represent de-replicated isolates, therefore, some of the isolates may have been isolated more than once, and although the isolates were selected based on morphological characteristics, it should be noted that someisolates may appear different on different media when in fact they are the same.

3.3 Screening for Antimicrobial compounds

3.3.1 Antimicrobial Overlay Assay

The antimicrobial overlay assay was used to identify antimicrobial activity from isolates associated with *Distaplia skoogi* and *Eudistoma sp.* 011RSASPN. The isolates were tested against 5 bacterial strains, 3 Gram-positive and 2 Gram-negative strains namely; *E. coli* 1699, *S. aureus*, *B. cereus*, *P. putida* and *M. smegmatis*. The antibacterial activity was indicated by a zone of clearance that formed around the colony.

UNIVERSITY of the
WESTERN CAPE

A total of 78 antimicrobial activity producing isolates were identified, with multiple bioactivities being displayed by 16 of the bacterial isolates (Figure 3.3). The high prevalence of activity against *E. coli* 1699 was not expected since the strain has been genetically engineered to be resistant to the majority of known antibiotics (refer to Appendix B for a table of resistance). This suggests that the natural products of these isolates are potentially novel. There were 33 isolates with antimicrobial activity associated with *Distaplia skoogi*, whereas, 45 isolates with antimicrobial activity were associated with *Eudistoma sp.* 011RSASPN.

http://etd.uwc.ac.za/

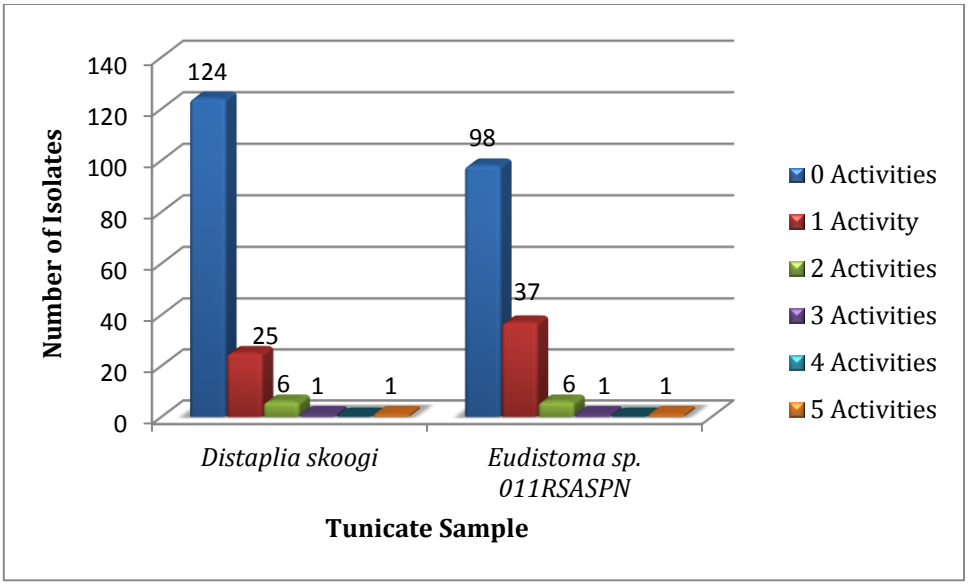


Figure 3.3: Graph representing the antimicrobial bioactivity of isolates associated with *Distaplia skoogi* and *Eudistoma sp.* 011RSASPN.

UNIVERSITY of the
WESTERN CAPE

Thereafter, an antimicrobial activity screen was performed for each isolate on four different media viz. ACM, GYM, TSA and ZBA; this was performed based on the One Strain Many Compounds (OSMAC) principle. This approach follows the reasoning that under different growth conditions, the same strain/isolate is able to produce different compounds. The media selected for OSMAC ranged from media that is high in nutrients and carbon sources to media low in nutrients and carbon sources. It was evident that on different media, the same isolates displayed different activity, and certain isolates that were initially screened and displayed no activity, displayed activity on a media that they were not previously grown or screened on (Figure 3.5). Figure 3.5 indicates the best media; this represents the type of medium that the isolate displayed the most activity on, in terms of the number of test strains it was active against.

http://etd.uwc.ac.za/

There were four isolates in total that displayed antimicrobial activity against the same test strain on more than one medium, they are as follows; PE12_11, PE12_69, PE12_79 and PE12_113. However, isolate PE12_11, displayed the most diverse activity profile, in that it displayed antimicrobial activity on 2 or more different media against each test strain. This could potentially indicate that this isolate, as well as the isolates mentioned above, produce more than one antimicrobial compound. Due to the multiple bioactivities of PE12 _11 based on the preliminary data, this isolate was selected for further analysis.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

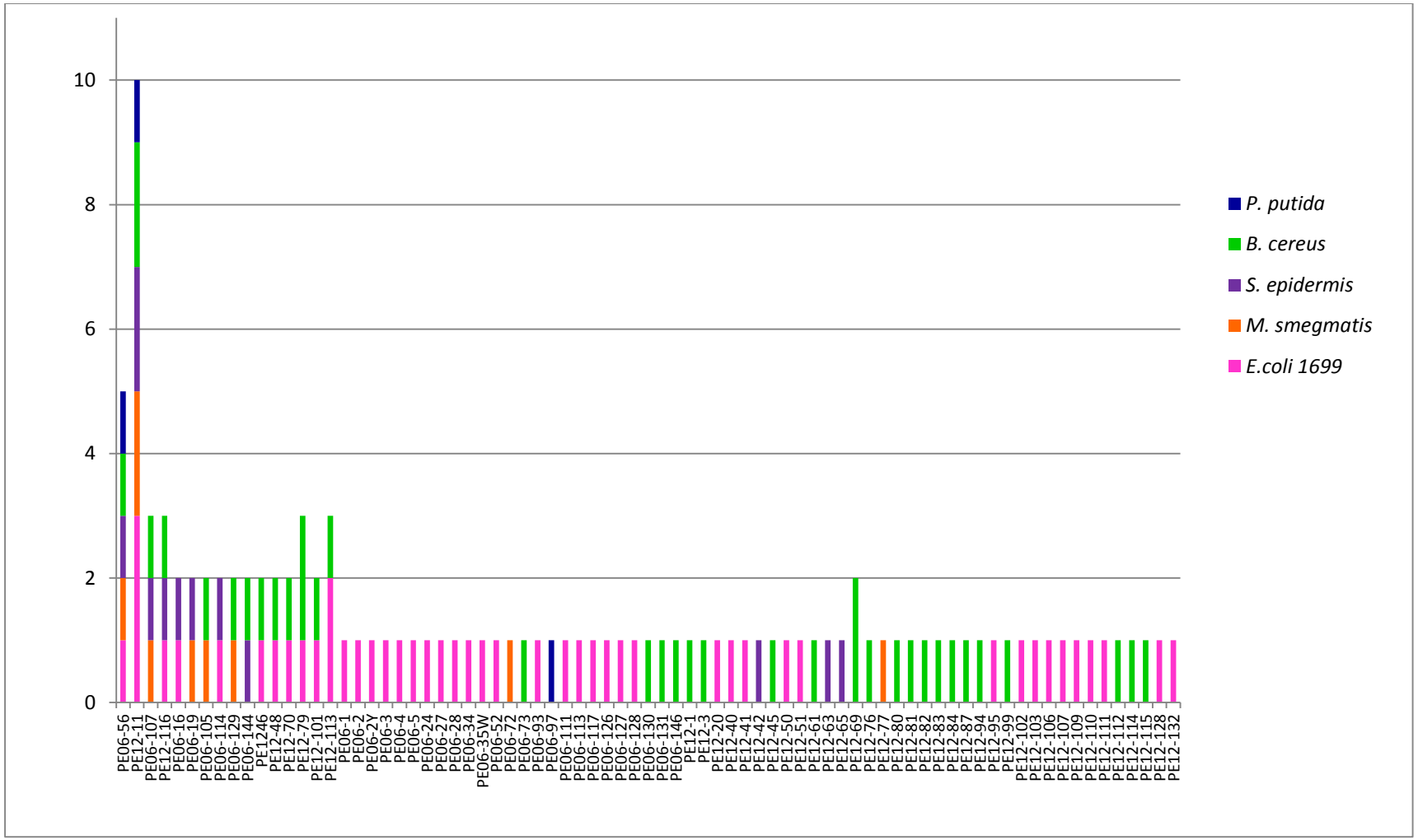


Figure 3.4: Graph summarising the isolates that displayed antimicrobial activity against the 5 different test strains when using the OSMAC approach. The graph indicates the activity (y-axis) of each isolate on their best medium.

http://etd.uwc.ac.za/

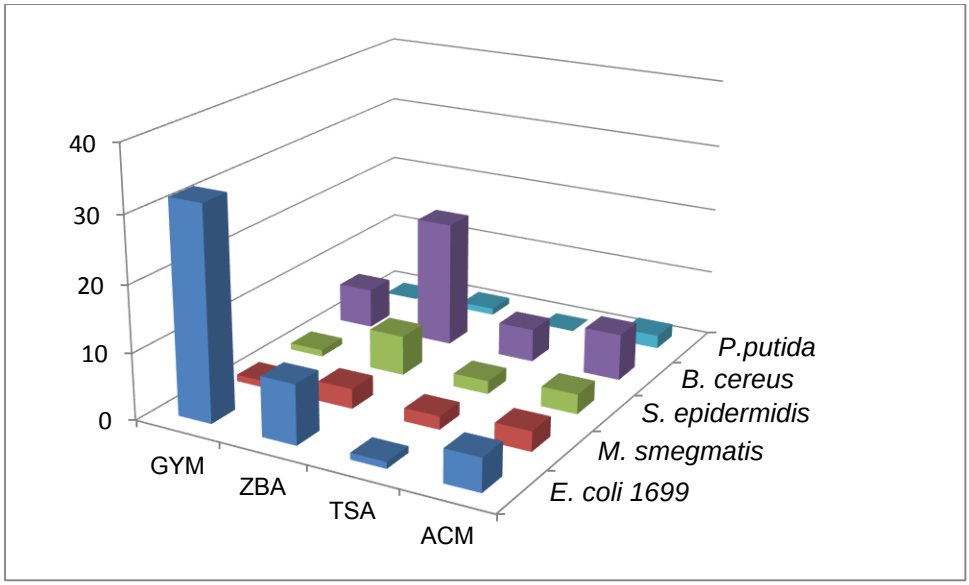


Figure 3.5: Graph representing the correlation between the 4 different media vs the activities in the total isolates using the OSMAC approach.

UNIVERSITY of the
WESTERN CAPE

The OSMAC approach works on the principle that by changing culture conditions of a microorganism, bioactivity can be induced due to the microorganism's stress response. The stress response causes the microorganisms to "switch-on" silent secondary metabolite genes thus producing antimicrobial compounds (Craney et al. 2013). Figure 3.5 represents the effect of medium related differences against the various test strains. From figure 3.5 we can see that GYM medium as well as ZBA medium produced the highest response in terms of antimicrobial activity within the isolates. These two medium types as explained in section 3.2.2 are nutrient rich media.

http://etd.uwc.ac.za/

3.4 16S rRNA Gene Amplification

Phylogenetic characterisation and de-replication of the bioactive isolates from *Distaplia skoogi* and *Eudistoma sp.* 011RSASPN was determined by 16S rRNA gene sequencing. The closest related species was determined by a BLASTn analysis against the NCBI 16S rRNA gene sequence database (bacteria/archaea) presented in Table 3.1.

The partial 16S rRNA sequences were determined for 55% of the antimicrobial producing isolates. These isolates were selected based on their activity profiles, and the criteria that were selected for included: the number of activities displayed as well as the bioactivity profiles displayed on different media types for the same isolate. The antimicrobial producing isolates showed identities ranging from 96-100% identity to microorganisms on the NCBI database (Table 3.1).

UNIVERSITY of the
WESTERN CAPE

A study by Riesenfeld et al. (2008) indicates that *Pseudovibrio ascidiaceicola* F423 and *Pseudovibrio ascidiaceicola* strain F10102 are tunicate specific isolates. Their study showed that the common phyla associated with tunicates are Proteobacteria, Bacteroidetes, and Verrucomicrobia which accounted for 97% of the tunicate diversity. Their BLAST results indicated that tunicate-associated bacteria were most closely related to bacteria from marine environments, which included but was not limited to bacteria known to be tunicate- or sponge-associated.

It was observed that 25% of the isolates sequenced are represented by the *Bacillus* genus and 42% of the isolates sequenced are represented by the *Pseudovibrio* genus. This is expected since these microorganisms are commonly isolated from the marine environment (Perez-Matos et al. 2007).

http://etd.uwc.ac.za/

The bioactive strains isolated from *Distaplia skoogi* all belonged to the *Bacillaceae* family, with the exception of two of the isolates which belonged to the *Vibrionaceae* and *Rhodobacteraceae* families respectively. The majority of the isolates belonged to the genus *Bacillus*, that is found in various environments, due to their ability to form spores (Nicholson et al. 2000). Microorganisms within the *Bacillus* genus have been shown to produce antimicrobial compounds, including those isolated from marine invertebrates (Hentschel et al. 2001; Zhang et al. 2012).

Isolates PE6_129 and PE12_50 isolated from *Distaplia skoogi* and *Eudistoma sp* 011RSASPN showed an identity of 97% to *Lysinibacillus macroides* and 96% identity to *Pseudovibrio ascidiaceicola*, respectively. This implies that these isolates could represent a new species, which could be determined by complete sequencing of the entire 1.5 kb 16S rRNA gene in a further study (Lavy et al. 2014). The 16S rRNA gene sequence classification for identity is as follows; 97% at the species level, 95% at the genus level and 80% at the phylum level.

UNIVERSITY of the
WESTERN CAPE

The majority of the bioactive strains isolated from *Eudistoma sp.* 011RSASPN belonged to the genus *Pseudovibrio* which falls within the class of Alphaproteobacteria (Phylum-Proteobacteria). Members of this genus have been identified to be associated with marine invertebrates, including tunicates (Fukunaga et al. 2006) sponges (Enticknap et al. 2006), and corals (Koren and Rosenberg, 2006), although a few studies have reported *Pseudovibrio* rRNA gene sequences from free-living bacteria in seawater (Agogue et al. 2005).

http://etd.uwc.ac.za/

Table 3.1: Identification of antimicrobial producing strains associated with marine invertebrates using partial 16S rRNA gene sequence results sorted based on isolation media.

Sample ID	Isolation Media	Closest BLASTn Hit	% Related	Sequence length (bp)	Number of indicator strains, isolate is active against	Best Activity Media
PE06_105	172F	*Lysinibacillus fusiformis*	99%	1500	2	TSA
PE06_129	172F	*Lysinibacillus macroides*	97%	155	2	ZBA
PE06_27	ACM	*Bacillus weihenstephanensis*	100%	918	1	GYM
PE06_114	CHI	*Paracoccus carotinifaciens*	100%	576	2	ZBA
PE12_116	GYM	*Vibrio splendidus*	98%	634	3	GYM
PE12_20	PMM	*Pseudovibrio ascidiaceicola*	100%	634	1	GYM
PE12_11	R2A	*Peanibacillus polymyxa*	99%	553	5	ACM
PE12_109	SEM	*Pseudovibrio ascidiaceicola*	99%	521	1	ZBA
PE06_1	SNA	*Bacillus anthracis*	100%	797	1	GYM
PE06_4	SNA	*Bacillus cereus*	100%	917	1	GYM
PE12_40	SNA	*Lysinibacillus fusiformis*	100%	257	1	GYM

http://etd.uwc.ac.za/

PE12_41	SNA	*Ruegeria atlantica*	100%	654	1	GYM
PE12_46	SNA	*Pseudovibrio ascidiaceicola*	99%	581	2	GYM
PE06_97	SWA	*Vibrio splendidus*	98%	453	1	ZBA
PE06_56	TSA	*Bacillus mycoides*	99%	718	5	ACM
PE12_50	TSA	*Pseudovibrio ascidiaceicola*	96%	419	1	GYM
PE12_61	TSA	*Pseudovibrio ascidiaceicola*	99%	641	1	ZBA
PE12_79	ZBV	*Pseudovibrio ascidiaceicola*	99%	580	2	GYM
PE12_80	ZBV	*Pseudovibrio sp. P1MA4*	99%	1343	1	ZBA
PE12_81	ZBV	*Pseudovibrio japonicus*	99%	561	1	ZBA
PE12_82	ZBV	*Pseudovibrio ascidiaceicola*	99%	528	1	ZBA
PE12_99	ZBV	*Pseudovibrio sp. P1MA3*	99%	1351	1	ZBA

http://etd.uwc.ac.za/

The isolates in Table 3.1 represent de-replicated isolates; however, several of the isolates at the 16S gene rRNA level are identical but display different bioactivity profiles (Table 3.1 and Figure 3.5). Identification using 16S rRNA results alone are not sufficient to determine the identity of the isolates, however, this was used as a method of further de-replication of the isolates.

Although only the isolates shown to produce antibacterial activity are recorded in Table 3.1, an attempt was made to analyse the media-related effects on the isolation. The actinobacteria media (Modified 172F and GYM) as well as ACM medium isolated 3 *Bacillaceae* family bacteria namely; *Lysinibacillus* and *Bacillus* genera from the *D. skoogi* tunicate and 1 microorganism from the *Vibrionaceae* family which was isolated from *Eudistoma sp* 011RSASPN. The majority of the microorganisms isolated on SNA media, which is used for the isolation of Firmicutes (Section 3.2.2), belonged to the phylum Firmicutes with the exception of 2 microorganisms that belonged to the Proteobacteria phylum namely; *Pseudovibrio* and *Ruegeria*. TSA, which is also typical for the isolation of Firmicutes as well as other Gram-positive microorganisms, resulted in the isolation of a *Bacillus*. The remaining 2 microorganisms that were isolated on TSA belonged to the Proteobacteria phylum and were Gram-negative. These microorganisms were from the *Pseudovibrio* genus. The ZBV media, which is used for isolating Gram-negative bacteria, isolated only *Pseudovibrio* species, which are Gram-negative microorganisms. As stated above the majority of isolates were identified to belong to the *Pseudovibrio* genus, which is expected, as many *Pseudovibrio* species have been isolated predominantly from tunicates (Harrington et al. 2014). The majority of the media used in this study were able to isolate the microorganisms that they were expected to isolate. However, *Pseudovibrio*, *Vibrio* and *Bacillus* species were

http://etd.uwc.ac.za/

isolated using a number of different media, not just one specific one. It is therefore important to employ as many different media as possible in order to capture as many of the culturable representatives as possible. It is also important to note that one may fail to access the bioactivity diversity if isolations are based purely on morphology, since many of the isolates may appear to look the same on a specific medium, however they might all display different bioactivity patterns. It is therefore important in this kind of work to not dereplicate based on morphology but rather on bioactivity profiles.

3.5 Antimicrobial Extract Preparation

There are several methods that are used for the isolation and successive purification of antimicrobial peptides (Filira et al. 1990). The techniques used are generally dependant on the class of antimicrobial peptide as well as the impurity of the peptide (Kiyama et al. 1984). The most frequently used techniques are solvent extraction and ion-exchange chromatography, however, the standard method is High Pressure Liquid Chromatography (HPLC), but this is not always a viable option due to it being relatively expensive (Kamysz et al. 2004).

UNIVERSITY of the
WESTERN CAPE

Solvent Extraction – This extraction method is used to extract and purify extracellular antimicrobial metabolites. It is commonly used when the nature of the metabolites is unknown. The crude extracellular extract (culture supernatant) is treated with one or two solvents, and mixed for 1 hr after which the mixture either undergoes centrifugation with subsequent evaporation or the solvent is evaporated off the mixture immediately. Thereafter, the solvent-free extract is dissolved in distilled water. Commonly used solvents include but are not limited to, acetone, ethyl acetate,

http://etd.uwc.ac.za/

chloroform and methanol; these solvents are either used alone or in combination with each other.

Thin-Layer Chromatography (TLC) – A chromatography technique that is used to separate non-volatile mixtures (Harwood and Moody, 1989). TLC is carried out on a sheet of one of the following; glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, such as silica gel, aluminium oxide, or cellulose.

Thin-layer chromatography can be used to monitor the progress of a reaction, identify compounds as well as determine the purity of a compound or solution (Reich and Schibli, 2007).

High Pressure Liquid Chromatography – A highly sensitive technique that separates substances to a degree where chemical compounds that may have similar structures are differentiated.

UNIVERSITY of the
WESTERN CAPE

Solid Phase Extraction (SPE) - This method was developed for determining the concentration of synthetic, biological, and environmental samples before analysis by conventional methods. This method relies on chromatographic separation that can be utilized for various applications (Kamysz et al. 2004). There are four basic extraction principles that are applied during SPE; these include polar, non-polar, covalent interactions as well as ion-exchange chromatography. SPE has advantages over other methods of extraction due to its rapidity, reproducibility, and efficiency (Zeeuw, 1997).

Ion-Exchange Chromatography – This method separates compounds based on their charge. There are essentially three types of ion-exchange chromatography, Cation-exchange which selects for positively charged compounds, Anion-exchange which

http://etd.uwc.ac.za/

selects for negatively charged compounds and mixed bed ion exchange which selects for both positively and negatively charged compounds such as biological molecules (Weiss and Weiss, 2005).

Antimicrobial extracts were prepared in collaboration with the PharmaSea partner, Medina Foundation. The antimicrobial extracts were prepared from liquid cultures and screened against bio-medically relevant indicator strains. The screening identified the bioactivity displayed in Figure 3.3.

The antimicrobial extracts were prepared using both an ion-exchange resin (Amberlite MB-150) as well as a hydrophobic resin (Sepa SP207ss). The resins each have their own respective properties (Table 3.2). The Sepa resin was selective for hydrophobic compounds whereas, the Amberlite resin was selective for polar (both anionic as well as cationic) molecules/compounds. The chemical nature of Amberlite MB-150 and Sepa SP207ss are significantly different, Amberlite MB-150 resin is highly cationic and anionic and is an ion exchange resin that is suitable for ion exchange chromatography and has been used as a chelating resin, whereas Sepa SP207ss resin is a dense resin that is suitable for reverse phase chromatography and has been used for the extraction of large hydrophobic compounds (Sigma-Aldrich, 2006).

Antimicrobial activity was identified with the Sepa resin (Figure 3.3) as well as with the Amberlite resin.

http://etd.uwc.ac.za/

Table 3.2: Chemical differences between Amberlite MB-150 and Sepa SP207ss beads

Resin	Chemical Nature	Resin Type	Mesh Size	Capacity
Amberlite MB-150	Polystyrene	Mixed Bed Ion-Exchange	16-50	2 meq/ml
Sepa SP207ss	Polyaromatic	Hydrophobic Resin	20-60	1.18 g/ml

3.5.1 High Pressure Liquid Chromatography (HPLC) and Well Diffusion Antibiotic Assay of antimicrobial extract PE12_11

HPLC was used as another separation method and to fractionate the metabolites. The antimicrobial extracts were fractionated in order to ascertain which fractions displayed activity; it was also used to infer how many compounds were responsible for the antibacterial activities displayed.

UNIVERSITY of the
WESTERN CAPE

The antimicrobial extract PE12_11 was prepared in ZBA media and was fractionated via HPLC and 24 fractions (2 ml) were collected.

The well diffusion assay was used to determine which fraction of the PE12_11 extract displayed antimicrobial activity. There were six active fractions, namely, fraction 1, 2, 4, 13, 16 and 17, however, only fraction 13 consistently displayed antimicrobial activity each time. The change in antimicrobial activity is a common problem associated with drug discovery. There are many reasons as to why antimicrobial activity is inconsistent, since antimicrobial activity is produced in response to induced stress as well as being a protective mechanism (Strelkauskas et al. 2009). This could also be an indication that the compound is labile.

http://etd.uwc.ac.za/

3.6 Screening for bio-medically relevant compounds

Screening for bio-medically relevant antimicrobial activities was carried out by several PharmaSea partners. The screens included a range of microbial infective agents (anti-MRSA, anti-fungal) and anti-inflammatory activity assays to name a few. Antimicrobial extracts of each positive hit, i.e. the isolate that displayed antimicrobial activity, were prepared from cultures grown in four different media, namely GYM, TSA, ZBA and ACM and subsequently screened by the PharmaSea partners. These four media were selected since the preliminary screening results (3.3.1) showed that these media resulted in the induction of the most antimicrobial activities. In addition to these preliminary data obtained by a member on the PharmaSea project, it was demonstrated that when growing the same isolate on these four media, different antimicrobial activity profiles were observed.

Isolates PE06_34 and PE06_105 are particularly interesting, since their antimicrobial extracts were demonstrated to be inhibitory to Methicillin Resistant *Staphylococcus aureus* (MRSA). MRSA is a highly drug resistant strain of *S. aureus* and is a major cause of infections leading to a high mortality rate worldwide (Witte et al. 2007). *S. aureus* has been recognized as one of the major causes of infections in humans and is a leading cause of many nosocomial infections (Witte et al. 2007). The emergence of MRSA has made these infections almost impossible to treat, in that the resistance to methicillin antibiotics indicates resistance to all β-lactam antibiotics (Hryniewicz, 1999). Due to the severity, the growing incidence of MRSA infections as well as the multidrug resistance that this strain possesses, efficacious infection control measures are required. This necessitates the development of novel antibiotics that are able to inhibit MRSA.

http://etd.uwc.ac.za/

The isolates PE06_34 and PE06_105, displayed an inhibitory effect on MRSA. The level of inhibition indicated that the antimicrobial extracts of these isolates can potentially be used as antibiotics to combat MRSA infections. Furthermore, since little evidence of cytotoxicity was exhibited by these extracts, it could infer that the extracts from isolates PE06_34 and PE06_105 prepared in ACM media are non-toxic to human cells; however, the purified compound may display different results and may be toxic to human cells.

http://etd.uwc.ac.za/

Table 3.3: A summary of the isolates that demonstrated bio-medically relevant bioactivity and the growth media which induced the activities

ORGANISM	Extract Media	MEDINA ASSAYS					MarBio Assays		KEY	
		E.coli - MB2884	*S. aureus*- MRSA- MB5393	*P. aeruginosa* - PA01	*A. fumigatus* ATCC 46645	*C. albicans* MY1055	Toxicity	Anti-Inflammatory	MEDINA ASSAYS	
PE06-105	ZBA	0	0	0	2	0	2	0	0	NO INHIBITION
PE06-105	TSA	0	0	0	2	0	2	0	1	SLIGHT INHIBITION
PE06-105	ACM	0	2	0	0	0	0	0	2	INHIBITION
PE06-111	GYM	0	0	0	2	2	0	0	**TOXICITY**	
PE06-111	ACM	0	0	0	2	0	0	0	0	NOT TOXIC TO HUMAN CELLS
PE06-126	ZBA	0	0	0	2	1	0	0	1	SLIGHT TOXICITY TO HUMAN CELLS
PE06-126	TSA	0	0	0	2	2	0	0	2	VERY TOXIC TO HUMAN CELLS
PE06-129	ACM	0	0	0	2	0	0	0	**ANTI-INFLAMMATORY**	
PE06-19	ACM	0	0	0	2	2	0	0	0	NO ACTIVITY
PE06-28	ACM	0	0	0	2	0	0	0	1	SLIGHT ACTIVITY
PE06-34	GYM	0	0	0	2	2	0	0	2	HAS ACTIVITY
PE06-34	ACM	0	2	0	2	0	0	0		
PE12-101	ACM	0	0	0	2	0	0	0		
PE12-102	ZBA	0	0	0	2	0	0	0		
PE12-106	GYM	0	0	0	2	2	0	0		
PE12-106	ACM	0	0	0	2	0	0	0		
PE12-107	ACM	0	0	0	2	0	0	0		
PE12-128	TSA	0	0	0	2	0	0	0		
PE12-46	TSA	0	0	0	2	0	2	0		
PE12-50	GYM	0	0	0	2	0	0	0		
PE12-50	TSA	0	0	0	2	0	2	0		
PE12-95	GYM	0	0	0	2	2	0	0		

http://etd.uwc.ac.za/

A majority of the isolates and their respective extracts displayed anti-fungal activity against *Aspergillus fumigatus* ATCC 46645, with seven of the isolates also displaying anti-fungal activity against *Candida albicans* MY1055. The emergence of life-threatening fungal infections has caused an increased need for novel anti-fungal agents. *Candida albicans* causes infections such as candidiasis in immunocompromised individuals which often proves to be life threatening (Fung et al. 1986; Onishi et al. 2000).

Aspergillus fumigatus is ubiquitous in nature, occurring naturally in soil as well as plants. It is known to be a human pathogen causing many diseases. These diseases include, allergic reactions and invasive aspergillosis, a condition that has been reported to cause morbidity as well as mortality. Resistance to treatment methods such as the drug class azoles has been limited, however, over recent years' multi-resistance to azoles has been detected as the main reason for treatment failure. Therefore, finding novel antifungals, specifically with a different mode of action to the azoles, is of importance (Faria-Ramos et al. 2014).

UNIVERSITY of the
WESTERN CAPE

The data revealed that none of the isolates displayed any anti-inflammatory properties. The cytoxicity values indicate that the extracts are potentially safe to use, however the toxicity needs to be properly evaluated once the compound/s responsible for the activity have been identified and isolated. Extensive toxicity tests will have to be performed to determine the amount of compound within the extract that is safe to use.

http://etd.uwc.ac.za/

Chapter 4: Results and Discussion of PE12_11 - *Paenibacillus polymyxa*

4.1 Introduction

The *Paenibacillus* genus is known to produce antibiotics from the polymyxin class. This class of antibiotic was first discovered in 1947 and was used clinically from the 1950's to the 1970's (Velkov et al. 2013). Due to the neurotoxicity and nephrotoxicity that was displayed during intravenous use, the polymyxins were deemed unsafe for clinical use and were no longer produced (Velkov et al. 2013). Polymyxins display multiple activities against drug-resistant Gram-negative microorganisms such as *Acinetobacter baumannii*, *K. pneumonia* and *P. aeruginosa*. There are currently 2 polymyxins that are used clinically as a last-defence against extremely drug-resistant Gram-negative microorganisms namely; polymyxin B and polymyxin E, also known as colistin. Analogues of polymyxin B and colistin have been produced in order to improve their microbiological and toxicological activity profiles (Velkov et al. 2010).

UNIVERSITY of the
WESTERN CAPE

Due to the activity profile of PE12_11 as well as the partial 16S rRNA gene sequence data, it suggested that the antimicrobial compound being produced is possibly a polymyxin. However, the activity profile of PE12_11 also displayed activity against Gram-positive indicator microorganisms suggesting that this isolate could produce more than one active secondary metabolite. Dereplication is something that needs to be done at the beginning in a biodiscovery study, so as not to lose too much time looking for known compounds. As such, a bioinformatics analysis of secondary metabolite pathways in the *Paenibacillus* genus was conducted in parallel to a sequence-based screening of the PE12_11 pathways. Bioinformatics analysis, given all the tools available, can enable dereplication at the genomic level; instead of at the chemistry level (Weber et al. 2015). Furthermore, the genomics approach is able to provide one with information regarding the type of biosynthetic gene clusters

http://etd.uwc.ac.za/

present as well as infer any structural predictions about the natural products produced, from the corresponding informatics (Kleigrewe et al. 2015). In *P. polymyxa*, NRPS and NRPS-transAT PKS hybrids are most frequently encountered and are generally amenable to informatics-based deductions of structure (Kleigrewe et al. 2015). Genome mining for gene clusters that encode the biosynthetic pathways for microbial secondary metabolites has become an important methodology for novel compound discovery (Weber et al. 2015). antiSMASH has just launched version 3.0 of their online program which enables the user to search for biosynthetic gene clusters within their gene sequence. The improved version of antiSMASH allows one to dereplicate since their new algorithm, a variant of the ClusterBlast module, is able to identify similarities of previously identified clusters to any of 1172 clusters with known end products in their database (Weber et al. 2015). The number of whole microbial genomes and metagenomic data that is publicly available is exponentially increasing and due to this, genome mining has become an exceptionally attractive tool for novel drug discovery. This has steered the development of bioinformatic tools that are used for screening and identification of the genetic background of the bioactivities which include gene clusters responsible for the production of the novel compounds (Machado et al. 2015).

UNIVERSITY of the
WESTERN CAPE

An added advantage of a genomic based approach versus a chemistry one is that the majority of the clusters are silent under standard laboratory culture conditions and require induction (Seyedsayamdost, 2014). Several of the new bioinformatic tools have been designed so as to search specifically for PKS and NRPS clusters, since these structures are conserved. Therefore bioinformatic programs were designed in order to identify gene clusters that are silent under standard laboratory conditions. Apart from antiSMASH, there are several bioinformatic programs that

http://etd.uwc.ac.za/

enable the user to search for various biosynthetic gene clusters: such as BAGEL3, which is used to identify bacteriocins (Van Heel et al. 2013); NapDos which is able to identify keto-synthase (KS-domains) as well as condensation domains (C-domains) (Ziemert et al. 2012); and NP.search which identifies whole gene clusters which may potentially be comprised of several KS- and/or C-domains (Li et al. 2009). PRISM is able to identify biosynthetic gene clusters, predict genetically encoded NRPS and PKS (type I and II), and it can also be used for dereplicating identified natural products (Skinnider et al. 2015).

4.2 Bioinformatics analysis of gene clusters for *Paenibacillus sp.*

PE12_11, an isolate associated with the tunicate *Eudistoma sp.* 011RSASPN displayed multiple antimicrobial activities (section 3.3.1) and was identified to have 99% sequence identity to *Paenibacillus polymyxa* SC2. Previous studies have identified that *Paenibacillus polymyxa* strains are known to produce secondary metabolite genes that encode for antimicrobial activity (Choi et al. 2009).

Due to *P. polymyxa* being known for producing antimicrobial activities, it was important to establish early on in the study whether this strain contained a potentially novel secondary metabolite gene cluster. Polymyxin (encoded by a NRPS pathway) is a well-known antibiotic that is currently being used, therefore there was a need to identify whether the strain of *P. polymyxa* that was isolated from the *Eudistoma sp.* 011RSASPN was novel.

A bioinformatics analysis was carried out on the whole genome sequences of *Paenibacillus sp*. in order to compare the number of biosynthetic gene clusters prevalent in the closely related species.

http://etd.uwc.ac.za/

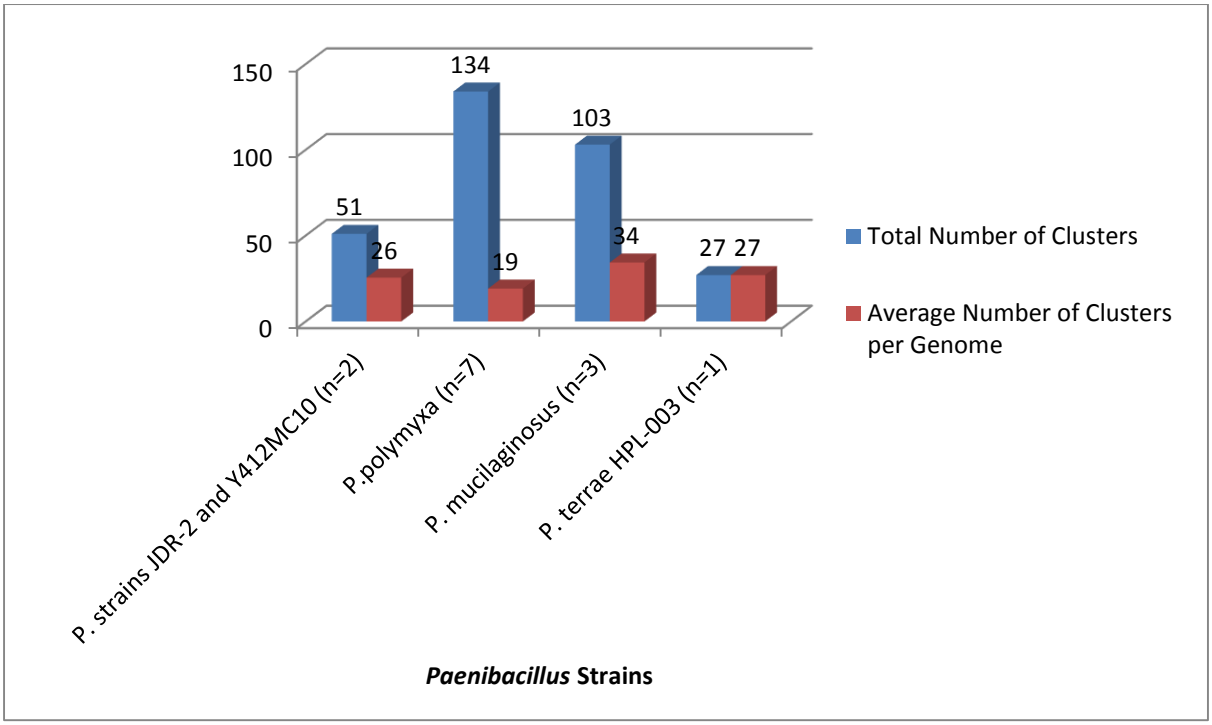


Figure 4.1: Quantitative analysis of biosynthetic gene clusters prevalent in the closely related *Paenibacillus* strains. *Paenibacillus* strains were selected based on their relatedness to the *Paenibacillus* isolate (PE12_11) that displayed multiple bioactivities. Genome mining was performed using antiSMASH2.0 software. The number of strains analysed for each *Paenibacillus* species were as follows; *Paenibacillus* strain JDR-2; n=1, *Paenibacillus* strain Y412MC10; n=1, *P. polymyxa*; n=7, *P. mucilaginosus*; n=3 and *P. terrae* HPL-003; n=1. The accession numbers for each strain analysed are as follows: *Paenibacillus* strain JDR-2; NC_012914.1, *Paenibacillus* strain Y412MC10; NC_013406.1; *P. polymyxa*; NC_014622.1, NC_014483.1, NC_017542.1, CP006872.1, CP006941.2, CP009909.1, CP010268.1; *P. mucilaginosus*; NC_017672.3, NC_016935.1, NC_015690.1 and *P. terrae* HPL-003; NC_016641.1.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

P. mucilaginosus contained the most biosynthetic gene clusters per genome (Figure 4.1) with *P. polymyxa* having the least number of clusters per genome; however, in Figure 4.2 *P. polymyxa* displays the most non-ribosomal peptide synthase (NRPS) gene clusters. It is also observed that *P. polymyxa* contains the most diverse range of biosynthetic gene clusters as compared to the other *Paenibacillus* species.

http://etd.uwc.ac.za/

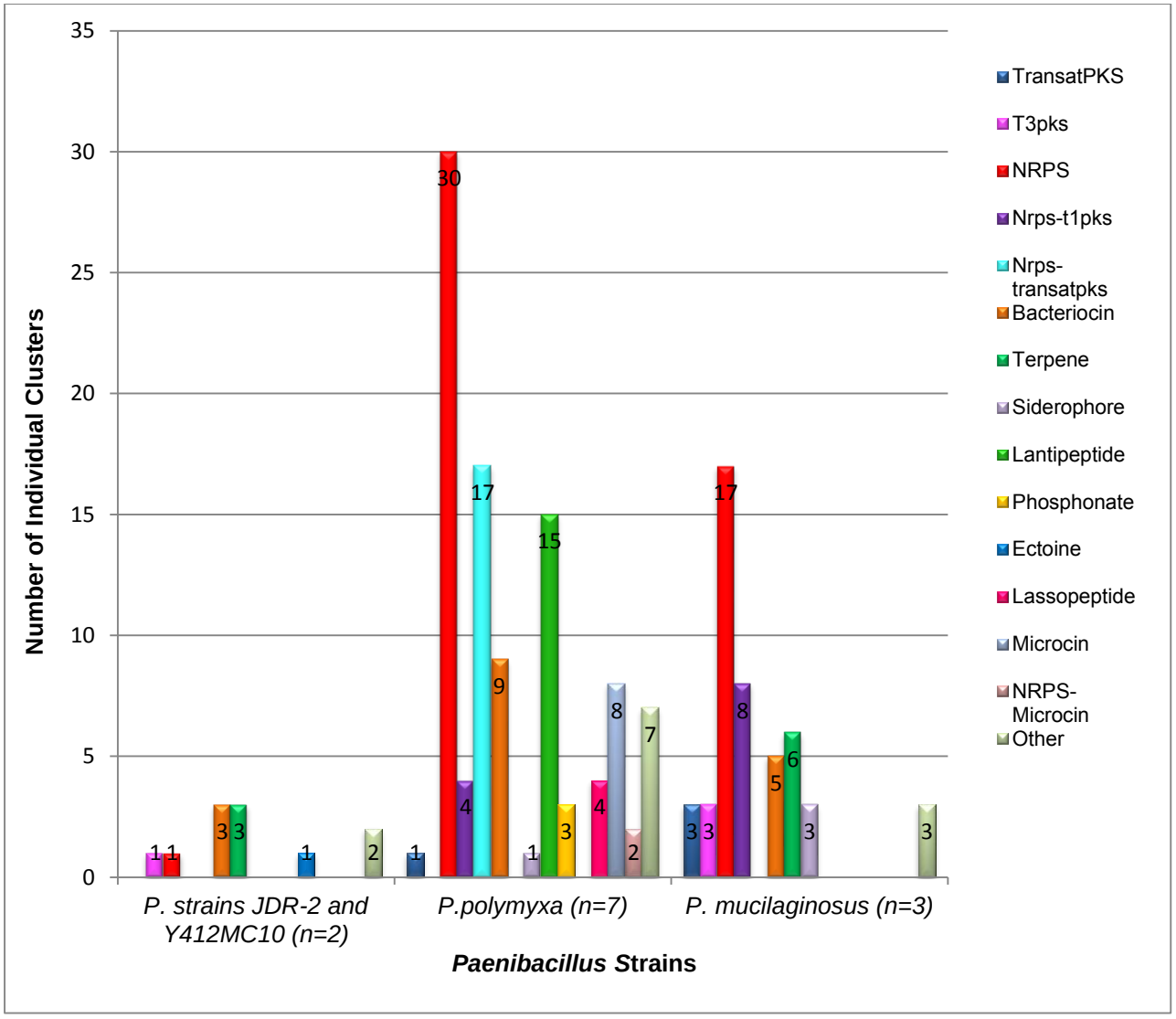


Figure 4.2: Qualitative analysis of the distribution of biosynthetic gene clusters prevalent in closely related *Paenibacillus* species. *Paenibacillus* strains were selected based on their relatedness to the *Paenibacillus* isolate that displayed multiple bioactivities. Genome mining was performed using antiSMASH2.0 software. The number of strains analysed for each *Paenibacillus* species were as follows; *Paenibacillus* strain JDR-2; n=1, *Paenibacillus* strain Y412MC10; n=1, *P. polymyxa*; n=7 and *P. mucilaginosus*; n=3. The accession numbers for each strain analysed are as follows: *Paenibacillus* strain JDR-2; NC_012914.1, *Paenibacillus* strain Y412MC10; NC_013406.1; *P. polymyxa*; NC_014622.1, NC_014483.1, NC_017542.1, CP006872.1, CP006941.2, CP009909.1, CP010268.1 and *P. mucilaginosus*; NC_017672.3, NC_016935.1, NC_015690.1.

http://etd.uwc.ac.za/

The bioinformatics analysis revealed that NRPS pathways were present in all the *Paenibacillus* genomes that were analysed and is the most abundant gene cluster in the *P. mucilaginosus* genomes as well as in the *P. polymyxa* genomes (Figure 4.3). The *P. mucilaginosus* species displays the second most diverse and abundant gene clusters. In addition to the NRPS gene cluster being abundant in the *P. polymyxa* species, the Nrps-transatpks was also found to be an abundant class within this species, with bacteriocins and lantipeptides having a similar abundance in *P. polymyxa*. Bacteriocins were the second most abundant class of secondary metabolite genes present in all the *Paenibacillus* genomes that were analysed.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

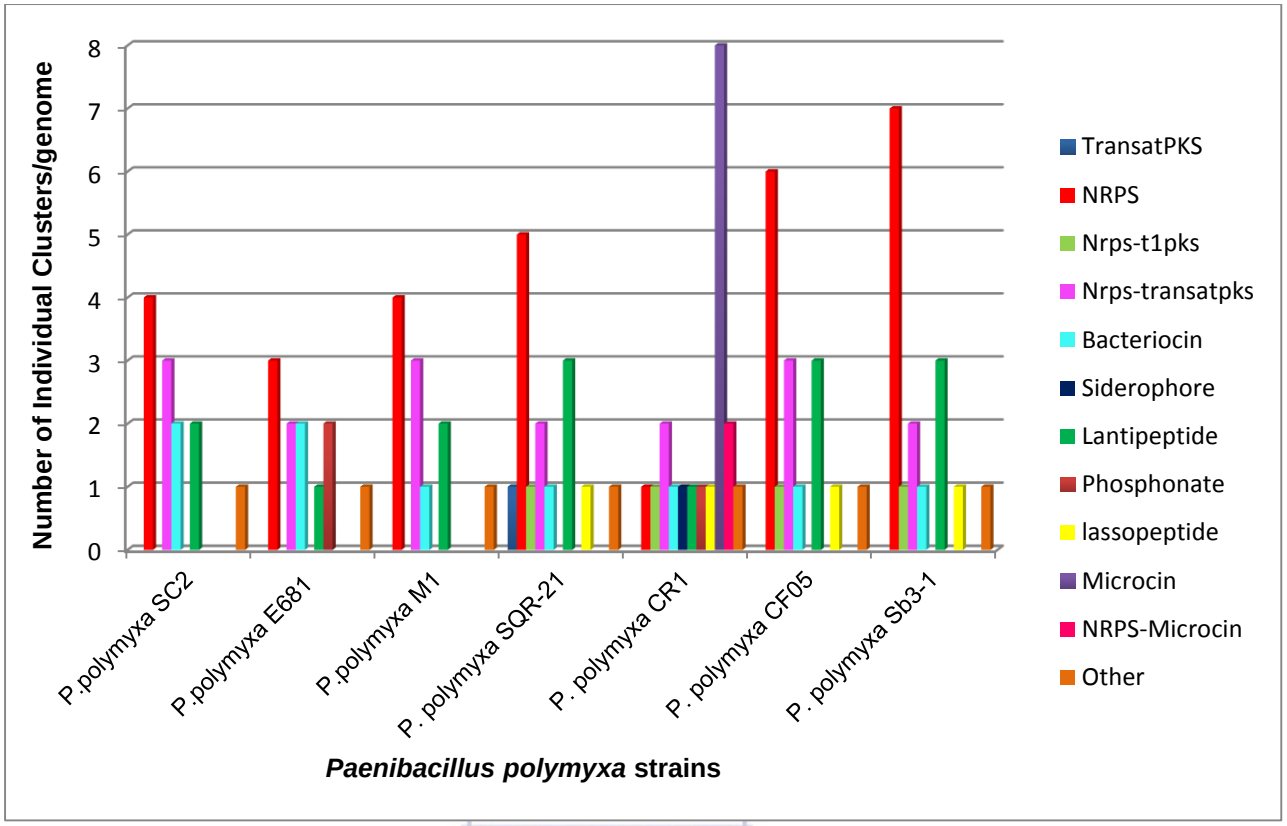


Figure 4.3: Quantitative analysis of biosynthetic gene clusters prevalent in closely related *Paenibacillus polymyxa* strains that have been shown to exhibit antimicrobial activity. *Paenibacillus polymyxa* strains were selected based on their relatedness to the *Paenibacillus* isolate PE12_11 that displayed multiple bioactivities. Genome mining was performed using antiSMASH2.0 software. The number of strains analysed for the *Paenibacillus polymyxa* species were n=7. The accession numbers for each strain analysed are as follows: *P. polymyxa*; NC_014622.1, NC_014483.1, NC_017542.1, CP006872.1, CP006941.2, CP009909.1 and CP010268.1.

A closer analysis of the *P. polymyxa* strains reveals that they are the only strains to exhibit NRPS-transatpks, microcins, NRPS-microcins as well as lantipeptides (Figure 4.2 and Figure 4.3). Bacteriocins were found to be present in all the *P. polymyxa* genomes that were analysed.

http://etd.uwc.ac.za/

It is interesting to note that the secondary metabolite genes within the *P. polymyxa* genomes that were analysed all belong to the same classes with relatively similar abundance within the genomes. One could infer that secondary metabolite genes of the same classes are generally evenly distributed within related strains of the same species.

It is well documented that *P. polymyxa* species are known to contain NRPS, PKS's as well as lantipeptides which have been demonstrated to be responsible for the production of antibiotics in microorganisms from the *Paenibacillus* genus (Wu et al. 2011). It is interesting to note that the latest four genomes to be completely sequenced (CP006872.1, CP006941.2, CP009909.1 and CP010268.1) all contain the gene cluster lassopeptides in relatively the same abundance. *P. polymyxa* CR1 has the least abundance of NRPS gene clusters, whilst having a very high abundance of microcins. It is also the only strain that contains a NRPS-microcin hybrid, and the prevalence of the hybrid is higher in abundance than the NRPS gene clusters. Due to the recent discovery and progress made with *P. polymyxa* it is evident that there is a lot that is still unknown about the various strains of *P. polymyxa*. The gene clusters that are present in these strains as well as the antibiotics that are produced. This evidence reinstates the need for more research to be done on the *P. polymyxa* strains.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

4.2.1 Summary of compounds identified and classified within the *P. polymyxa* species

Table 4.1: A summary of the compounds identified and characterized within the *P. polymyxa* strains (Adapted from the Novel Antibiotics Data Base: http://www.antibiotics.or.jp/journal/database/database-top.htm).

Compound	*P. polymyxa* Strain	Pathway	Activity Index	Year
Polymyxin P	T-39	NRPS	Antibacterial	1969
Jolipeptin	var. colistinus	NRPS	Antibacterial	1972
Gatavalin	var. colistinus	NRPS	Antifungal	1972
Polymyxin S1	Rs-6	NRPS	Antibacterial	1977
Polymyxin T1	E-12	NRPS	Antibacterial	1977
Tridecaptin A	AR-110	NRPS	Antibacterial	1978
Tridecaptin B	B-2	NRPS	Antibacterial	1978
Tridecaptin C	E-23	NRPS	Antibacterial	1978
Colistin pro-A	var. colistinus	NRPS	Antibacterial	1982
Colistin pro-B	var. colistinus	NRPS	Antibacterial	1982
Colistin pro-C	var. colistinus	NRPS	Antibacterial	1982
Fusaricidin A	KT-8	NRPS	Antifungal	1996
Fusaricidin B	KT-8	NRPS	Antifungal	1997
Fusaricidin C	KT-8	NRPS	Antifungal	1997
Fusaricidin D	KT-8	NRPS	Antifungal	1997
Polymyxin E7	var. colistinus	NRPS	Antibacterial	2001

There is a need to develop or identify diverse polymyxin derivatives in order to solve the issues relating to toxicity as well as resistance problems of polymyxins (Kim et al. 2015). According to Kim et al. (2015), no drug discovery platform has generated polymyxin derivatives that are non-toxic. A method that has been successful is the genetic engineering of the polymyxin synthase responsible for the antimicrobial activity (Kim et al. 2015).

http://etd.uwc.ac.za/

The Adenylation domain (A-domain) of the polymyxin A synthase from *P. polymyxa* E681 was substituted with the A-domain of *P. polymyxa* ATCC21830 in order to make a derivative of Polymyxin E which was then cloned into *Bacillus subtilis* BSK4dA for the production of polymyxin B and E (Kim et al. 2015).

4.2.2 PCR Screening for Secondary Metabolite Gene Clusters based on Bioinformatics Analysis

In order to determine whether the antibiotic produced was potentially novel, as well as to determine if isolate PE12_11 encodes more than 1 NRPS, as is the case for all other known strains, the genomic DNA was PCR screened for NRPS gene clusters. Genome sequencing is one way in which the pathways in PE12_11 could have been identified. However, given that the analysis showed that all *P. polymyxa* strains contain NRPS pathways, and that polymyxin itself is a NRP, pre-screening by PCR was a very useful alternative due to the conserved regions in the A domain of NRPSs, which are routinely used as targets for this type of screening. An expected 900bp product for the NRPS gene cluster (Figure 4.4) was obtained. This was used to identify whether these gene clusters were the same as the gene clusters that produce polymyxin, as this would aid in identifying whether PE12_11 also produces polymyxin.

http://etd.uwc.ac.za/

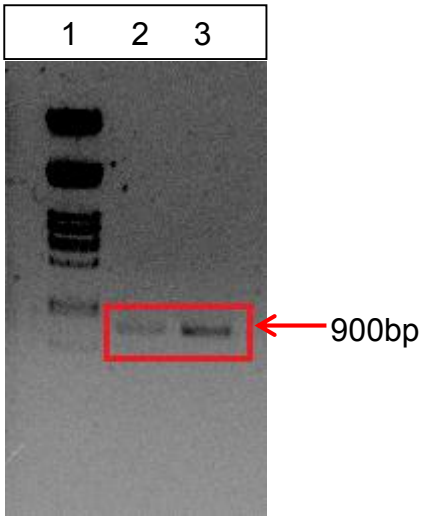


Figure 4.4: PCR amplification of NRPS gene cluster. Lane 1: λ-PstI DNA Ladder, Lane 2&3: PE12_11.

UNIVERSITY of the

The product was excised and cloned into pUC18 in order to be sequenced. Several transformants were picked and sequenced. Four of the clones were analysed. The sequence identity of the clones for the NRPS gene was determined using the Blastx tool, a summary of the results is in Table 4.2. Clone 1 showed 98% identity to *pmxA* AEZ51516.1. The *pmxA* gene cluster has been identified as being responsible for encoding the A-domain of the NRPS within *P. polymyxa* M1.

A study by Choi et al. (2009) indicated that the open reading frame of *pmxA* encodes for a polymyxin synthase and consists of an A-domain comprised of 4 modules as well as a C-domain. The four modules of the *pmxA* A-domain were inferred to be responsible for activating amino acid substrate specificities of leucine, threonine and diaminobutyric acid (Dab) (Choi et al. 2009).

http://etd.uwc.ac.za/

Table 4.2: Sequence identity of NRPS amplicons

Clone	Top Blast Hits	Amino Acid Identity (%)	Query Cover	Accession Number
1	PmxA [*Paenibacillus polymyxa*]	98	99	AEZ51516.1
	Non-ribosomal peptide synthase [Paenibacillus polymyxa]	97	68	WP_049825414.1
	Peptide Synthase [*Paenibacillus polymyxa*]	96	68	KEO76044.1
2	Synthase 2 Gramicidin S synthase II (*Paenibacillus polymyxa*)	96	69	WP_016324741.1
	Non-ribosomal peptide Synthase (*Paenibacillus polymyxa*)	96	69	WP_049825414.1
	Gramicidin synthase (*Paenibacillus polymyxa*)	96	77	WP_013312098.1
3	SocE [*Bacillus cereus*]	75	22	WP 001985485.1
4	SocE [*Bacillus cereus*]	91	28	WP 001985485.1

The sequenced based screening with NRPS primers revealed the presence of PmxA, which is a peptide synthase. It was also discovered that Gramicidin synthase, a peptide synthase and a NRP, was present in PE12_11.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

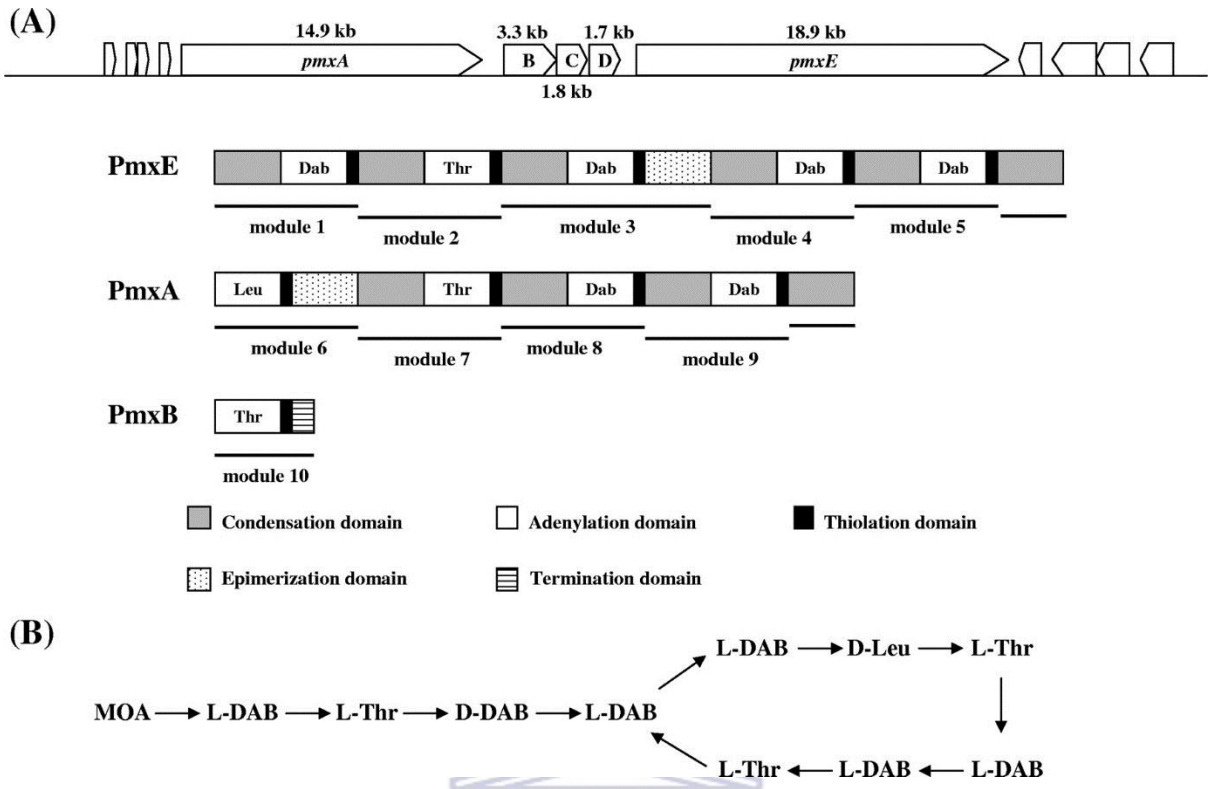


Figure 4.5: A schematic representation of the *pmx* gene cluster. A: A representation of the genetic structure of *pmx* genes (Top) and the domain organization of the Pmx enzymes (Bottom) and B: Indicates the primary structure of polymyxin A (Adapted from Choi et al. 2009).

UNIVERSITY of the
WESTERN CAPE

PmxA is an open reading frame that along with PmxB and PmxE encodes a polymyxin synthase (Figure 4.5). PmxA has been reported to be comprised of 4,953 amino acids, which contains four modules and a C-domain (Choi et al. 2009).

SocE or Store-operated Ca^{2+} entry is a calcium entry mechanism that is found in all cell types. It is responsible for regulating various cellular functions as well as determining the refilling of intracellular calcium stores. A reason for this result could also be due to the primers being degenerate; resulting in non-specific amplification.

http://etd.uwc.ac.za/

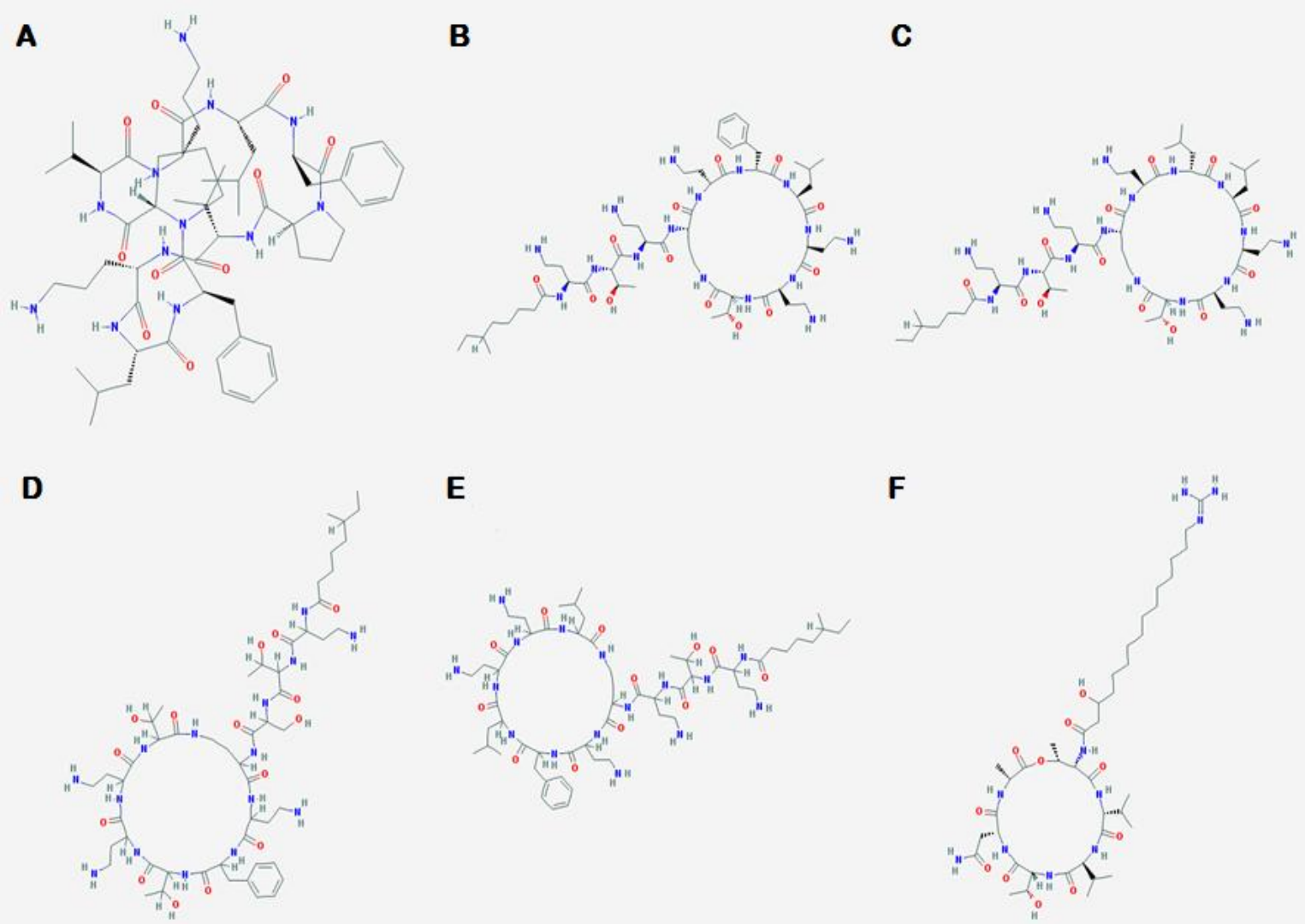


Figure 4.6: Structure of antibacterial and antifungal compounds from *P. polymyxa*. A: Gramacidin S; B: Polymyxin B; C: Polymyxin E7; D: Polymyxin S1; E: Polymyxin T1 and F: Fusaricidin A (Compound Structures Adapted from http://pubchem.ncbi.nlm.nih.gov/).

Peptide synthases are comprised of multifunctional polypeptide chains, which have been known to exceed 500 kDa, and form the backbone of templates for the sequential addition of amino acids to peptides. Gramicidin S (Figure 4.6) is synthesised by a NRPS in a two-step process. The first step involves the synthesis of the multienzyme complex in which amino acid-activating domains catalyse the adenylation of the constituent amino acids as well as the thioester formation.

http://etd.uwc.ac.za/

The amino acid-activating domain is comprised of roughly 600 residues that have a high homology with surrounding domains; sequential domains are then separated by a region consisting of 500 amino acids that form a nonhomologous region.

The second process involves the organization of the domains in a colinear sequence with the homologous amino acids in the peptide chain. The peptide chain is then transferred successively from one amino acid-activating domain to the next, where amino acids are added by the formation of a peptide bond (Zhang et al. 1995).

The cloning results indicate that there is more than one NRPS pathway within PE12_11, which is consistent with the analysis presented in Figure 4.3. where it is evident that several NRPS pathways are present within *P. polymyxa* strains.

http://etd.uwc.ac.za/

Chapter 5: Conclusions and Recommendations

There is currently a worldwide health crisis taking place, which is the increasing occurrence of drug-resistant pathogens within clinical settings (Lammie and Hughes, 2016). These drug-resistant pathogens have negative effects on patients as well as the overall healthcare system. Drug-resistant pathogens are increasingly becoming more difficult to treat. Due to this, the mortality rate associated with drug-resistant pathogens is increasing. This has an impact on the economy, since employees are staying out of work for extended periods of time due to inefficient treatment. This then results in increased strain being put on industry as well as the economy (Cosgrove, 2006). There are very limited treatment options available for overcoming drug-resistant infections which is coupled with the deterioration of novel drug discovery. Due to these factors the urgency of discovering novel drug candidates that are able to combat these infections is evident (Lammie and Hughes, 2016).

UNIVERSITY of the
WESTERN CAPE

Natural products are generally produced through secondary metabolism within organisms such as fungi, plants and bacteria. Natural products are a pivotal resource in novel drug discovery platforms in that they are chemically and biologically diverse. The chemical and biological diversity includes the production of anti-microbial, anti-fungal, anti-cancer as well as anti-inflammatory activities (Wang et al. 2012; Arasu et al. 2013).

During the period in which novel antibiotic discovery was prevalent, many of the antibiotics were isolated from terrestrial fungi and bacteria, and the importance of the natural products isolated from microorganisms was evident. Since this time, the number of novel drugs discovered from terrestrial habitats has decreased

http://etd.uwc.ac.za/

tremendously; this has led researchers to look for alternative sources of novel drugs such as the marine environment (Sarker et al. 2007; Prabhu et al. 2011).

Marine invertebrates such as corals, tunicates and sponges encompass the largest diversity within the marine environment (Leal et al. 2012). These invertebrates are an important resource of novel drug discovery platforms in that they have been shown to produce novel natural products. However, there is a supply and demand problem with using marine invertebrates as a source of novel drug candidates since the amount of compound that is produced by the invertebrates is generally found at a low concentration, therefore large amounts of biomass is required to produce sufficient compound (Proksch et al. 2002).

Marine invertebrates have been found to form symbiotic relationships with microorganisms. These microorganisms are interestingly chemically and biologically diverse and have been shown to produce natural products that are highly similar in structure as well as function to natural products that have been isolated from the symbiotic invertebrate (Piel et al. 2004). This discovery has led researchers to conclude that the true producers of these natural products are in fact the symbiotic microorganisms. Microorganisms are able to offer a sustainable alternative source of natural products, especially coupled to the fact that microorganisms can be grown in large quantities in order to upscale the amount of compound required (Wilson et al. 2014).

The emphasis of this study was focused on marine tunicates endemic to the South African coastline which is surrounded by two distinct ocean currents (cold Benguela and warm Mozambique currents) resulting in high marine biodiversity (Griffiths et al. 2010). Marine tunicates from the South African environment present a fascinating

http://etd.uwc.ac.za/

area of study because it has been largely unexplored and could prospectively result in novel secondary metabolites (Skariyachan et al. 2014; Steinert et al. 2015). The aim of this study was to screen microbial symbionts from two South African Tunicates namely; *Distaplia skoogi* and *Eudistoma sp.* 011RSASPN, for antimicrobial activity as well as to identify the secondary metabolite genes responsible for the antimicrobial activity.

5.1 Isolation of microbial symbionts from *Distaplia skoogi* and *Eudistoma sp.* 011RSASPN

Conventional methods of microbiology were used to isolate microbial symbionts from the tunicates (Section 3.2.2). A broad range of media was selected for bacterial isolation in order to access the full potential of the microbial and chemical diversity within the tunicate samples. *Distaplia skoogi* yielded 157 morphologically distinct colonies, whereas 143 morphologically distinct colonies were isolated from *Eudistoma sp.* 011RSASPN. The tunicate derived bacterial isolates were screened for anti-microbial activity against 5 diverse indicator strains, 3 Gram-positive and 2 Gram-negative strains namely; *E. coli* 1699, *S. aureus*, *B. cereus*, *P. putida* and *M. smegmatis*. It should be noted that *E. coli* 1699 is a multi-drug resistant engineered strain. This strain was engineered to be resistant to 50 of the most common antibiotics. Therefore, inhibitory activity against this strain is particularly significant as it indicates that those isolates are potentially chemically diverse and could have a novel mechanism of action.

The initial screening occurred on the media in which the isolates were isolated from. Thereafter, secondary screening techniques were employed using the OSMAC

http://etd.uwc.ac.za/

principle (Section 1.5) in which the isolates were screened on the following four different media ACM, GYM, TSA and ZBA (Section 3.3.1). Following the OSMAC approach the activity profiles of the isolates changed in that strains that had previously displayed no activity, now displayed activity. Isolate PE12_11 displayed the most diverse activity profile on more than one media type. It was inferred, due to the activity profile, that PE12_11 could potentially produce more than one antimicrobial compound. Further analysis on PE12_11 was performed in which the 16S rRNA gene was identified as having 99% homology to *Paenibacillus polymyxa*. A bioinformatic review revealed that the predominant secondary metabolite pathway present in *P. polymyxa* is the NRPS pathway. This was then compared with the PCR screen performed on PE12_11 in which it was evident that more than one NRPS pathway was identified (Section 4.2.2). The sequenced based screening with NRPS primers revealed the presence of *pmxA*, which codes for a peptide synthase responsible for the production of polymyxin. It was also discovered that the gene for Gramicidin synthase, a peptide synthase responsible for the production of a NRP, was present in PE12_11. Thus it is expected that PE12_11 produces 2 well-known antibiotics. Polymyxin's mechanism of action is due to it disrupting the bacterial cell membrane through the interaction with phospholipids within the cell membrane. Due to its mechanisms of action, it is selectively toxic to Gram-negative bacterial species. Polymyxins are used as a last resort antibiotic against multiple drug resistant strains since they have been found to be nephrotoxic as well as neurotoxic. These strains include *Pseudomonas aeruginosa* and carbapenemase-producing Enterobacteriaceae. Gramicidin synthase is used clinically against both Gram-positive as well as Gram-negative bacterial species with its mechanisms of action being the disruption of the lipid membrane as well as the enhancement of the

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

permeability of the bacterial cytoplasmic membrane (Gause and Brazhnikova, 1944; Prenner et al. 1997).

The result of the sequence based screening of PE12_11 correlates with the activity profile of PE12_11 which indicates that more than one antibacterial compound is potentially being produced, which coupled with the HPLC data (Section 3.5) in which different activity profiles were identified for different fractions of the compound, further substantiates this. In order to substantiate these claims as well as to ascertain whether any other compounds are produced with similar activity against the test strains, the compound as well as the different HPLC fractions produced by PE12_11 should undergo Mass-Spectrometry or Nuclear Magnetic Resonance (NMR), as this will enable one to identify the presence of polymyxin and gramicidin, and whether any other antibacterial secondary metabolite is also produced.

Once the compound has been identified further analysis will determine whether this strain produces anything else that is novel. Further sequence/genomics based screening will enable one to quickly ascertain if there are any other pathways worth looking at. Thereafter, one can potentially ensure that the compound is sustainably produced and avoid the supply bottleneck through heterologous expression in a suitable and non-susceptible host organism.

5.2 The benefits of using a culture based approach

The culture based approach afforded an opportunity to isolate as well as identify various bacterial species from the South African marine environment. To date, there has been a lack of research that has been carried out on national marine resources and this study, while not extensive, has enabled an insight into the biodiversity that

http://etd.uwc.ac.za/

thrives within a niche environment in the Algoa Bay. The culture based approached resulted in two potentially novel species being found, isolates PE06_129 and PE12_50 isolated from *Distaplia skoogi* and *Eudistoma sp.* 011RSASPN showed an identity of 97% to *Lysinibacillus macroides* and 96% identity to *Pseudovibrio ascidiaceicola*, respectively. However, in order for this inference to be substantiated the complete sequence of the 16S rRNA genes needs to be obtained. Therefore despite the great plate count anomaly (most of the microbes seen under the microscope cannot currently be grown under laboratory conditions), culture based approaches are still valid for the identification of diverse marine species. The culture based approached can be improved in order to target more novel species by selecting specific criteria as well as culture conditions in which the isolates are cultured.

UNIVERSITY of the
WESTERN CAPE

5.3 The importance of dereplication

Dereplication is an important process to carry out early on in a bioprospecting or biodiscovery study. Dereplication ensures that one can avoid processing and testing the same isolates, which also aids in the prevention of wasting valuable time and resources on known compounds. Before dereplication was carried out, prioritisation was performed early on in this study using an initial bioactivity based assay. This ensured that replicated isolates were not further analysed, thereby reducing the number of isolates that were taken further into the study. The remaining active isolates were then dereplicated and identified by 16s rRNA gene sequencing. Dereplication can easily be carried out by using a parallel approach such as a bioactivity-guided as well as a genome-guided approach. Bioactivity-guided assays are relatively cost effective and can be carried out in a few days. The bioactivity

http://etd.uwc.ac.za/

assays are able to give an indication as to which isolates contain the same activity, and together with isolate morphology as well as 16S rRNA gene sequencing data can easily identify the replicates in the study. The genome-guided approach is more effective in identifying replicates; however, it takes more time to achieve results. The genome-guided approach can be carried out using full genome sequencing as a means to overcome the limitations of PCR screening. Full genome sequencing is more informative and one is able to make predictions of the compound structure.

Due to one of the isolates, PE12_11, displaying activity against all the indicator strains early on in the study, this strain was primarily focused on and further studies were carried out on this strain before any 16S rRNA gene sequence data was carried out and before secondary dereplication was performed. It was only after the 16S rRNA gene sequence results were analysed and the PCR screening was performed that it was identified that the isolate that displayed potentially novel bioactivity was in fact not novel and a well-known species with well-known compounds and activity was isolated. This just shows the importance of dereplication as well as the danger of focusing on the “best” strain based on one test. Although further testing of this compound could identify a novel variant, the chances are unlikely. Therefore, it is advisable that all isolates with multiple activity be treated equally as this will increase the likelihood of finding a novel compound.

UNIVERSITY of the
WESTERN CAPE

5.4 Has the SA marine environment anything to offer?

In 2015, Davies-Coleman and Veale embarked on international collaborations with regards to mandelalides and cephalostatins, which are produced by the tube worm and ascidian respectively, drawing attention to the South African marine environment

http://etd.uwc.ac.za/

as well as our drug discovery platforms. Numerous parallel studies were conducted in my laboratory by colleagues, where numerous other sponges and tunicates were sampled and it was identified that many isolates demonstrated potent activities against fungi, yeast and MRSA. Within this study, two isolates, PE06_34 and PE06_105, demonstrated antimicrobial activity against MRSA. Due to the growing incidence of MRSA infections as well as the multidrug resistance that this strain possesses, efficacious infection control measures are required. This necessitates the development of novel antibiotics that are able to inhibit MRSA.

Numerous isolates and their respective extracts displayed anti-fungal activity against *Aspergillus fumigatus* ATCC 46645, with seven of the isolates also displaying anti-fungal activity against *Candida albicans* MY1055. The emergence of life-threatening fungal infections has caused an increased need for novel anti-fungal agents. *Candida albicans* causes infections such as candidiasis in immunocompromised individuals which often prove to be life threatening. In finding novel anti-fungal agents this can be alleviated or reduced (Fung et al. 1986; Onishi et al. 2000).

UNIVERSITY of the
WESTERN CAPE

Based on the wide array of the activities that have been discovered from isolates from tunicates within the South African marine environment, it is evident that this environment has a lot to offer and that there is a lot that can still be explored.

http://etd.uwc.ac.za/

References

Adrian TE. 2007. Novel marine-derived anti-cancer agents. Curr Pharm Des **13:**3417-3426.

Agogue H, Casamayor EO, Bourrain M, Obernosterer I, Joux F, Herndl GJ, Lebaron P. 2005. A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems. FEMS Microbiol Ecol **54:**269-280. doi:S0168-6496(05)00099-1.

Altschul SF, Gish W. 1996. [27] Local alignment statistics. Meth Enzymol **266:**460-480.

Aoki M, Hashimoto K, Watanabe H. 1989. The intrinsic origin of bioluminescence in the ascidian, *Clavelina miniata*. Biol Bull **176:**57-62.

Arasu MV, Duraipandiyan V, Ignacimuthu S. 2013. Antibacterial and antifungal activities of polyketide metabolite from marine Streptomyces sp. AP-123 and its cytotoxic effect. Chemosphere **90:**479-487. doi:http://dx.doi.org/10.1016/j.chemosphere.2012.08.006.

Arnison PG, Bibb MJ, Bierbaum G, Bowers AA, Bugni TS, Bulaj G, Camarero JA, Campopiano DJ, Challis GL, Clardy J. 2013. Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. Nat Prod Rep **30:**108-160.

Austin MB, Noel JP. 2003. The chalcone synthase superfamily of type III polyketide synthases. Nat Prod Rep **20:**79-110.

Bader J, Mast-Gerlach E, Popović M, Bajpai R, Stahl U. 2010. Relevance of microbial coculture fermentations in biotechnology. J Appl Microbiol **109:**371-387.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Baker P, Kennedy J, Morrissey J, O'Gara F, Dobson A, Marchesi J. 2010. Endoglucanase activities and growth of marine-derived fungi isolated from the sponge Haliclona simulans. J Appl Microbiol **108:**1668-1675.

Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E, Weber T. 2013. antiSMASH 2.0--a versatile platform for genome mining of secondary metabolite producers. Nucleic Acids Res **41:**W204-12. doi:10.1093/nar/gkt449.

Blunt J. W, Copp B. R, Keyzers R. A, Munro M. H, Prinsep M. R. 2015. Marine natural products. Natural product reports **32:**116-211.

Bode HB, Bethe B, Höfs R, Zeeck A. 2002. Big effects from small changes: possible ways to explore nature's chemical diversity. ChemBioChem **3:**619-627.

Boopathy N, Kathiresan K. 2011. Anticancer drugs from marine flora: an overview. J Oncol **2010**. doi:10.1155/2010/214186.

Bourlat SJ, Nielsen C, Economou AD, Telford MJ. 2008. Testing the new animal phylogeny: a phylum level molecular analysis of the animal kingdom. Mol Phylogenet Evol **49:**23-31.

Branch GM, Griffiths CL, Branch ML, Beckley LE. 1994. Two oceans. A guide to the marine life of southern Africa.

Bromley CL, Parker-Nance S, De la Mare J, Edkins AL, Beukes DR, Davies-Coleman MT. 2013. Halogenated oxindole and indoles from the South African marine ascidian *Distaplia skoogi*. S Afr J Chem **66:**00-00.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Burighel P, Caicci F, Zaniolo G, Gasparini F, Degasperi V, Manni L. 2008. Does hair cell differentiation predate the vertebrate appearance? Brain Res Bull **75:**331-334.

Chait R, Shrestha S, Shah AK, Michel J, Kishony R. 2010. A differential drug screen for compounds that select against antibiotic resistance. PLoS One **5:**e15179.

Choi SK, Park SY, Kim R, Kim SB, Lee CH, Kim JF, Park SH. 2009. Identification of a polymyxin synthase gene cluster of *Paenibacillus polymyxa* and heterologous expression of the gene in *Bacillus subtilis*. J Bacteriol **191:**3350-3358. doi:10.1128/JB.01728-08.

Cosgrove SE. 2006. The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. Clin Infect Dis **42 Suppl 2:**S82-9. doi:CID38026.

UNIVERSITY of the
WESTERN CAPE

Craney A, Ahmed S, Nodwell J. 2013. Towards a new science of secondary metabolism. J Antibiot **66:**387-400.

Cushnie TT, Cushnie B, Lamb AJ. 2014. Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. Int J Antimicrob Agents **44:**377-386.

Davidson BS. 1993. Ascidians: producers of amino acid-derived metabolites. Chem Rev **93:**1771-1791.

Davidson SK, Allen SW, Lim GE, Anderson CM, Haygood MG. 2001. Evidence for the biosynthesis of bryostatins by the bacterial symbiont "Candidatus Endobugula sertula" of the bryozoan *Bugula neritina*. Appl Environ Microbiol **67:**4531-4537.

http://etd.uwc.ac.za/

Davies-Coleman MT. 2010. Natural products research in South Africa: end of an era on land or the beginning of an endless opportunity in the sea?. S Afr J Chem **63:**105-113.

Davies-Coleman MT, Beukes DR. 2004. Ten years of marine natural products research at Rhodes University. S Afr J Sci **100:**540-543.

Davies-Coleman MT, Veale CG. 2015. Recent Advances in Drug Discovery from South African Marine Invertebrates. Marine drugs **13:**6366-6383.

de Zeeuw RA. 1997. Drug screening in biological fluids the need for a systematic approach. Journal of Chromatography B: Biomedical Sciences and Applications **689:**71-79. **De Voogd NJ, Cleary DF.** 2008. An analysis of sponge diversity and distribution at three taxonomic levels in the Thousand Islands/Jakarta Bay reef complex, West-Java, Indonesia. Mar Ecol **29:**205-215.

UNIVERSITY of the
WESTERN CAPE

De Vries DJ, Beart PM. 1995. Fishing for drugs from the sea: status and strategies. Trends Pharmacol Sci **16:**275-279.

Dolušić E, Larrieu P, Meinguet C, Colette D, Rives A, Blanc S, Ferain T, Pilotte L, Stroobant V, Wouters J. 2013. Indoleamine 2, 3-dioxygenase inhibitory activity of derivatives of marine alkaloid tsitsikammamine A. Bioorg Med Chem Lett **23:**47-54.

Donia MS, Fricke WF, Partensky F, Cox J, Elshahawi SI, White JR, Phillippy AM, Schatz MC, Piel J, Haygood MG. 2011. Complex microbiome underlying secondary and primary metabolism in the tunicate-Prochloron symbiosis. Proceedings of the National Academy of Sciences **108:**E1423-E1432.

http://etd.uwc.ac.za/

Drewes SE. 2012. Natural products research in South Africa: 1890–2010. S Afr J Sci **108(5-6):**17-24.

Enticknap JJ, Kelly M, Peraud O, Hill RT. 2006. Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. Appl Environ Microbiol **72:**3724-3732.

Faria-Ramos I, Farinha S, Neves-Maia J, Tavares PR, Miranda IM, Estevinho LM, Pina-Vaz C, Rodrigues AG. 2014. Development of cross-resistance by Aspergillus fumigatus to clinical azoles following exposure to prochloraz, an agricultural azole. BMC microbiology **14:**1.

Farrelly V, Rainey FA, Stackebrandt E. 1995. Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl Environ Microbiol **61:**2798-2801.

UNIVERSITY of the
WESTERN CAPE

Filira F, Biondi L, Cavaggion F, Scolaro B, Rocchi R. 1990. Synthesis of O-glycosylated tuftsins by utilizing threonine derivatives containing an unprotected monosaccharide moiety. Int J Pept Protein Res **36:**86-96.

Firn R. 2010. Nature's chemicals: the natural products that shaped our world. Oxford University Press Oxford, UK.

Fischbach MA, Walsh CT. 2006. Assembly-line enzymology for polyketide and non-ribosomal peptide synthase peptide antibiotics: logic, machinery, and mechanisms. Chem Rev **106:**3468-3496.

http://etd.uwc.ac.za/

Freeman MF, Vagstad AL, Piel J. 2016. Polytheonamide biosynthesis showcasing the metabolic potential of sponge-associated uncultivated 'Entotheonella'bacteria. Curr Opin Chem Biol **31:**8-14.

Freeman MF, Gurgui C, Helf MJ, Morinaka BI, Uria AR, Oldham NJ, Sahl HG, Matsunaga S, Piel J. 2012. Metagenome mining reveals polytheonamides as posttranslationally modified ribosomal peptides. Science **338:**387-390. doi:10.1126/science.1226121.

Fujii I, Watanabe A, Sankawa U, Ebizuka Y. 2001. Identification of Claisen cyclase domain in fungal polyketide synthase WA, a naphthopyrone synthase of *Aspergillus nidulans*. Chem Biol **8:**189-197.

Fukunaga Y, Kurahashi M, Tanaka K, Yanagi K, Yokota A, Harayama S. 2006. *Pseudovibrio ascidiaceicola* sp. nov., isolated from ascidians (sea squirts). Int J Syst Evol Microbiol **56:**343-347.

UNIVERSITY of the
WESTERN CAPE

Fung JC, Donta ST, Tilton RC. 1986. *Candida* detection system (CAND-TEC) to differentiate between *Candida albicans* colonization and disease. J Clin Microbiol **24:**542-547.

Gause G, Brazhnikova M. 1944. Gramicidin S and its use in the treatment of infected wounds. Nature **154:**703.

Greenbaum D, Luscombe NM, Jansen R, Qian J, Gerstein M. 2001. Interrelating different types of genomic data, from proteome to secretome: 'oming in on function. Genome Res **11:**1463-1468. doi:10.1101/gr.207401.

http://etd.uwc.ac.za/

Griffiths CL, Robinson TB, Lange L, Mead A. 2010. Marine biodiversity in South Africa: An evaluation of current states of knowledge. PLoS One **5:**e12008.

Gurgui C, Piel J. 2010. Metagenomic approaches to identify and isolate bioactive natural products from microbiota of marine sponges, p. 247-264. *In* Anonymous Metagenomics. Springer.

Hamada T, Sugawara T, Matsunaga S, Fusetani N. 1994. Polytheonamides, unprecedented highly cytotoxic polypeptides from the marine sponge *Theonella swinhoei* 2. Structure elucidation. Tetrahedron Lett **35:**609-612.

Harrington C, Reen FJ, Mooij MJ, Stewart FA, Chabot J, Guerra AF, Glöckner FO, Nielsen KF, Gram L, Dobson AD. 2014. Characterisation of Non-Autoinducing Tropodithietic Acid (TDA) Production from Marine Sponge *Pseudovibrio* Species. Marine drugs **12:**5960-5978.

UNIVERSITY of the
WESTERN CAPE

Harvey AL, Edrada-Ebel R, Quinn RJ. 2015. The re-emergence of natural products for drug discovery in the genomics era. Nature Reviews Drug Discovery **14:**111-129.

Harwood LM, Moody CJ, Harwood LM. 1989. Experimental organic chemistry: principles and practice. Blackwell Scientific Oxford, United Kingdom.

Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C, Hacker J. 2001. Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. FEMS Microbiol Ecol **35:**305-312.

Hildebrand M, Waggoner LE, Liu H, Sudek S, Allen S, Anderson C, Sherman DH, Haygood M. 2004. bryA: an unusual modular polyketide synthase gene from

http://etd.uwc.ac.za/

the uncultivated bacterial symbiont of the marine bryozoan *Bugula neritina*. Chem Biol **11:**1543-1552.

Holland LZ. 2007. Developmental biology: a chordate with a difference. Nature **447:**153-155.

Hopwood DA, Sherman DH. 1990. Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annu Rev Genet **24:**37-62.

Hryniewicz W. 1999. Epidemiology of MRSA. Infection **27:**S13-S16.

Janssen A, Chin NL, Scheffer JJ, Svendsen AB. 1986. Screening for antimicrobial activity of some essential oils by the agar overlay technique. Pharmacy World & Science **8:**289-292.

Jensen PR, Fenical W. 1994. Strategies for the discovery of secondary metabolites from marine bacteria: ecological perspectives. Annual Reviews in Microbiology **48:**559-584.

Jiménez JT, Šturdíková M, Šturdík E. 2010. Bioactive marine and terrestrial polyketide and peptide secondary metabolites and perspectives of their biotechnological production. Acta Chimica Slovaca **3:**103-119.

Kamysz W, Okrój M, Lempicka E, Ossowski T, Lukasiak J. 2004. Fast and efficient purification of synthetic peptides by solid-phase extraction. Acta Chromatographica 180-186.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri P, Davis MI, Edeen PT. 2008. A quantitative analysis of kinase inhibitor selectivity. Nat Biotechnol **26:**127-132.

Karleskint G, Turner R, Small J. 2012. Introduction to marine biology. Cengage Learning.

Kennedy J, O'leary N, Kiran G, Morrissey J, O'gara F, Selvin J, Dobson A. 2011. Functional metagenomic strategies for the discovery of novel enzymes and biosurfactants with biotechnological applications from marine ecosystems. J Appl Microbiol **111:**787-799.

Kennedy J, Codling CE, Jones BV, Dobson AD, Marchesi JR. 2008. Diversity of microbes associated with the marine sponge, *Haliclona simulans*, isolated from Irish waters and identification of polyketide synthase genes from the sponge metagenome. Environ Microbiol **10:**1888-1902.

Kennedy J, Flemer B, Jackson SA, Lejon DP, Morrissey JP, O'Gara F, Dobson AD. 2010. Marine metagenomics: new tools for the study and exploitation of marine microbial metabolism. Marine drugs **8:**608-628.

Kennedy J, Marchesi JR, Dobson AD. 2007. Metagenomic approaches to exploit the biotechnological potential of the microbial consortia of marine sponges. Appl Microbiol Biotechnol **75:**11-20.

Khosla C, Kapur S, Cane DE. 2009. Revisiting the modularity of modular polyketide synthases. Curr Opin Chem Biol **13:**135-143.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Kim S, Park S, Choi S, Park S. 2015. Biosynthesis of Polymyxins B, E, and P Using Genetically Engineered Polymyxin Synthases in the Surrogate Host *Bacillus subtilis*. J.Microbiol.Biotechnol **25:**1015-1025.

Kim TK, Fuerst JA. 2006. Diversity of polyketide synthase genes from bacteria associated with the marine sponge *Pseudoceratina clavata*: culture-dependent and culture-independent approaches. Environ Microbiol **8:**1460-1470.

Kittakoop P, Mahidol C, Ruchirawat S. 2014. Alkaloids as important scaffolds in therapeutic drugs for the treatments of cancer, tuberculosis, and smoking cessation. Current topics in medicinal chemistry **14:**239-252.

Kiyama S, Fujii N, Yajima H, Moriga M, Takagi A. 1984. Studies on peptides. Int J Pept Protein Res **23:**174-186.

Kleigrewe K, Almaliti J, Tian IY, Kinnel RB, Korobeynikov A, Monroe EA, Duggan BM, Di Marzo V, Sherman DH, Dorrestein PC. 2015. Combining mass spectrometric metabolic profiling with genomic analysis: a powerful approach for discovering natural products from cyanobacteria. J Nat Prod **78:**1671-1682.

Koren O, Rosenberg E. 2006. Bacteria associated with mucus and tissues of the coral *Oculina patagonica* in summer and winter. Appl Environ Microbiol **72:**5254-5259. doi:72/8/5254.

Kühl, M., L. Behrendt, E. Trampe, K. Qvortrup, U. Schreiber, S. M. Borisov, I. Klimant, and A. W. Larkum. 2012. Microenvironmental ecology of the chlorophyll b-containing symbiotic cyanobacterium *Prochloron* in the didemnid ascidian *Lissoclinum patella*. Frontiers in Microbiology **3:**402.

http://etd.uwc.ac.za/

Kusari S, Hertweck C, Spiteller M. 2012. Chemical ecology of endophytic fungi: origins of secondary metabolites. Chem Biol **19:**792-798.

Lammie SL, Hughes JM. 2016. Antimicrobial Resistance, Food Safety, and One Health: The Need for Convergence. Annual Review of Food Science and Technology **7:**287-312.

Lavy A, Keren R, Haber M, Schwartz I, Ilan M. 2014. Implementing sponge physiological and genomic information to enhance the diversity of its culturable associated bacteria. FEMS Microbiol Ecol **87:**486-502. doi:10.1111/1574-6941.12240.

Leal MC, Puga J, Serôdio J, Gomes NC, Calado R. 2012. Trends in the discovery of new marine natural products from invertebrates over the last two decades–where and what are we bioprospecting?. PLoS One **7:**e30580.

UNIVERSITY of the
WESTERN CAPE

Levinton JS. 2001. Marine biology: function, biodiversity, ecology. New York Oxford University Press.

Li MH, Ung PM, Zajkowski J, Garneau-Tsodikova S, Sherman DH. 2009. Automated genome mining for natural products. BMC Bioinformatics **10:**1.

López-Legentil S, Erwin PM, Turon M, Yarden O. 2015. Diversity of fungi isolated from three temperate ascidians. Symbiosis **66:**99-106.

Machado H, Sonnenschein EC, Melchiorsen J, Gram L. 2015. Genome mining reveals unlocked bioactive potential of marine Gram-negative bacteria. BMC Genomics **16:**158.

http://etd.uwc.ac.za/

Manske RH, Shin KH. 1965. The alkaloids of papaveraceous plants: liii. Eschscholtzia californica cham. Canadian Journal of Chemistry **43:**2180-2182.

Marahiel MA, Stachelhaus T, Mootz HD. 1997. Modular peptide synthases involved in non-ribosomal peptide synthase peptide synthesis. Chem Rev **97:**2651-2674.

McGuire J, Hasskare H. 2007. Pharmaceuticals, General Survey. Ullmann's Encyclopedia of Chemical Technology.

Muscholl-Silberhorn A, Thiel V, Imhoff JF. 2008. Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. Microb Ecol **55:**94-106.

Newman DJ, Cragg GM. 2015. Endophytic and epiphytic microbes as "sources" of bioactive agents. Frontiers in Chemistry **3:**34.

UNIVERSITY of the
WESTERN CAPE

Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev **64:**548-572.

Norrander J, Kempe T, Messing J. 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101-106.

Nyström M, Folke C. 2001. Spatial resilience of coral reefs. Ecosystems **4:**406-417.

Onishi J, Meinz M, Thompson J, Curotto J, Dreikorn S, Rosenbach M, Douglas C, Abruzzo G, Flattery A, Kong L, Cabello A, Vicente F, Pelaez F, Diez MT, Martin I, Bills G, Giacobbe R, Dombrowski A, Schwartz R, Morris S, Harris G,

http://etd.uwc.ac.za/

Tsipouras A, Wilson K, Kurtz MB. 2000. Discovery of novel antifungal (1,3)-beta-D-glucan synthase inhibitors. Antimicrob Agents Chemother **44:**368-377.

Parker-Nance, S. 2013**.** Ascidians of South Africa: A Historical. The Biology of Ascidians 278.

Pawlik JR. 1993. Marine invertebrate chemical defenses. Chem Rev **93:**1911-1922.

Pearce CJ, Doyle TW, Forenza S, Lam KS, Schroeder DR. 1988. The biosynthetic origins of rebeccamycin. J Nat Prod **51:**937-940.

Penesyan A, Kjelleberg S, Egan S. 2010. Development of novel drugs from marine surface associated microorganisms. Marine drugs **8:**438-459.

Pérez-Matos AE, Rosado W, Govind NS. 2007. Bacterial diversity associated with the Caribbean tunicate *Ecteinascidia turbinata*. Antonie Van Leeuwenhoek **92:**155-164.

UNIVERSITY *of the*
WESTERN CAPE

Pettit RK. 2011. Small-molecule elicitation of microbial secondary metabolites. Microbial biotechnology **4:**471-478.

Phelan R, O'Halloran J, Kennedy J, Morrissey J, Dobson A, O'Gara F, Barbosa T. 2012. Diversity and bioactive potential of endospore-forming bacteria cultured from the marine sponge *Haliclona simulans*. J Appl Microbiol **112:**65-78.

Philippe H, Derelle R, Lopez P, Pick K, Borchiellini C, Boury-Esnault N, Vacelet J, Renard E, Houliston E, Quéinnec E. 2009. Phylogenomics revives traditional views on deep animal relationships. Current Biology **19:**706-712.

http://etd.uwc.ac.za/

Piel J, Hui D, Wen G, Butzke D, Platzer M, Fusetani N, Matsunaga S. 2004. Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. Proc Natl Acad Sci U S A **101:**16222-16227. doi:0405976101.

Prabhu AS, Ananthan G, Hussain SM, Balasubramanian T. 2011. Antibacterial activity of Ascidian *phallusia arabica* against human clinical isolates. J Appl Pharma Sci **1:**143-145.

Prenner EJ, Lewis RN, Neuman KC, Gruner SM, Kondejewski LH, Hodges RS, McElhaney RN. 1997. Nonlamellar phases induced by the interaction of gramicidin S with lipid bilayers. A possible relationship to membrane-disrupting activity. Biochemistry **36:**7906-7916.

Proksch P, Edrada R, Ebel R. 2002. Drugs from the seas–current status and microbiological implications. Appl Microbiol Biotechnol **59:**125-134.

Rateb ME, Houssen WE, Harrison WT, Deng H, Okoro CK, Asenjo JA, Andrews BA, Bull AT, Goodfellow M, Ebel R. 2011. Diverse metabolic profiles of a Streptomyces strain isolated from a hyper-arid environment. J Nat Prod **74:**1965-1971.

Reich E, Schibli A. 2007. High-performance thin-layer chromatography for the analysis of medicinal plants. Thieme 227-232.

Reysenbach A, Pace N, Robb F, Place A. 1995. Archaea: a laboratory manual—thermophiles. Cold Spring Harb.Protoc **16:**101-107.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Riesenfeld CS, Murray AE, Baker BJ. 2008. Characterization of the microbial community and polyketide biosynthetic potential in the palmerolide-producing tunicate Synoicum adareanum. J Nat Prod **71:**1812-1818.

Sanchez C, Zhu L, Brana AF, Salas AP, Rohr J, Mendez C, Salas JA. 2005. Combinatorial biosynthesis of antitumor indolocarbazole compounds. Proc Natl Acad Sci U S A **102:**461-466. doi:0407809102.

Sarker SD, Nahar L, Kumarasamy Y. 2007. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the< i> in vitro</i> antibacterial screening of phytochemicals. Methods **42:**321-324.

Schmidt EW, Donia MS, McIntosh JA, Fricke WF, Ravel J. 2012. Origin and variation of tunicate secondary metabolites. J Nat Prod **75:**295-304.

Schupp P, Poehner T, Edrada R, Ebel R, Berg A, Wray V, Proksch P. 2003. Eudistomins W and X, two new β-carbolines from the Micronesian tunicate *Eudistoma sp*. J Nat Prod **66:**272-275.

Schwarzer D, Finking R, Marahiel MA. 2003. Non-ribosomal peptide synthase peptides: from genes to products. Nat Prod Rep **20:**275-287.

Seyedsayamdost MR. 2014. High-throughput platform for the discovery of elicitors of silent bacterial gene clusters. Proc Natl Acad Sci U S A **111:**7266-7271. doi:10.1073/pnas.1400019111.

Shenkar N, Swalla BJ. 2011. Global diversity of *Ascidiacea*. PLoS One **6:**e20657. doi:10.1371/journal.pone.0020657.

http://etd.uwc.ac.za/

Skariyachan S, G Rao A, Patil M, Saikia B, Bharadwaj K, Rao G. 2014. Antimicrobial potential of metabolites extracted from bacterial symbionts associated with marine sponges in coastal area of Gulf of Mannar Biosphere, India. Lett Appl Microbiol **58:**231-241.

Skinnider MA, Dejong CA, Rees PN, Johnston CW, Li H, Webster AL, Wyatt MA, Magarvey NA. 2015. Genomes to natural products PRediction Informatics for Secondary Metabolomes (PRISM). Nucleic Acids Res gkv1012.

Slyusarev G, Cherkasov A. 2008. Structure and supposed feeding mechanisms of the plasmodium of *Intoshia linei* (Orthonectida). Invertebrate zoology **5**(1): 47-51.

So A, Gupta N, Brahmachari S, Chopra I, Munos B, Nathan C, Outterson K, Paccaud J, Payne D, Peeling R. 2011. Towards new business models for R&D for novel antibiotics. Drug Resistance Updates **14:**88-94.

UNIVERSITY of the
WESTERN CAPE

Speight MR, Henderson PA. 2010. Marine ecology: concepts and applications. Wiley-Blackwell, Oxford, 276.

Stachowicz JJ, Lindquist N. 2000. Hydroid defenses against predators: the importance of secondary metabolites versus nematocysts. Oecologia **124:**280-288.

Staveness D, Abdelnabi R, Schrier AJ, Loy BA, Verma VA, DeChristopher BA, Near KE, Neyts J, Delang L, Leyssen P. 2016. Simplified Bryostatin Analogues Protect Cells from Chikungunya Virus-Induced Cell Death. J Nat Prod **79** (4): 675-679.

Steinert G, Taylor MW, Schupp PJ. 2015. Diversity of Actinobacteria Associated with the Marine Ascidian *Eudistoma toealensis*. Marine Biotechnology 1-9.

http://etd.uwc.ac.za/

Strelkauskas A, Strelkauskas J, Moszyk-Strelkauskas D. 2009. Microbiology: a clinical approach. Garland Science New York, NY, USA.

Tianero MDB, Kwan JC, Wyche TP, Presson AP, Koch M, Barrows LR, Bugni TS, Schmidt EW. 2015. Species specificity of symbiosis and secondary metabolism in ascidians. The ISME journal **9:**615-628.

Trindade M, van Zyl LJ, Navarro-Fernández J, Elrazak AA. 2015. Targeted metagenomics as a tool to tap into marine natural product diversity for the discovery and production of drug candidates. Frontiers in microbiology **6:**890.

Tsukimoto M, Nagaoka M, Shishido Y, Fujimoto J, Nishisaka F, Matsumoto S, Harunari E, Imada C, Matsuzaki T. 2011. Bacterial production of the tunicate-derived antitumor cyclic depsipeptide didemnin B. J Nat Prod **74:**2329-2331.

Ueoka R, Uria AR, Reiter S, Mori T, Karbaum P, Peters EE, Helfrich EJ, Morinaka BI, Gugger M, Takeyama H. 2015. Metabolic and evolutionary origin of actin-binding polyketides from diverse organisms. Nature chemical biology **11:**705-712.

van Heel AJ, de Jong A, Montalban-Lopez M, Kok J, Kuipers OP. 2013. BAGEL3: Automated identification of genes encoding bacteriocins and (non-) bactericidal posttranslationally modified peptides. Nucleic Acids Res **41:**W448-53. doi:10.1093/nar/gkt391.

Velho-Pereira S, Furtado I. 2012. Antibacterial activity of halophilic bacterial bionts from marine invertebrates of Mandapam-India. Indian journal of pharmaceutical sciences **4:**331-338.

http://etd.uwc.ac.za/

Velkov T, Roberts KD, Nation RL, Thompson PE, Li J. 2013. Pharmacology of polymyxins: new insights into an 'old' class of antibiotics. Future microbiology **8:**711-724.

Vizcaino JA, Sanz L, Cardoza RE, Monte E, Gutierrez S. 2005. Detection of putative peptide synthase genes in *Trichoderma* species: application of this method to the cloning of a gene from *T. harzianum* CECT 2413. FEMS Microbiol Lett **244:**139-148.

Wada H, Makabe KW, Nakauchi M, Satoh N. 1992. Phylogenetic relationships between solitary and colonial ascidians, as inferred from the sequence of the central region of their respective 18S rDNAs. Biol Bull **183:**448-455.

Walmsley TA, Matcher GF, Zhang F, Hill RT, Davies-Coleman MT, Dorrington RA. 2012. Diversity of bacterial communities associated with the Indian Ocean Sponge *Tsitsikamma favus* that contains the bioactive pyrroloiminoquinones, Tsitsikammamine A and B. Marine Biotechnology **14:**681-691.

Walsh CT, Chen H, Keating TA, Hubbard BK, Losey HC, Luo L, Marshall CG, Miller DA, Patel HM. 2001. Tailoring enzymes that modify non-ribosomal peptide synthase peptides during and after chain elongation on NRPS assembly lines. Curr Opin Chem Biol **5:**525-534.

Wang W, Kim H, Nam S, Rho BJ, Kang H. 2012. Antibacterial Butenolides from the Korean Tunicate *Pseudodistoma antinboja*. J Nat Prod **75:**2049-2054.

Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Muller R, Wohlleben W, Breitling R, Takano E, Medema MH. 2015.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res **43:**W237-43. doi:10.1093/nar/gkv437.

Weiss J, Weiss T. 2005. Handbook of Ion Chromatography, Third, Completely Revised and Enlarged Edition. WILEY-VCH Verlag, Weinheim.

Wess G, Urmann M, Sickenberger B. 2001. Medicinal chemistry: challenges and opportunities. Angewandte Chemie International Edition **40:**3341-3350.

Williams DH, Stone MJ, Hauck PR, Rahman SK. 1989. Why are secondary metabolites (natural products) biosynthesized? J Nat Prod **52:**1189-1208.

Wilson K. 1987. Preparation of genomic DNA from bacteria. Current protocols in molecular biology 2-4.

Wilson MC, Mori T, Rückert C, Uria AR, Helf MJ, Takada K, Gernert C, Steffens UA, Heycke N, Schmitt S. 2014. An environmental bacterial taxon with a large and distinct metabolic repertoire. Nature **506:**58-62.

Witte W, Strommenger B, Stanek C, Cuny C. 2007. Methicillin-resistant Staphylococcus aureus ST398 in humans and animals, Central Europe. Emerging Infectious Diseases **13**(2):255-258.

Wu X, Qian C, Fang H, Wen Y, Zhou J, Zhan Z, Ding R, Li O, Gao H. 2011. Paenimacrolidin, a novel macrolide antibiotic from Paenibacillus sp. F6-B70 active against methicillin-resistant Staphylococcus aureus. Microbial Biotechnology **4:**491-502.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Zhang L, An R, Wang J, Sun N, Zhang S, Hu J, Kuai J. 2005. Exploring novel bioactive compounds from marine microbes. Curr Opin Microbiol **8:**276-281.

Zhang JH, Quigley NB, Gross DC. 1995. Analysis of the syrB and syrC genes of *Pseudomonas syringae pv. syringae* indicates that syringomycin is synthesized by a thiotemplate mechanism. J Bacteriol **177:**4009-4020.

Ziemert N, Podell S, Penn K, Badger JH, Allen E, Jensen PR. 2012. The natural product domain seeker NaPDoS: a phylogeny based bioinformatic tool to classify secondary metabolite gene diversity. PloS one **7:**e34064.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Appendices

Appendix A: Isolation and growth media used in this study

Culture media used in this study are listed in table A1 and table A2; the media were autoclaved at 121°C for 20 minutes. The agar was added at a concentration of 15 g/L for the preparation of solid media and the pH was adjusted using 32% HCl and 10 M NaOH.

Table A1: Isolation and culture media used in this study

Media	Constituents	Quantity/L of dH_20
Activated charcoal Medium (pH 7.0)	HEPES	2.38 g
	Sodium pyruvate	3 g
	Yeast extract	0.1 g
	Soybean peptone	3 g
	NaNO3	0.34 g
	K2HPO4	0.1 g
	MgSO4* 7 H2O	0.15 g
	Activated charcoal	3 g
Ashby's nitrogen free medium (pH 7.5)	Mannitol	15 g
	K2HPO4	0.3 g
	MgSO4* 7 H2O	0.3 g
	10% (w/v) Sodium molybdate solution	0.1 g
	10% (w/v) FeCl3	0.05 g
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
BG 11 (pH 7.5)	Solution 1 - NaNO3 (15 g/L)	100 ml
	Solution 2 - K2HPO4 (2 g/L)	10 ml
	Solution 3 - MgSO4*7 H2O (3.75 g/L)	10 ml
	Solution 4 - CaCl2*2 H2O (1.8 g/L)	10 ml
	Solution 5 - Citric acid (0.3 g/L)	10 ml
	Solution 6 - Ammonium ferric citrate (0.3 g/L)	10 ml
	Solution 7 - Na2EDTA (0.05 g/L)	10 ml
	Solution 8 - Na2CO3 (1 g/L)	10 ml

http://etd.uwc.ac.za/

	Wolfe's mineral solution	1 ml
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
Glucose-Yeast-Malt (GYM)	D-Glucose	4 g
	Yeast extract	4 g
	Malt extract	10 g
	CaCO3	2 g
	NaCl	24 g
	MgCl2	5.3 g
	KCl	0.7 g
	CaCl2	0.1 g
Modified 172F (pH 7.5)	Glucose	10 g
	Yeast extract	5 g
	Starch	10 g
	Tryptone	5 g
	MgSO4* 7 H2O	2 g
	CaSO4 * 2 H2O	2 g
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
	CaCl2	0.075 g
Oatmeal (ISP Media 3)	Jungle oats easy (no sugar)	40 g
Planctomycetes medium	Glucose	1 g
	(NH4)2SO4	0.25 g
	Peptone	0.15 g
	Yeast extract	0.15 g
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
	CaCl2	0.075 g
Pringsheim's Cyanobacteria specific medium	KNO3	0.2 g
	(NH4)2 HPO4	0.02 g
	MgSO4* 7 H2O	0.01 g
	CaCl2 * 2 H2O	0.005 g
	FeCl2	0.5 g
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
	CaCl2	0.075 g
Reasoners 2 agar (pH 7.2)	Casein acid hydrolysate	0.5 g
	Dextrose	0.5 g
	K2HPO4	0.3 g

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

	MgSO4 anhydrous	0.024 g
	Proteose peptone	0.5 g
	Sodium pyruvate	0.3 g
	Starch soluble	0.5 g
	Yeast extract	0.5 g
Seawater agar	dH2O	250 ml
	Sea water	750 ml
	Wolfe's mineral solution	5 ml
	Wolfe's vitamin solution	10 ml
Shivji's nutrient agar (pH 7.5)	Peptone	5 g
	Beef extract	3 g
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
	CaCl2	0.075 g
Sponge extract medium	Sponge extract	10 g
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
	CaCl2	0.075 g
Tryptocase soya agar (pH7.5)	TSB	3 g
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
	CaCl2	0.075 g
Tryptocase soya agar (pH4.5)	TSB	3 g
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
	CaCl2	0.075 g
Zobell 1/4 strength (ZBA)	Yeast extract	1.25 g
	Peptone	3.75 g
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
	CaCl2	0.075 g
Zobell + Crystal Violet (ZBV)	Yeast extract	1.25 g
	Peptone	3.75 g
	NaCl	18 g
	MgCl2	2 g
	KCl	0.525 g
	CaCl2	0.075 g
	Crystal violet	0.00000001%

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Table A2: General purpose media used in this study

Media	Constituents	Quantity/L of dH_20
Luria Bertani Broth (LB)	Tryptone	10 g
	Yeast	5 g
	NaCl	10 g
Sloppy agar (semi-solid LB)	Tryptone	10 g
	Yeast extract	5 g
	NaCl	10 g
	Agar	7.5 g

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Appendix B: List of antibiotics to which the multi-drug resistant E. coli 1699 exhibits resistance

Table B1: List of antibiotics to which the multi-drug resistant *E. coli* 1699 exhibits resistance

Compound	MIC (ug/ml)	Target/MOA	Antibiotic classification
A54145CB-181234	> 512	membrane	
Calcimycin (A23187)	64	membrane	ionophore
Daptomycin	> 512	membrane	lipopeptide
Gramicidin	128	membrane	polypeptide
Polymyxin B	1	membrane	polypeptide (cationic)
Ampicillin	> 256	cell wall	aminopenicillin
Aztreonam	≤ 0.03	cell wall	monocyclic beta-lactam
Cephalosporin C	64	cell wall	cephalosporin
Penicillin G	> 256	cell wall	beta-lactam
Ristocetin	> 512	cell wall	aminoglycoside
Teicoplanin	> 512	cell wall	glycopeptide
Vancomycin	512	cell wall	glycopeptide
Aclacinomycin A	> 512	DNA interaction	anthracycline
Actinomycin A	> 256	DNA interaction	polypeptide (toxic)
Actinomycin D	256	DNA interaction	polypeptide (toxic)
Bleomycin A2	> 64	DNA interaction	glycopeptide
Coumermycin A1	64	DNA interaction	aminocoumarin
Daunorubicin	> 256	DNA interaction	anthracycline
Gliotoxin	32	DNA interaction	mycotoxin
Mitomycin C	1	DNA interaction	aziridine-containing
Streptonigrin	2	DNA interaction	quinone-containing
Streptozotocin	> 64	DNA interaction	glucosamine
Chromomycin A3	> 512	gyrase	glycoside
Nalidixic Acid	> 256	gyrase	naphthyridone
Novobicin	> 256	gyrase	aminocoumarin
Apramycin	64	protein synthesis	aminoglycoside
Dibekacin	> 256	protein synthesis	aminoglycoside
Gentamycin	128	protein synthesis	aminoglycoside
Kanamycin	> 256	protein synthesis	aminoglycoside
Kasugamycin	> 64	protein synthesis	aminoglycoside

http://etd.uwc.ac.za/

Neomycin	16	protein synthesis	aminoglycoside
Netilmycin	128	protein synthesis	aminoglycoside
Streptomycin	> 256	protein synthesis	aminoglycoside
Streptothricin	256	protein synthesis	aminoglycoside
Tobramycin	> 256	protein synthesis	aminoglycoside
Puromycin	> 64	protein synthesis	aminonucleoside
Spectinomycin	> 256	protein synthesis	aminocyclitol
Chloramphenicol		protein synthesis	acetamide
Chlortetracycline		protein synthesis	tetracyclide
Erythromycin	64	protein synthesis	macrolide
Lincomycin	512	protein synthesis	lincosamide
Spiramycin	256	protein synthesis	macrolide
Tetracycline	> 256	protein synthesis	tetracycline
Thiostrepton	> 512	protein synthesis	oligopeptide
Tylosin	512	protein synthesis	macrolide
Virginamycin	64	protein synthesis	streptogramin
Rifampin	> 256	RNA polymerase	rifamycin
Rifamycin SV	> 64	RNA polymerase	rifamycin
Albomycin	high	iron metabolism	cyclic polypeptide
Trimethoprim	> 400	DHFR	diaminopyrimidine

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/