Marine bacteria as a potential source for novel antimicrobial compounds

UNIVERSITY *of the*
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By

ELLEN KELEBOGILE SEGOPA

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Bellville

Supervisor: Prof. Marla Trindade

Co-supervisor: Prof. Francois Halleen

Declaration

I, Ellen Kelebogile Segopa, declare that "**Marine bacteria as a potential source for novel antimicrobial compounds**" is my own work, that it has not been submitted for any degree or examination in any other tertiary institution, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

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Abstract

The high rate of rediscovery of known compounds has led to a decline in the discovery of novel natural products. The high biodiversity of organisms growing in extreme conditions such as oceans has led to the increased interest by researchers for their use as a source of novel natural products. Marine bacteria are known for their extensive biosynthetic capacity to produce diverse natural products, which are suitable for various biotechnology applications such as in agriculture, for treatment of fungal plant pathogens, and as antibiotics, for treatment of bacterial infections.

This study aimed at discovering novel secondary metabolites from marine bacteria previously associated with novel marine invertebrate species endemic to the South African coast. The methodologies used in this study included a bioassay guided fractionation coupled to genome sequencing and mining. For the bioassay guided fractionation approach, the study first focused on screening marine bacteria for antimicrobial activity when cultured on 4 different media, against fungal strains previously shown to be virulent olive trunk pathogens. In parallel, the bacterial isolates with the most inhibitory activity against the fungal pathogens were also screened for antimicrobial activity against 4 indicator strains including Gram-negative *Escherichia coli* 1699 (*E. coli*), *Pseudomonas putida*, and Gram-positive *Staphylococcus epidermidis* ATCC14990, and *Bacillus cereus* ATCC10702. One of the marine bacterial isolates, PE6-126, showed diverse antimicrobial activity including antibacterial and antifungal activity against the tested strains.

The genome sequencing data revealed that this isolate was *B. cereus* based on the average nucleotide identity (ANI) (>99%) to reference strains. antiSMASH analysis of the genome revealed nine predicted secondary metabolite clusters including bacteriocins (2), non-ribosomal peptide synthetase (NRPS) (2), siderophore (1), sactipeptide (1), betalactone (1), linear azol(in)e-containing peptides (LAP) - bacteriocin (1) and a terpene (1). Some of these pathways had low to no sequence similarity to known pathways, indicating the potential of these pathways to produce novel compounds. One of the pathways showed very high sequence similarity to the thuricin CD pathway in *Bacillus thuringiensis*. Considering that thuricin CD has been reported to have antimicrobial activity against *B. cereus* (ATCC1072), it was hypothesised that it could also be produced by PE6-126. However, the antimicrobial extract from PE6-126 was tested for sensitivity to proteinase K and heat treatment, which thuricin CD is known to be sensitive to. The results revealed that the antimicrobial activity was not lost after treatment, implying that a different metabolite could be responsible for the anti-*B. cereus*

activity. In addition, PE6-126 initially displayed antimicrobial activity against a multi-drug resistant *E. coli* 1699, suggesting some of the antimicrobial compound/(s) produced by this strain could potentially be novel. The bioassay-guided fractionation approach coupled to Liquid Chromatography Mass Spectrometry (LC-MS) did not lead to identification of the antimicrobial compound/(s), therefore it remains a question whether the secondary metabolite pathways predicted by antiSMASH lead to the production of the active compound/(s).

The results from this study showed that even well studied species have the potential to synthesize as yet undescribed compounds, based on the novelty of some of the pathways. This study highlights the importance of employing a genome-guided approach in drug discovery, as there may be many novel compounds to discover from biosynthetic pathways that have not yet been characterised. Further research is needed to identify the antimicrobial compound/(s) produced by PE6-126.

Keywords: Marine natural products, antimicrobial activity, olive trunk fungal pathogens, bioassay guided fractionation, genome mining, antiSMASH.

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Abbreviations

ACM	Activated charcoal medium
ANI	Average nucleotide identity
AntiSMASH	Antibiotics and secondary metabolites analysis shell
ARC	Agricultural research council
BGC	Biosynthetic gene cluster
CONTIG	Contiguous
°C	Degree Celsius
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
GYM	Glucose yeast malt
g	Gravitational force
g	Grams
HPLC	High performance liquid chromatography
IMBM	Institute for Microbial Biotechnology and Metagenomics
kDa	Kilo Dalton
LAP	Linear azol (in)e-containing peptides
LB	Luria Bertani
LC-MS	Liquid chromatography mass spectrometry
µL	Microlitre
mL	Millilitre
mg/mL	Milligram per millilitre
MiGA	Microbial Genomes Atlas Online
min	Minutes
NMR	Nuclear magnetic resonance
Rt	Retention time
RAST	Rapid Annotation using Subsystem Technology
NRPS	Non-ribosomal peptide synthetase
NRPs	Non-ribosomal peptide (s)
OD	Optical density

OSMAC	One Strain Many Compounds
PDA	Potato dextrose agar
PCR	Polymerase chain reaction
PKS	Polyketide synthase
SM	Secondary metabolite
TSA	Tryptic soy agar
UV	Ultra violet
WGS	Whole genome sequencing
ZBA	Zobell agar

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Chapter 1 - Literature review

1.1. Introduction

Natural products have been used successfully for decades as a source of most medicines (Harvey, 2008; Pettit, 2011). Natural sources such as plants, animals or microorganisms which include bacteria, algae and fungi are known to produce bioactive metabolites which control pathogens (Patridge *et al.*, 2016). Bacterial infectious diseases affecting human health are becoming a major challenge worldwide. The global health crisis caused by multi-drug resistance, particularly for the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa and Enterobacter*) pathogens which encompass both Gram-positive and Gram-negative species has reduced treatment options of many bacterial infections (Rice, 2008; Santajit and Indrawattana, 2016). Due to the overuse and inappropriate prescription of antimicrobials, these bacterial pathogens have developed different antimicrobial resistance mechanisms which places a significant burden on healthcare systems (Santajit and Indrawattana, 2016). Furthermore, there is also a reduction in drug discovery programs of novel antibiotics. The three major reasons which led to this decline is that the screening process of antimicrobial compounds resulted in rediscovery of already known compounds from the same ecological niches, high toxicity of the compounds to the host and the expensive cost associated with drug development (Wong, Oliver and Linington, 2012; Lewis, 2013). Thus, there is a significant need to develop novel antimicrobial compounds with diverse structures and novel mechanism of action for broad-spectrum applicability to target a variety of pathogens.

Plant diseases caused by fungal pathogens are also a major threat in the agricultural industry. One of the issues is trunk disease pathogens particularly in olives, which have recently been identified as a concern to the quality and quantity of olives produced globally (Carlucci *et al.*, 2015). Important fungal olive trunk pathogens which cause these diseases globally includes members of the *Botryosphaeriaceae* spp., *Diaporthaceae*, *Diatrypaceae*, *Togniniaceae*, *Pleurostomataceae* and *Didymellaceae* (Taylor, Hale and Hartill, 2001; Phillips, 2005; Moral *et al.*, 2010, 2017; Cloete *et al.*, 2011; Carlucci *et al.*, 2013, 2015; Úrbez-Torres *et al.*, 2013). Olive trunk pathogens have not been extensively studied and thus, there is limited literature reported on olive trunk diseases. There is currently no available treatment for the control of olive trunk fungal pathogens, therefore it is essential to develop novel antifungal compounds in order to manage these diseases and sustain the quality and yield of olives.

One of the natural sources, which is increasingly studied for novel bioactive compounds are the microorganisms from the marine environment. This is because the marine environment is

known to have marine microorganisms that produce bioactive metabolites with unique and complex structures (Haefner, 2003; Bérdy, 2005; McArthur *et al.*, 2008; Bhatnagar and Kim, 2010; Mayer *et al.*, 2011; Altmann, 2017). Since the marine environment is relatively underexplored as compared to the terrestrial environment, it is a great source of novel natural products with potential applications in pharmaceuticals, biotechnology and agriculture (Haefner, 2003; Harvey, 2008; Bhatnagar and Kim, 2010). Marine natural products derived from invertebrates and their associated microorganisms have shown a wide array of chemical diversity and bioactivity including antibacterial, antifungal and anticancer properties (Gerwick and Moore, 2012; Hu *et al.*, 2015; Altmann, 2017). Among microorganisms, marine bacteria are the most prolific sources of novel secondary metabolites (Bhatnagar and Kim, 2010) and account for a high number of drugs in clinical trials or approved for pharmaceutical applications (Gerwick and Moore, 2012). In contrast to the search for novel pharmaceutical compounds, there is limited exploration of marine natural products as antifungals especially in control of plant pathogens (Peng *et al.*, 2016; El-Hossary *et al.*, 2017). The marine environment, with its chemical diversity still holds enormous potential to provide leads for developing natural products with antifungal activity.

For the purpose of this study, the review will focus on marine natural products from bacteria and their bioactive properties with focus on antimicrobial activity. Furthermore, different classes of secondary metabolites produced by marine bacteria are discussed and also the use of bioassay guided isolation and genome mining approaches in the context of natural product discovery.

1.2. Natural Products in drug discovery

Natural products can be defined as any unmodified natural material or compounds produced by living organisms such as plants, microorganisms and animals from which semisynthetic structures can be derived (Pettit, 2011; Xiong *et al.*, 2013). Natural products have been considered as a source of leads for the development of drugs. Furthermore, natural products are known to have a range of therapeutic uses as anti-cancer, anti-viral, anti-diabetic, anti-inflammatory, and antimicrobial agents (Gullo *et al.*, 2006; Newman and Cragg, 2012; Xiong *et al.*, 2013). Historically, terrestrial plants were represented as major sources of natural products, however microorganisms have become recognised as a source of natural products given the number of antibiotics discovered from them (Altmann, 2017). Since the discovery of penicillin, which was isolated from the fungus *Penicillium rubens*, the research on natural products from microbes expanded and many other antibiotics such as erythromycin,

vancomycin, and cephalosporins were discovered (Bérdy, 2005; Sekurova, Schneider and Zotchev, 2019). These microbial derived natural products have since been used in medicine, agriculture, food industry, and scientific research. From the year 2000, approximately 77% of the approved antibiotics originated from microbes (Patridge *et al.*, 2016).

Although natural products from microbes have been discovered from the terrestrial environment and used successfully for many years in the pharmaceutical industry, repeated rediscovery of already known compounds became a major challenge (Tulp and Bohlin, 2005; Lewis, 2013; Sekurova, Schneider and Zotchev, 2019). Therefore, research has shifted to the marine environment for biodiscovery of novel natural products.

1.3. Marine natural products; the ocean as a rich source for novel natural products

More than 70% of planet earth is covered by the ocean and is known to harbour rich species diversity (Haefner, 2003; Anand *et al.*, 2006; Gerwick and Moore, 2012; Petersen, Kellermann and Schupp, 2020). The marine environment consists of a wide diversity of microbial species and ecosystems; however, it still remains an untapped source (Xiong *et al.*, 2013; Petersen, Kellermann and Schupp, 2020). Natural products from marine organisms are structurally complex and different from terrestrial organisms (Gerwick and Moore, 2012). This is probably due to the extreme conditions (low temperature, alkaline pH, high salinity, high pressure, dissolved oxygen, and lack of nutrients) specific to the ocean (Bhatnagar and Kim, 2010; Carvalho and Fernandes, 2010). The produced compounds also protect the organisms against non-biological features like high temperatures and high light intensities (Petersen, Kellermann and Schupp, 2020).

Marine organisms such as algae, tunicates, molluscs, sponges, soft corals, and microbes have been the most studied in the research of bioactive compounds. From some of these organisms, natural compounds have been developed successfully into therapeutics (**Table 1.1**).

Table 1.1: Examples of marine natural products that are FDA-Approved or in clinical trial phases with details of their chemical class, isolation source, therapeutic area of application and status. Adapted from Gerwick and Moore, 2012 and Petersen, Kellermann and Schupp, 2020.

Compound	**Chemical class**	**Source**	**Predicted biosynthetic source**	**Therapeutic area**	**Status**
Cytarabine	Nucleoside	Sponge	Bacterium	Cancer	FDA Approved
Vidarabine	Nucleoside	Sponge	Bacterium	Antiviral	FDA Approved
Ziconotide	Peptide	Cone Snail	Mollusc	Chronic pain	FDA Approved
Trabectedin	Alkaloid	Tunicate	Bacterium	Cancer	FDA Approved
Brentuximab vedotin	Antibody drug conjugate	Mollusc	Cyanobacterium	Cancer	FDA Approved
Eribulin mesylate	Macrolide	sponge	Bacterium	Cancer	FDA Approved
Omega-3-acid ethyl ester	Omega-3 fatty acid	Fish	Microalgae	Hypertriglyceridemia	FDA Approved
Plinabulin	Diketopiperazine	Fungus	Fungus	Cancer	Phase Ⅲ
Plitidepsin	Depsipeptide	Tunicate	Bacterium	Cancer	Phase Ⅲ
Bryostatin	Macrolide lactone	Bryozoans	Bacterium	Alzheimer's	Phase Ⅱ
Plocabulin	Macrolide lactone	Sponge	Sponge	Cancer	Phase Ⅱ
Marizomib	Beta-lactone-gamma	Bacterium	Bacterium	Cancer	Phase Ⅲ

The first FDA-approved drug derived from the marine environment was ziconotide, an analgesic agent isolated from a marine cone snail and is used for chronic pain management in patients (**Table 1.1**) (Gerwick and Moore, 2012; Altmann, 2017). Trabectedin (Yondelis®, Madrid, Spain) is another marine natural product which was isolated from a marine tunicate, *Ecteinascidia turbinata* and is used for treatment of soft tissue sarcoma (D'Incalci *et al.*, 2014; Hu *et al.*, 2015). Besides FDA approved drugs, other marine natural products have either reached the pre-clinical phase or are at the clinical phase of investigation (Abdelmohsen *et al.*, 2017; Petersen, Kellermann and Schupp, 2020).

Several natural products from marine invertebrates were shown to have structural similarities to those of compounds produced by their microbial symbionts, which led to the hypothesis that the microorganisms associated with the invertebrates are likely to be the true producers of these compounds or play a role in the biosynthesis of the compounds (Gerwick and Moore, 2012). The hypothesis was proved to be correct by Sudek et al, (2007), who showed that bryostatin, polyketides used for the treatment of Alzheimer's disease, and as anticancer agents, were synthesized by modular type 1 polyketide synthases (PKS-1) found in the *Endobugula sertula* bacterial symbiont of the marine bryozoan *Bugula neritina* (Sudek *et al.*, 2007). Furthermore, it was observed that after treatment of the *B. neritina* colonies with antibiotics, the number of the *E. sertula* was also reduced and this consequently led to reduced levels of bryostatin production. This evidence therefore suggested that *E. sertula* is likely to be the biosynthetic source of bryostatin.

Another study focused on the compound trabectedin (ET-743), which is an anticancer agent isolated from the tunicate *Ecteinascedia turbinata* (Rinehart, 2000). ET-743 is structurally similar to bacterial derived compounds, saframycin A, saframycin Mx1 and saframycin B isolated from *Streptomyces lavendulae, Myxococcus xanthus* and *Pseudomonas fluorescence*, respectively. Based on this, it was hypothesized that ET-743 is a product of marine bacterial symbionts. Through the use of metagenomic sequencing of the total DNA from the tunicate/microbial consortium, a 35kb contig containing the majority of the putative non-ribosomal peptide (NRP) biosynthetic pathway for ET-743 was obtained and this pathway was similar to the biosynthetic pathway of previously characterized saframycin A isolated from *S. lavendulae* (Rath *et al.*, 2011). These results suggested that the genes likely originated from the bacteria *Candidatus Endoecteinascidia frumentensis* and not from the tunicate due to the codon usage of the bacterium (Rath *et al.*, 2011). Since studies have confirmed that invertebrate associated microbes are responsible for producing bioactive secondary metabolites, research

has expanded its interest to microbes associated with invertebrates. For the purpose of this study, marine bacteria were of interest and will be discussed as a potential source of novel natural products.

1.4. Marine bacteria as producers of novel natural products

The marine environment has proven to be a rich source of microbial species diversity that is distributed in the shallow and deep marine waters, deep sea hydrothermal vents, and coral reef ecosystems (Gerwick and Moore, 2012; Petersen, Kellermann and Schupp, 2020). In most cases the marine bacteria are found to be associated with surfaces of macroorganisms such as sponges, algae, corals and tunicates, living in symbiotic relationships with these macroorganisms (Jensen and Fenical, 1994; Hu *et al.*, 2015). Marine bacteria are emerging as an exciting group for discovery of novel classes of biotechnology applications due to their high biodiversity and the missing gap of knowledge on biosynthesis of natural products (Jensen and Fenical, 1994; Sekurova, Schneider and Zotchev, 2019). Among the microorganisms, bacteria are the most prolific biological sources which account for a large number of drugs undergoing clinical trials and approved pharmaceuticals (Gerwick and Moore, 2012). Although there is a high diversity of microbial species, more than 99% of bacteria cannot be cultured under standard laboratory conditions (Gerwick and Moore, 2012). This led to the development of molecular techniques such as 16S rRNA analysis and metagenomics, which has made it possible to classify microorganisms based on their molecular phylogeny (Petersen, Kellermann and Schupp, 2020). Studies using phylogenetic analysis of the 16S rRNA genes showed that the major phyla in the ocean are Alpha proteobacteria, Gamma proteobacteria, Actinobacteria, Bacteroidetes and Cyanobacteria. Most research has focused on *Actinomycetes* particularly *Streptomyces*, which are well known as major sources of secondary metabolites and account for approximately 80% of the known antibiotics (Jensen and Fenical, 1994; Bérdy, 2005).

In this thesis, the production of antimicrobial activity from a marine bacterium was assessed. Specifically, activity against a range of olive trunk fungal pathogens and against a panel of Gram-positive and Gram-negative bacteria was investigated. The next two sections will introduce the need for novel compounds from the marine environment in order to combat fungal and bacterial pathogens.

1.4.1. Marine natural products as potential antibacterial agents

In the global search to contribute toward discovery of novel antimicrobials to combat antibiotic resistant strains, marine natural products have shown promising results. Many researchers have

isolated and identified novel antibacterial compounds against drug-resistant pathogens from marine bacteria (Hu *et al.*, 2015; Abdelmohsen *et al.*, 2017). For example, the bioactive compound lynamicin A, isolated from the marine actinomycete was shown to effectively inhibit both several Gram-negative and Gam-positive bacterial pathogens including methicillin-resistant (MRSA) *Staphylococcus aureus* and vancomycin resistant (VRE) *Enterococcus faecium* (McArthur *et al.*, 2008). A novel antibacterial peptide, bogorol A, was isolated from a marine *Bacillus* and displayed antimicrobial activity against antibiotic resistant pathogens including MRSA, VRE, *E. coli*, *Burkholderia cepacia* and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Barsby *et al.*, 2001). In another study, antibacterial activity of a novel triazolopyrimidine antibiotic, essramycin, isolated from the culture broth of the marine *Streptomyces* sp., isolate Merv8102, showed potent activity against the bacteria *E. coli* (ATCC 10536), *P. aeruginosa* (ATCC 10145), *Bacillus subtilis* (ATCC 6051), *S. aureus* (ATCC 6538), and *Micrococcus luteus* (ATCC 9341) (El-Gendy *et al.*, 2008). Recently, isatin was isolated from the marine bacterium *Pseudoalteromonas rubra* TKJD 22 associated with a marine tunicate. This compound exhibited antibacterial activity against MDR pathogens including MDR *E. coli*, MDR Extended-Spectrum Beta-Lactamase *E. coli* and MDR *Bacillus cereus*, *Micrococcus luteus*, and *Bacillus megaterium* (Ayuningrum *et al.*, 2019). It is evident from literature that marine natural products are abundant and could potentially serve as a source of novel antibacterial agents to combat multi-drug resistant microbes.

1.4.2. Marine natural products as potential antifungal agents

In contrast to the search for novel bioactive compounds for pharmaceutical application, not as much effort has been focused on exploring marine natural products for use as antifungal compounds. However, several compounds with antifungal activity have been isolated from marine microbes, invertebrates and algae (El-Hossary *et al.*, 2017). Within these groups, sponges are the leading sources of antifungal compounds, followed by bacteria, which in some cases are found to be associated with the sponges (**Figure 1.1**) (El-Hossary *et al.*, 2017). Researchers have isolated compounds from microbes and invertebrates, and shown that the extracts exhibit antifungal activity against both human and plant fungal pathogens. In one study, the marine actinomycete *Streptomyces hydrogenans,* demonstrated antifungal activity against *Candida albicans,* and *Pectinotrichum llanense* (Sulakshana *et al.*, 2018). Tareq *et al.* (2015) isolated two compounds; ieodoglucomide, and ieodoglycolipid from the ethyl acetate extract of a marine derived *Bacillus licheniformis*. These compounds were evaluated for

antifungal activity against the plant pathogens *Botrytis cinerea* and *Colletotrichum acutatum*, as well as against the human pathogen *C. albicans*, and were shown to inhibit fungal growth.

Haliangicin, a novel metabolite isolated from a culture broth of a marine myxobacterium showed antifungal activity against both human and plant pathogens, *Aspergillus niger*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Fusarium* sp. and *Mucor hiemalis* (Fudou, Iizuka and Yamanaka, 2001). Although studies have shown antifungal activity of marine bacterial secondary metabolites on other plant pathogens such as *Botrytis cinerea*, which infects more than 200 plant species (van Baarlen *et al.*, 2007); *Rhizobacter solani*, which causes root rot of many plant hosts (Fatima *et al.*, 2009); *Aspergillus niger*, which is a plant and human pathogen; and *Colletotrichum acutatum*, causing olive anthracnose (Úrbez-Torres *et al.*, 2013; Tareq *et al.*, 2015), to date there are no natural products from the marine environment which are used as fungicides in the agriculture industry (Peng *et al.*, 2016).

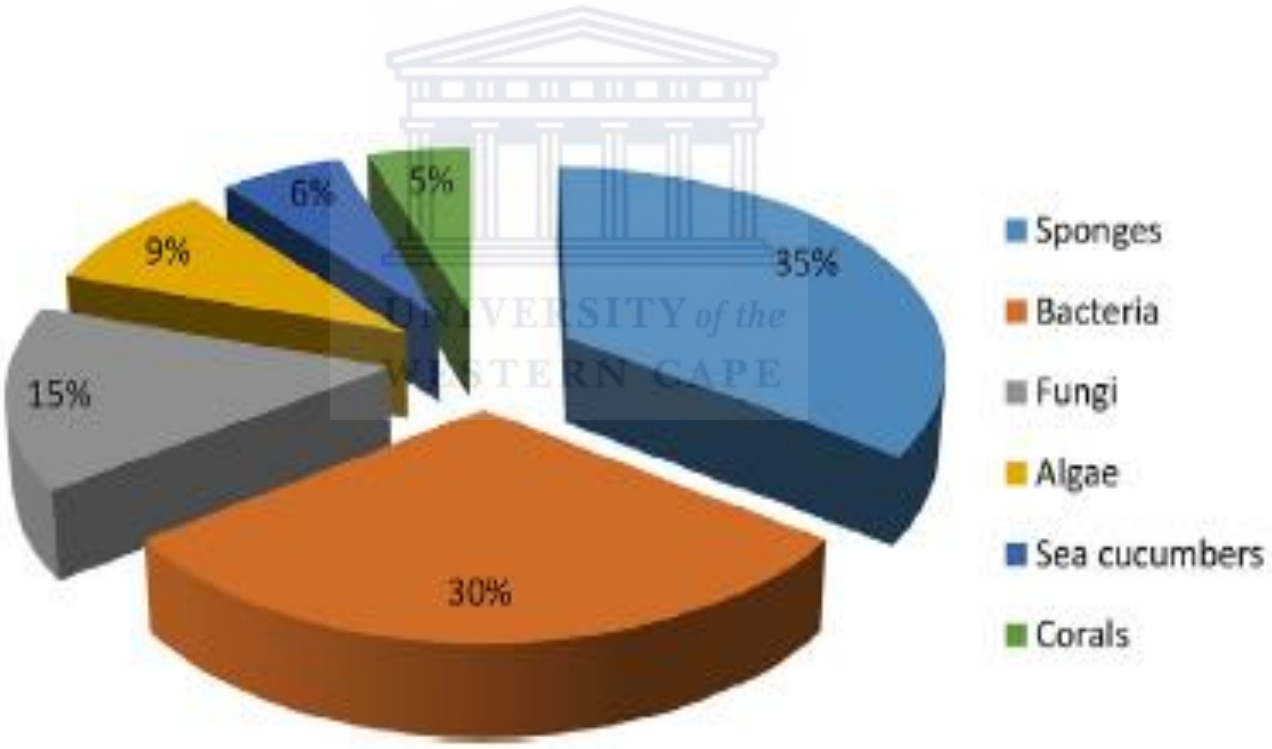

Figure 1.1: The percentage distribution of marine natural products with antifungal activity isolated from marine organisms. Taken from (El-hossary *et al*. 2017).

1.5. Secondary metabolites produced by marine bacteria

According to Ruiz *et al.*, (2010) secondary metabolites (SM) are a group of low molecular weight compounds with a size of approximately 3 KDa or less, which are synthesized by microorganisms as a mechanism for adapting to unfavourable conditions such as low nutrient availability, unfavourable pH conditions and salinity (Carvalho and Fernandes, 2010; O'Brien and Wright, 2011). The production of secondary metabolites by microorganisms therefore imparts a selective advantage against competitors for resources, and chemical defence against predators (Coleman *et al.*, 2011; O'Brien and Wright, 2011; Mutawila *et al.*, 2016; Petersen, Kellermann and Schupp, 2020).

Marine bacteria have received growing attention as the sources for bioactive metabolites and have a capacity to increase the number of marine natural products in clinical trials (Waters *et al.*, 2010). There are several advantages of sourcing secondary metabolites from marine bacteria; they are a natural and renewable source for producing secondary metabolites, easy to large scale by fermentation and have a comparatively simple genomic organization (Kasanah and Hamann, 2004; Waters *et al.*, 2010; Altmann, 2017). It is therefore possible to manipulate biosynthetic pathways to improve the concentration of the bioactive metabolite through heterologous expression in other hosts or through genetic engineering of the native strain (Jensen and Fenical, 1994; Waters *et al.*, 2010). This can circumvent the issue of biomass supply from invertebrates, which is often not economically sustainable in drug discovery (Waters *et al.*, 2010).

Secondary metabolites derived from marine bacteria are represented by different chemical classes that are both structurally and functionally diverse, including alkaloids, siderophores, terpenes, glycosides, peptides and others with a wide range of bioactivities (Jensen and Fenical, 1994; Duarte *et al.*, 2012; Blunt *et al.*, 2017). Classes of secondary metabolites produced by bacteria relevant to this study are discussed below.

1.5.1. Classes of secondary metabolites

1.5.1.1. Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria that are biologically active against other bacteria and closely related species (Shelburne *et al.*, 2007; Yang *et al.*, 2014; Mathur *et al.*, 2017). Lactic acid Bacteria (LAB) are widely studied bacteriocin producers, an example is nisin which has been used in the food industry as a preservative (Delves-Broughton, 1996). Although LAB bacteriocins are mostly studied, other

Gram-positive and Gram-negative bacteria are also capable of producing bacteriocins, which are promising alternatives to antibiotics and can be used as food preservatives, pathogen control agents, and pharmaceuticals (Yang *et al.*, 2014). However, for the purpose of this study, bacteriocin classes belonging to the Gram-positive bacteria will be discussed.

There are 3 major classes of bacteriocins from Gram-positive bacteria, namely; class I, class II and class III (Alvarez-Sieiro *et al.*, 2016; Mathur *et al.*, 2017), examples are listed in **Table 1.2**. These classes are divided based on size, modes of action, inhibitory spectrum, and immunity mechanisms (Bindiya and Bhat, 2016). Class I bacteriocins are small post translationally modified active peptides (RiPPs) that are less than 28 amino acids long (<5 kDa) (Yang *et al.*, 2014). This class is categorized into 7 subclasses according to the modification differences (Arnison *et al.*, 2013). Examples include the linear peptide (nisin), labrynthopeptins (labrynthopeptin A2) (Meindl *et al.*, 2010) and sactibiotics (thuricin CD, thurincin H and subtilosin A) (Kawulka *et al.*, 2004; Arnison *et al.*, 2013). All these classes undergo enzyme modification during biosynthesis that result in a variety of structures, and amino acid length. The biosynthetic mechanism of this class involves synthesis of a precursor peptide, consisting of a leader peptide and a core peptide. These are further processed by modification enzymes such as cyclases, and dehydratases. Finally, the leader peptide is proteolytically removed thereby releasing the active core peptide (Arnison *et al.*, 2013).

Class II bacteriocins (<10 kDa) are heat-stable membrane-active peptides which do not undergo post-translational modifications (Cotter, Ross and Hill, 2013; Bindiya and Bhat, 2016). This group is further divided into 4 subgroups; namely class IIa, class IIb, class IIc and class IId. Class IIa consists of listeria active and pediocin like peptides with a narrow antimicrobial activity spectrum, especially against Gram-positive bacteria (Cotter, Ross and Hill, 2013; Alvarez-Sieiro *et al.*, 2016). Class IIb comprises of bacteriocins which function based on the concerted activity of 2 peptides. Class IIc are circular peptide bacteriocins and class IId bacteriocins are linear non-pediocin like single peptides (Bindiya and Bhat, 2016).

Class III consists of large unmodified bacteriocins (>10 kDa) that are bacteriolytic and thermolabile (Alvarez-Sieiro *et al.*, 2016). This group has two subtypes; type IIIa which consists of bacteriolytic enzymes which lyse cell walls of susceptible bacterial strains and type IIIb bacteriocins which are non-lytic bacteriocins (Alvarez-Sieiro *et al.*, 2016; Bindiya and Bhat, 2016).

Table 1.2: Examples of different classes of bacteriocins from Gram-positive bacteria. Adapted from Bindiya and Bhat, 2016.

	Bacteriocins	**Class /type**	**Size (kDa)**	**Examples**
Gram-positive bacteria	Class I	Type A Type B Type C Type D	<5	Nisin Mersacidin Lacticin 3147 Subtilisin A Streptolysin S
	Class II	Class A- antilisterial Class B- synergistic	<10	Pediocin PA1 Carnobacteriocin B2
	Class III	Class A- bacteriolytic enzymes Class B- nonlytic peptides	>10	Lysostaphin Helveticin

For the purpose of this study, two subclasses of class 1 bacteriocins which are a promising source of new lead structures for development of novel antimicrobials will be discussed further.

1.5.1.1.1. Sactipeptides - subclass 2

Sactipeptides are a group of peptides with unusual sulphur to α-carbon crosslinks that are produced by radical S-adenosylmethionine (SAM) proteins (Murphy *et al*., 2011). To date, sactipeptides which have been isolated and characterized include, subtilosin A (Kawulka *et al.*, 2004), sporulation killing factor (Skf) (Liu *et al.*, 2010), thurincin H (Sit *et al.*, 2011), and thuricin CD (Rea *et al.*, 2010). The best studied sactipeptide is subtilisin A, produced by *Bacillus subtilis.* Subtilin A has broad antimicrobial activity against *Enterococcus faecium*, *Listeria monocytogenes,* and *Streptococcus pyogenes* (Shelburne *et al.*, 2007). Thuricin CD is a narrow spectrum 2 component sactipeptide (trnα and trnβ) produced by *Bacillus thuringiensis* DPC 6431 and has antimicrobial activity against *Clostridium difficile,* which causes diarrhoea in humans (Rea *et al.*, 2010). This makes sactipeptides promising for use in the medical industry. The biosynthetic gene clusters of all known sactipeptide are common, and have an operon containing structural genes, immunity proteins, transporters, and radical S adenosyl-methionine enzymes with a conserved region (**Figure 1.2**) (Flühe and Marahiel, 2013). All known sactipeptides are from members of the *Bacillus* genus, however genome mining studies have revealed putative sactipeptide gene clusters from other species such as *Clostridium,*

Bacteroidetes sp. and *Petrotoga mobilis* (Murphy *et al.*, 2011). In another study by Zhao (2016), the presence of sactipeptide gene clusters in bacterial genomes was investigated and results from this study identified thuricin CD, and thurincin H in the genomes of *Bacillus thuringiensis*, and *Bacillus cereus*. This sactipeptide subclass is of interest since it is a new emerging class of ribosomal assembled and postranslationally modified peptides that display diverse bioactivities.

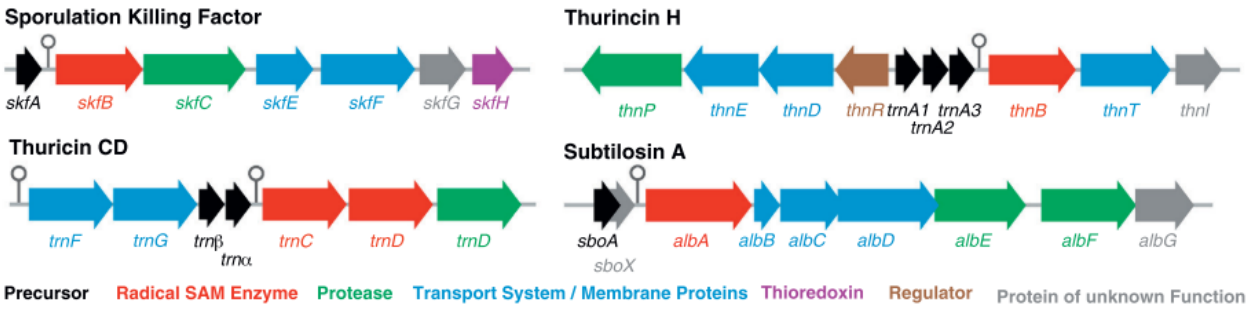

Figure 1.2: Schematic representation of the sactibiotic bacteriocin gene cluster. Each operon has structural genes, transporter genes, modification and maturation genes, one radical SAM enzyme per precursor peptide and immunity genes. (Taken from Flühe and Marahiel, 2013).

1.5.1.1.2. Linear azol (in)e-containing peptides (LAP's) - subclass 4

Linear azol(in)e-containing peptides form a subgroup of RiPPs consisting of combinations of heterocyclic rings of oxazoles and thiazoles, which result from cyclization of serine and threonine (Alvarez-Sieiro *et al.*, 2016). Prominent groups of natural products derived from this group includes streptolysin S produced by *Streptococcus pyogenes*, and microcin B17 produced by *E. coli* (Heddle *et al.*, 2001; Cox, Doroghazi and Mitchell, 2015). The characterized LAP's are plantazolicin A, and plantazolicin B produced by soil bacterium *Bacillus amyloliquefaciens* (Scholz *et al.*, 2011; Arnison *et al.*, 2013; Banala, Ensle and Süssmuth, 2013; Lopes *et al.*, 2017). Studies have reported that plantazolicin A has antimicrobial activity against several *Bacillus* species and *Micrococcus luteus* (Scholz *et al.*, 2011).

1.5.1.2. Non-ribosomal peptides

Non-ribosomal peptides (NRPs) constitute a structurally diverse family of natural products with different secondary metabolite classes ranging from siderophores, biosurfactants, antibiotics, pigments, immunosuppressants, and anticancer agents (Martínez-Núñez and López, 2016). NRPs consist of various linear, cyclic, and branched structures, which are

generated by large multi domain enzymes known as non-ribosomal peptide synthetases (NRPS). Within the bacteria domain, Proteobacteria, Actinobacteria, Firmicutes, and Cyanobacteria are the major producers of NRPs (Martínez-Núñez and López, 2016). Members of the *Bacilli*, and *Actinomyces* genus are the major producers of non-ribosomal peptides (Finking and Marahiel, 2004). Genome mining studies have estimated that 88% of *Bacillus* species genomes have known or putative gene clusters of NRPS, indicating that they are a potentially rich source of antimicrobials (Zhao, 2016). Many NRPs are used in various environmental and pharmaceutical industries. In the medical field, daptomycin and bacitracin are used as antibiotics. Cyclosporine A is used an immunosuppressant, and bleomycin A, which has cytostatic activity, is used in patients undergoing cancer therapy (**Figure 1.3**) (Martínez-Núñez and López, 2016). Secondary metabolites encoded by non-ribosomal peptide synthetase pathways are increasingly receiving attention due to their diverse complex structures and vast range of bioactivities applicable to human medicine, crop protection, and environmental restoration (Martínez-Núñez and López, 2016; Agrawal *et al.*, 2017).

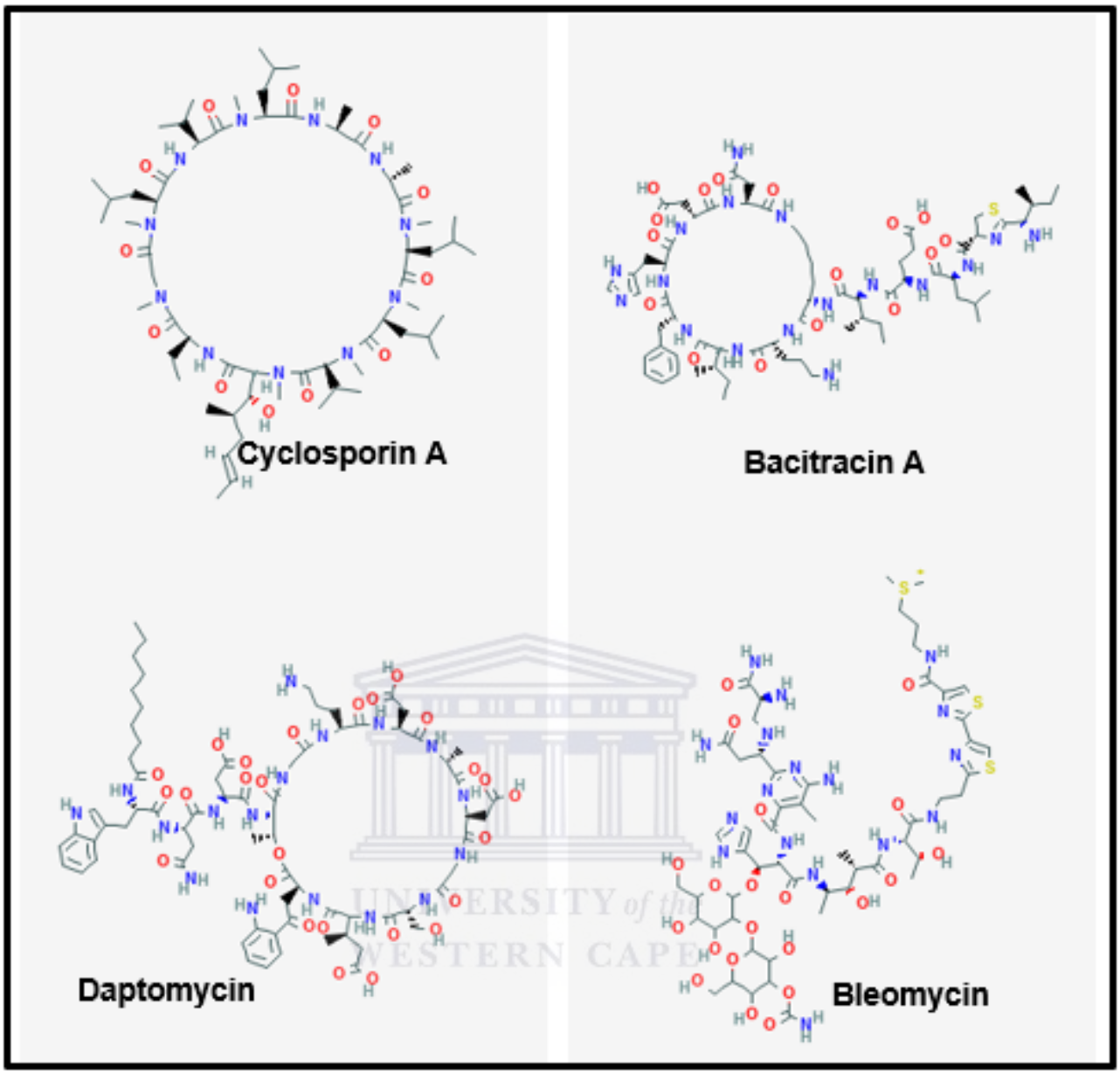

Figure 1.3: Structures of some of the NRPs used commercially as antibiotics. Taken from https://pubchem.ncbi.nlm.nih.gov/compound; Accessed online 16 April 2020.

1.6. Techniques used in discovery of novel bioactive marine natural products

Over the past years, the natural product discovery process has evolved and led to advances in strategies for detection, isolation and development of bioactive compounds relevant to multiple industrial applications. This section discusses two strategies currently employed in the process of natural product discovery, namely; the bioassay guided isolation, and whole genome sequencing and mining technique (Corre and Challis, 2007; Duarte *et al.*, 2012).

1.6.1. Bioassay guided fractionation as a technique for natural product discovery

In natural product discovery, bioassay guided fractionation has been successfully used for identification of bioactive compounds. This standard procedure involves multiple steps which aid in identification, and isolation of the bioactive compound. The steps followed include (i) investigation of the bioactive compound using a series of bioassays such as well diffusion assays, microtiter well plate assay or a dual inoculation assay, (ii) extracting the compounds of interest by employing different solvents of different polarities, and testing for bioactivity, (iii) continuous fractionation of the bioactive extract using chromatographic separations such as HPLC for the isolation of a pure compound, (iv) chemical analysis, and structural characterization of the bioactive compound using spectroscopic techniques such as LC-MS and Nuclear magnetic resonance (NMR) (**Figure 1.4**) (Duarte *et al.*, 2012; Weller, 2012; Malviya and Malviya, 2017).

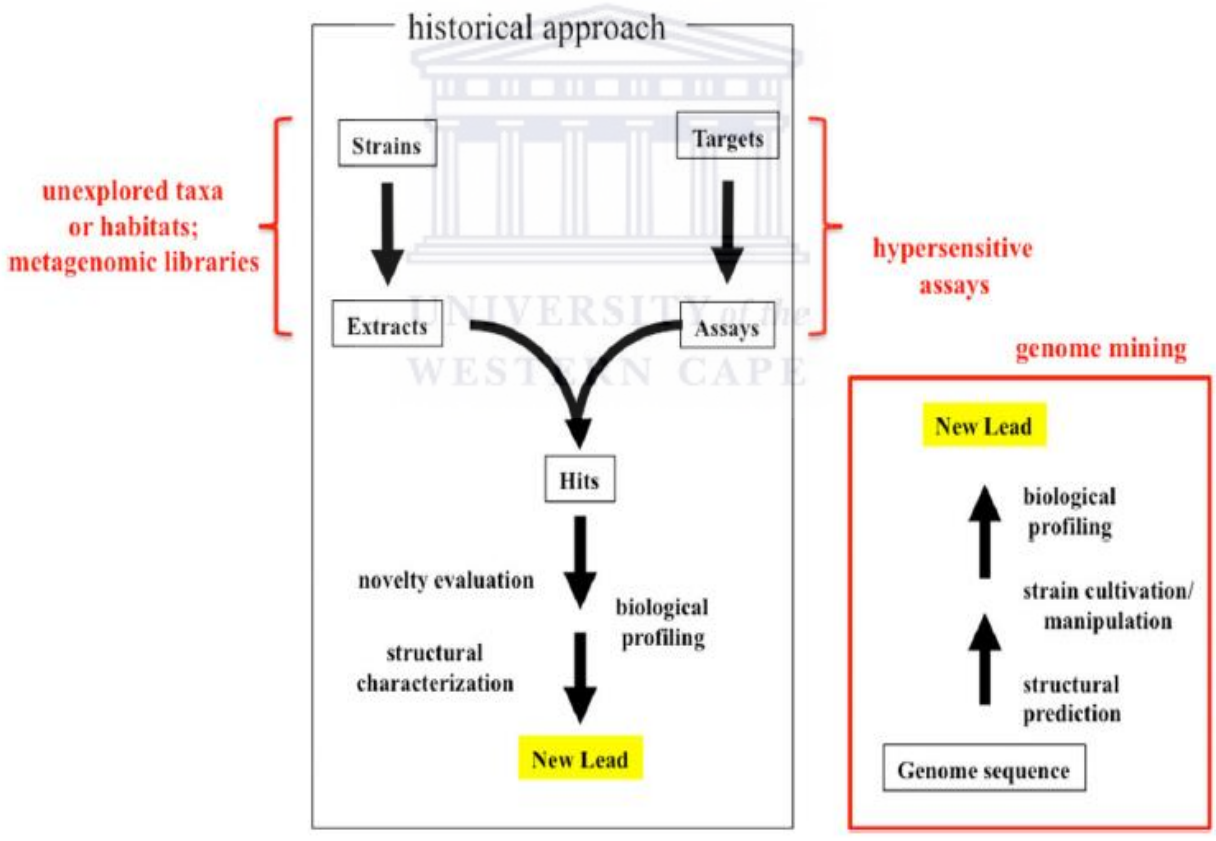

Figure 1.4: Historical approach of drug discovery of microbial natural products vs. recent advances in drug discovery. (Taken from Monciardini *et al.*, 2014).

This process ultimately links the observed bioactivity to the isolated compound by excluding fractions with no bioactivity (Monciardini *et al.*, 2014). Bioassay guided isolation is however, expensive, time consuming, and rediscovery of known compounds is still a major challenge (Zhao, 2011; Machado *et al.*, 2015; Malviya and Malviya, 2017; Li and Lou, 2018). Furthermore, loss of activity during fractionation and isolation is also a possibility with this technique (Inui *et al.*, 2012). In order to circumvent the issue of rediscovery of bioactive compounds, analytical tools such as HPLC, LC-MS, NMR and Ultra violet (UV) spectrophotometry can be used for de-replication by providing useful structural, and chemical information of compounds (Harvey, Edrada-Ebel and Quinn, 2015). Although these tools can be used to predetermine whether a compound is novel, they only compare based on physicochemical properties (chromatography retention times, mass to charge ratios UV-Vis profiles and NMR chemical shifts) and not on biological properties (Lang *et al.*, 2008; Wong, Oliver and Linington, 2012). This means that if a compound is structurally similar to a well-known characterized compound, it may be disregarded and may not take into consideration the potential novel biological activity of the compound.

1.6.2. Genome mining as a tool for natural product discovery

The rapid development of genome sequencing technologies, and advanced computational analyses of DNA sequences has increased the potential for natural product discovery. Over 292,565 sequenced bacterial genomes are now available (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/), which has drastically increased the knowledge and understanding of the genes involved in secondary metabolite biosynthesis (Gerwick and Moore, 2012). Genome mining uses the microbe's genomic information to determine whether it has potential to produce novel compounds. The genes encoding biosynthetic pathways for secondary metabolite are organised into biosynthetic gene clusters (BGCs) that generally code for multidomain enzymes such as polyketide synthases (PKS), and non-ribosomal peptide synthetases (Weber, 2014; Sekurova, Schneider and Zotchev, 2019). Through this genome mining approach, these putative BGCs are identified in the genome sequence, analyses of these clusters can provide information on the predicted chemical class of the SM synthesized by the enzymes encoded by the gene clusters and of silent BGCs (Weber, 2014; Sekurova, Schneider and Zotchev, 2019). Various online bioinformatic tools have been developed for detecting and analysing of these secondary metabolite pathways. Some examples of the online tools include antiSMASH (https://antismash.secondarymetabolites.org), BAGEL3 (http://bagel.molgenrug.nl/), Rapid

Annotation Subsystem Technology (RAST) (van Heel *et al.*, 2013; Overbeek *et al.*, 2014; Blin *et al.*, 2019), and PRISM (Skinnider *et al.*, 2017). antiSMASH for instance, is used as a tool for genome mining of gene clusters encoding biosynthetic pathways for secondary metabolites such as, terpenes, bacteriocins, siderophores, polyketides, and NRPs. This rapid web server allows DNA sequence files of different formats (FASTA, EMBL and Genbank) to be uploaded and thereafter biosynthetic gene clusters can be predicted, which can ultimately show the putative secondary metabolite. All these bioinformatic tools combined with homology- based searches, and phylogenetic analyses offer enormous potential to predict the production of interesting and novel secondary metabolites by microorganisms (Xiong *et al.*, 2013). This ultimately serves as a rapid method to avoid rediscovery of already known compounds, and also aids in the selection of the isolates to prioritize for further characterization, bulk extraction, chemical analysis and structure elucidation of the compound.

Unlike conventional bioactivity screening, whole genome sequence data analysis has revealed that microorganisms have far greater potential to synthesize and produce novel, and complex secondary metabolites (Li and Lou, 2018). For example, only 5 metabolites were identified from *Streptococcus coelicolor* A3 by conventional bioactivity assays, however, genome analysis showed that *S. coelicolor* had the potential to produce 20 additional natural products (Bentley *et al.*, 2002; Aigle *et al.*, 2014). In the case of the marine actinomycete *Salinispora tropica*, bioinformatic analysis revealed that this bacterium has approximately 9.9% of its genes dedicated to natural product biosynthesis (Udwary *et al.*, 2007). The majority of the gene clusters were novel, indicating that it likely produces novel natural products, however its biosynthetic potential was not fully identified by traditional bioactivity screening and chemical analysis (Udwary *et al.*, 2007). This is because there are silent secondary metabolite pathways, which are not expressed in laboratory fermentation conditions; in this case the compounds cannot be produced or the metabolites are produced in very low concentration despite the fact that the genome contains all the gene clusters responsible for biosynthesis (Zhao, 2011; Aigle *et al.*, 2014; Blin *et al.*, 2019; Sekurova, Schneider and Zotchev, 2019) For this reason, WGS as a natural product discovery approach has advantages over conventional bioactivity screening since it does not depend on the expression of secondary metabolites. Furthermore, WGS and mining can predict a variety of novel secondary metabolite classes in the genomes prior to isolation of the compound and also offers a linkage to structural information. Whole genome sequencing and mining studies has also highlighted the potential of well-known microbes to produce novel natural products.

1.7. Research aims and objectives

Marine bacteria exhibit great potential in producing natural products with diverse biotechnological applications. A collection of approximately 3000 marine invertebrate-associated bacteria from the oceans along the South African coast was established by the IMBM as one of the aims of a collaborative European Union-funded project "PharmaSea". In 2017, 35 sponge-associated bacterial isolates with antibacterial activity were identified from this collection and twelve of these exhibited antimicrobial activity against the multi-drug resistant *E. coli* 1699 strain (Matobole *et al.*, 2017). Screening for antifungal activity and other relevant biomedical activities also identified strains associated with tunicates that produced activities against *Aspergillus fumigatus* and *Candida albicans* (Hoosen, 2016). Therefore, the aim of the study was to screen marine bacteria for antimicrobial activity, isolate and characterize the novel antimicrobial compound/(s) with potential biotechnology applications. In order to achieve this aim, the objectives of the study were as follows:

1) To screen marine bacteria previously shown to exhibit antifungal and antibacterial activity. Previous studies by IMBM demonstrated antifungal activity of these marine bacterial strains against *Aspergillus fumigatus* and *Candida albicans*. Therefore, the antifungal activity of marine bacterial strains was evaluated against virulent olive trunk fungal pathogens. In parallel, antibacterial activity of the strain with broad spectrum activity was investigated against bacterial strains closely related to opportunistic pathogens and an engineered multi-drug resistant strain *E. coli* 1699.

2) To sequence the genome of the most active microbial isolate and identify biosynthetic gene clusters responsible for producing the secondary metabolites.

3) To isolate and characterize microbial extract of the strain using a bioassay-guided fractionation approach coupled to LC-MS.

1.8. References

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Chapter 2 - Screening of marine bacteria against virulent olive trunk fungal pathogens and bacterial indicator strains

2.1. Introduction

Recently, studies have identified pathogens which cause olive trunk diseases (Moral *et al.*, 2010; Carlucci *et al.*, 2013, 2015; Úrbez-Torres *et al.*, 2013). These pathogens are known to invade the plant's woody parts mainly through wounds caused by pruning, scars and stomata (Serra, Mannoni and Ligios, 2008; Rolshausen *et al.*, 2010; Cloete *et al.*, 2011), especially during spore dispersal in rainy weather (Munkvold and Marois, 1993). However, pruning wound susceptibility and protection of olive trees from these trunk pathogens is unknown.

There is no available registered treatment reported in literature for olive trunk pathogens. However, in other hosts which are affected by the same diseases caused by trunk pathogens, management is through pruning wound protectants, use of chemical fungicides and/or biocontrol agents (Rolshausen *et al.*, 2010; Amponsah *et al.*, 2012; Mutawila *et al.*, 2016). Chemical treatment has been used to control trunk pathogens in other hosts, although there are drawbacks associated with this treatment. This includes the breakdown of chemical fungicides especially during rainfalls, and environmental risks of these chemicals (Munkvold and Marois, 1993; van Niekerk *et al.*, 2010). Pruning wound protection by biological control agents offers an alternative to the use of harmful chemicals and provides more sustainable long-term control of trunk disease pathogens (Munkvold and Marois, 1993).

One of the mechanisms involved in biocontrol activity of biocontrol agents is their ability to produce secondary metabolites (Ferreira, Matthee and Thomas, 1990; Shwab and Keller, 2008). Marine bacterial secondary metabolites are chemically and structurally diverse (**Section 1.4**), therefore they may be suitable as potential sources of environmentally friendly novel antifungal compounds which can be developed as pruning wound protectants. The marine environment has gained interest for research on discovering novel bioactive compounds (Bhatnagar and Kim, 2010). Marine bacteria have been recognised as good sources for potential antimicrobials due to the number of novel compounds with multiple biological activities such as antimicrobial, antiviral, anticancer, antioxidant and antifouling activities (Anand *et al.*, 2006; Abdelmohsen *et al*., 2017; Ayuningrum *et al*., 2019).

Another global issue is that there has been an increase in the number of multi-drug resistant pathogens (bacteria, fungi and parasites) over the years (**Section 1.1**) (Bhatnagar and Kim, 2010; Meena and Kanwar, 2015; Jones *et al.*, 2017). Furthermore, there are no novel chemical classes of antibiotics which have been successfully introduced into clinical practise over the

recent decades. Therefore, it would also be valuable to discover and develop novel bioactive compounds from marine bacteria which can also be used to combat microbial drug resistance.

Generally, screening for natural products with antimicrobial activity involves methods such as diffusion, bioautography and dilution methods (Balouiri, Sadiki and Ibnsouda, 2016). In order to identify bacteria that potentially produce bioactive compounds, extraction of these compounds and subsequent testing against the pathogens is essential. Many studies have been accomplished based on dual inoculation of microorganisms such as bacteria and fungi (Svetlana Živkovic *et al.*, 2010; Bertrand *et al.*, 2014). These techniques are dependent on diffusion of the antimicrobial agent into the medium (Balouiri, Sadiki and Ibnsouda, 2016). As part of ongoing efforts to discover bioactive antimicrobial compounds from marine bacteria, the aim of this chapter was to evaluate the antimicrobial activity of 13 marine bacterial strains which were previously isolated from marine invertebrates by former IMBM researchers. In this chapter, marine bacteria were tested for their potential to inhibit mycelial growth of fungal trunk disease pathogens using a dual inoculation assay. In the case of bacterial indicator strains, the antibacterial activity was determined using a well diffusion assay technique. The results obtained were used to select the marine bacterial isolates with the most antagonism and to isolate the antimicrobial compound using a bioassay guided fractionation approach complemented with genome mining (presented in **Chapter 3**).

2.2. Materials and Methods

2.2.1. Strains and cultivation

The 13 marine bacterial isolates used in this study were part of the IMBM culture collection at the University of the Western Cape, Cape Town, South Africa (**Table 2.1**). The bacterial strains used were associated with endemic marine invertebrates, collected by SCUBA diving (2013) from Algoa Bay in Port Elizabeth, Eastern Cape, South Africa (34°00'40.6"S 25°43'11.7"E). These marine bacterial isolates were selected based on exhibited antibacterial and antifungal activities from previous screening conducted by the IMBM. Bacteria from fresh glycerol stocks of the IMBM culture collection were cultivated on Zobell agar (ZBA), trypticase soy agar (TSA), activated charcoal media (ACM) and glucose yeast malt media (GYM) at room temperature (23°C) for 2 days (**Appendix A1 - A4)**. The 9 fungal strains used in this study (**Table 2.2**) were from the Agricultural Research Council (ARC) Infruitec-Nietvoorbij fungal culture collection, Stellenbosch, South Africa fungal culture collection and were shown to be virulent olive trunk pathogens (van Dyk, 2020). The strains were revived by culturing on potato

dextrose agar plates (PDA) (**Appendix A5**) and incubated at room temperature (23°C). Plates inoculated with fast growing fungi (ID669, PMM2093, PMM2090, CSN182) were incubated for 3-4 days at a temperature of 23°C, those with moderate and slow growth for 7 (CSN343) and 14-21 (ID318**,** CSN946**,** CSN418) days, respectively. For antibacterial screening, 4 indicator strains were used, including Gram-negative *E. coli* 1699, and *Pseudomonas putida*, and Gram-positive *Bacillus cereus* ATCC10702, and *Staphylococcus epidermidis* ATCC14990. The test strains were cultivated from glycerol stocks, streaked on Luria-Bertani (LB) media agar plates (**Appendix A7**) and incubated overnight at 37°C. *E. coli* 1699 (Cubist, USA) was selected because it has been genetically engineered for resistance against 52 known antibiotics (**Appendix C**).

Table 2.1: Marine bacterial isolates evaluated for antifungal activity.

Bacterial strain	**Strain ID**
NS*	PE05-34
NS	PE6-19
NS	PE06-34
Bacillus cereus	PE6-126
NS	PE8-10
NS	PE8-149A
NS	PE8-77B
NS	PE12-95
Pseudovibrio ascidiaceicola	PE12-106
NS	PE13-163
Pseudovibrio ascidiaceicola	PE14-07
Pseudovibrio ascidiaceicola	PE14-12
Pseudovibrio sp.	PE14-104

*NS - represents the bacterial isolates which were not sequenced for identification.

Table 2.2: Olive fungal trunk pathogens screened against for antagonism.

Fungal pathogen	**Strain ID**
Eutypa lata	ID318
Neofusicoccum sp. nov.	PMM2090
Neofusicoccum capensis	ID396
Diaporthe foeniculina	CSN343
Diplodia seriata	PMM2093
Neofusicoccum vitifusiforme	CSN182
Neofusicoccum stellenboschiana	ID669
Phaeoacremonium africanum	CSN946
Phaeoacremonium parasiticum	CSN418

2.2.2. Dual inoculation assay

The antifungal activity of the marine bacterial isolates was tested against the fungal strains listed in **Table 2.2**. A dual inoculation assay technique was utilized to screen for antifungal activity against the virulent olive trunk pathogens. In order to determine if the pathogens were capable of growing on the media, prior to this assay, fungal trunk pathogens were cultivated on the media (ACM, GYM, TSA and ZBA) used for the dual inoculation assay for 7 days at 23°C. The marine bacteria were streaked out on one side of agar plates containing their respective growth media (ACM, GYM, TSA and ZBA) (**Table 2.3**). Following inoculation of the marine bacterial isolate on their respective media, the cultures were incubated at room temperature (23°C) for 14 days to allow for secondary metabolite production. After 14 days of bacterial growth, a mycelial plug (5 mm diameter) obtained from an actively growing margin of a 7-day fungal culture, was inoculated 2 cm away (mycelium side facing down) from streaked bacterial colonies on their respective media plates (ACM, GYM, TSA and ZBA). The dual inoculated plates were further incubated for 5 days at 23°C and the diameter of fungal mycelial growth was measured. Control plates included 5 mm fungal mycelial plugs inoculated on the centre of clean ZBA, TSA, ACM and GYM plates without the bacterial culture. Fungal growth measurements were conducted when mycelial growth on the control plates had reached the periphery of the Petri dish. Fungal mycelial growth diameter was recorded as the mean diameter of radial growth from two perpendicular points on the plate minus the original 5 mm of the fungal mycelial plug used for inoculation. For every experimental unit, the measurements

were averaged between the 3 replicates. All assays were performed in triplicate and the experiment was repeated twice for reproducibility.

Table 2.3: Media used for marine bacterial strains and fungal trunk pathogens in the dual inoculation assay.

	Culture Media			
Bacterial Isolate ID	**TSA**	**ZBA**	**GYM**	**ACM**
PE14-07	+	+	-	-
PE6-126	+	+	-	-
PE8-149A	+	-	-	-
PE14-104	-	+	-	-
PE06-34	-	-	+	-
PE13-163	-	+	-	-
PE05-34	-	-	+	-
PE12-95	-	-	+	-
PE12-106	-	-	+	-
PE8-77B	-	-	-	+
PE14-12	-	+	-	-
PE06-19	-	-	-	+
PE8-10	-	-	+	-

+: represents the culture media the bacterial isolate was grown on, -: bacterial isolate was not grown on that media.

2.2.3. Preparation of ethyl acetate extracts for bioassays

The marine bacterial isolate which showed antimicrobial activity from the screening results, named PE6-126, was selected for extraction of secondary metabolites. PE6-126 was grown in 2 L Erlenmeyer flasks containing 500 mL of ZBA liquid broth for 14 days at 23°C. After two weeks, the cultures were centrifuged at 4032 x *g* for 20 min. The culture supernatant was then extracted with equal volumes of analytical reagent (AR) grade ethyl acetate (Merck (Pty) Ltd) and incubated for 24 hours on a rotary shaker (125 rpm) to ensure maximum exposure to the ethyl acetate. The mixture was separated on a separation funnel and thereafter the ethyl acetate fraction was evaporated to dryness under reduced pressure at 35°C using a rotary evaporator (IKA RV10 basic). The remaining brown residue in the flask was resuspended in 5 mL methanol and the methanol was evaporated in a drying oven at 35°C. The extracts were stored

as a dried pellet at 4°C. Prior to the antimicrobial assays, the extract was re-suspended in 20% Dimethyl sulfoxide (DMSO) and filtered through a 0.22 µm syringe filter.

2.2.4. Well diffusion assay

In order to evaluate the antifungal and antibacterial activity of the extract, the agar well diffusion assay was used. This assay is based on the principle that the compound in the extract will diffuse through the agar and inhibit growth of the test strain (Balouiri, Sadiki and Ibnsouda, 2016). Fungal strains were grown for 7 days at 23°C on PDA prior to being used in the assay. A 5 mm fungal mycelial plug was aseptically transferred from the fungal plate to the centre of a clean PDA plate. Three wells (0.5 cm) were made 2 cm away from the fungal mycelial plug and 100 µL of extract added in the wells. The plates were incubated for 5 days at room temperature (23°C).

For the antibacterial screening, wells were created in the centre of Luria agar (LA) plates using a sterile cork borer, thereafter 100 µL of the extract was added to each well. The solution was allowed to diffuse for approximately 2-3 hours into the agar prior to plating of the indicator strains. Bacterial indicator strains were cultured in 10 mL LB broth and incubated on a shaking incubator (135 rpm) at 37°C for 16 hours. For the assay, the OD (600 nm) of the 16-hour old culture was adjusted to OD range of 0.30-0.35. One hundred microliters (100 µL) of this bacterial dilution was spread plated on the prepared LA plate using a sterile spreader. The control was a 20% DMSO solution pipetted into a separate well and the plates were incubated at 37°C for 16 hours after which growth was observed. A clear zone with no microbial growth around the well of interest was scored as antimicrobial activity.

2.2.5. Data analysis for dual inoculation assays

The collected data from the dual inoculation assay was subjected to Analysis of Variance (ANOVA) using GLM (General Linear Models) in order to determine the significant differences (p <0.05) between the means of the experimental group. ANOVA was done per individual pathogen and trial, thereafter the two trials were combined. Percentage inhibition was calculated in terms of the pathogen and its respective medium control. In the case where data from the 2 trials were different, a weighted analysis was done. Levene's test for homogeneity of variances of the combined trial data was performed for each pathogen. In order to get a normal distribution of the data the major outliers were discarded. This procedure was performed using statistical software version 9.2 (SAS Institute Inc. 1999).

2.3. Results & discussion

2.3.1. Cultivation

In order to determine whether the media used to culture marine bacteria would be suitable for culturing olive fungal trunk pathogens as well, fungal strains were grown on all the media used for the dual inoculation assay. From this preliminary experiment, the results showed that all the media (TSA, ZBA, GYM and ACM), supported growth of both the marine bacteria as well as growth of the fungal pathogens. Therefore, all the antifungal screenings were performed on these 4 media.

2.3.2. Dual inoculation assay

The dual inoculation assay was performed in order to assess the potential of marine bacteria to inhibit different genera, namely, *Botryosphaeria, Diaporthe, Eutypa and Phaeoacremonium* which are known to be virulent trunk pathogens (Cloete *et al.*, 2011; Gramaje *et al.*, 2012; Carlucci *et al.*, 2015; Van Dyk, 2020). This assay involves cultivation of two or more microorganisms in the same cultivation conditions to induce the production of secondary metabolites. A number of studies have demonstrated that the dual inoculation assay is one of the techniques used for discovery of novel bioactive natural products. A study by Bester (2006) showed that in dual cultures of *B. subtilis* isolated from grapevine pruning wounds, several trunk pathogens, including members of *Botryosphaeriaceae* (*D. seriata*, *N. australis*, *N. parvum*), *Phaeoacremonium* (*P. viticola) and Phaeomoniella (P. chlamydosporia*) were inhibited (Bester, 2006). In one study, rhizosphere *Streptomyces* from *Araucaria angustifolia* showed various degrees of inhibition when co-cultivated with the trunk pathogen *N. parvum* (Dalmas *et al.*, 2013). In another study by Ferreira *et al.* (1991), *Bacillus subtilis* isolated from grapevine wood inhibited mycelial growth of *Eutypa lata* on PDA when co-cultivated. All these studies support the hypothesis that dual culturing is one method which may stimulate the production of inhibitory compounds onto the media, thereby reducing mycelial growth of pathogens.

In this study, the results obtained from the dual inoculation assay demonstrated that 7 out of 13 bacterial strains had antifungal activity against 6 fungal pathogens on selected media (**Table 2.4**; **Figures 2.1-2.4**). Observations from the assay included inhibition of the fungal mycelial growth, whereby fungal mycelia did not grow close to the bacterial streaking, this resulted in mycelial growth diameter less than in the control plate. In the control plate containing only the fungal pathogen, the fungal mycelial growth diameter was higher than in the dual inoculated

plates. In the case where mycelial growth of the pathogen grew over the bacterial colony, this was recorded as no inhibition. PE6-126 had the highest inhibitory effects on most fungal pathogens, with either little or no mycelial growth. PE14-07, PE14-12 and PE13-163 also showed inhibitory effect against the fungal pathogens.

Table 2.4: Seven bacterial strains which had inhibitory effects on mycelial growth of fungal pathogens on selected media (ACM, GYM, TSA and ZBA).

	OLIVE TRUNK PATHOGENS								
Bacterial isolate ID	**CSN343** ***D. foeniculina***	**ID396** ***N. capensis***	**CSN182** ***N. vitifusiforme***	**ID669** ***N. stellenboschiana***	**PMM2090** ***Neofusicoccum*** **sp. nov**	**PMM2093** ***D. seriata***	**CSN418** ***P. parasiticum***	**1D318** ***E. lata***	**CSN946** ***P. africanum***
PE06-126 (TSA)	√	-	√	-	-	-	-	-	-
PE06-126 (ZBA)	√	-	√	-	√	√	-	√	√
PE14-104 (ZBA)	√	-	√	-	-	√	-	-	√
PE13-163 (ZBA)	√	-	√	-	√	√	-	-	√
PE14-07 (ZBA)	√	-	-	-	-	-	-	-	√

PE14-12 (ZBA)	√	√	√	√	-	-	√	√	-
PE06-19 (ACM)	√	-	√	-	-	-	-	-	√
PE08-77B (ACM)	√	-	√	-	-	-	-	-	-
PE5-34 (GYM)	-	-	-	-	-	-	-	-	-
PE-6-34 (GYM)	-	-	-	-	-	-	-	-	-
PE8-10 (GYM)	-	-	-	-	-	-	-	-	-
PE12-95 (GYM)	-	-	-	-	-	-	-	-	-
PE12-106 (GYM)	-	-	-	-	-	-	-	-	-

√ represents the olive trunk pathogens which were inhibited by marine bacterial isolates in the dual inoculation assay; - indicates that there was no fungal inhibition by the marine bacterial strain.

From the 4 different types of media used for the assay, the fungal isolates displayed varying mycelial growth inhibition depending on the type of medium used for the bacterial cultivation. For example, when the bacterial isolate PE6-126 was cultivated on TSA, ZBA and ACM media, activity against CSN343 was observed (**Figure 2.1-2.3)**, whereas high mycelial growth was observed in GYM media **(Figure 2.4**). Interestingly, none of the marine bacteria conferred antifungal activity when GYM media was used, whereas most bacteria which were cultivated on ZBA had antifungal activity.

Both GYM and ZBA are nutrient rich media, although different results were observed on these two media. On GYM, there was high fungal mycelial growth in dual inoculated plates and the control plates, this suggested that there was no antifungal activity. In ZBA, most fungal isolates had little or no mycelial growth and this suggested that the bacteria were producing an antifungal compound. This media composition probably contained an ingredient which triggers secondary metabolite production. However, based on these results there was also a possibility that these marine bacteria depleted a particular metabolite in the media while being incubated for two weeks without the fungal isolate, therefore this would result in low fungal mycelial growth in the dual inoculated plates as compared to the control plates with no bacteria inoculated. There was an exception to this by one of the fungal pathogens (ID396) which had a high mycelial growth on ZBA media as well **(Figure 2.5)**.

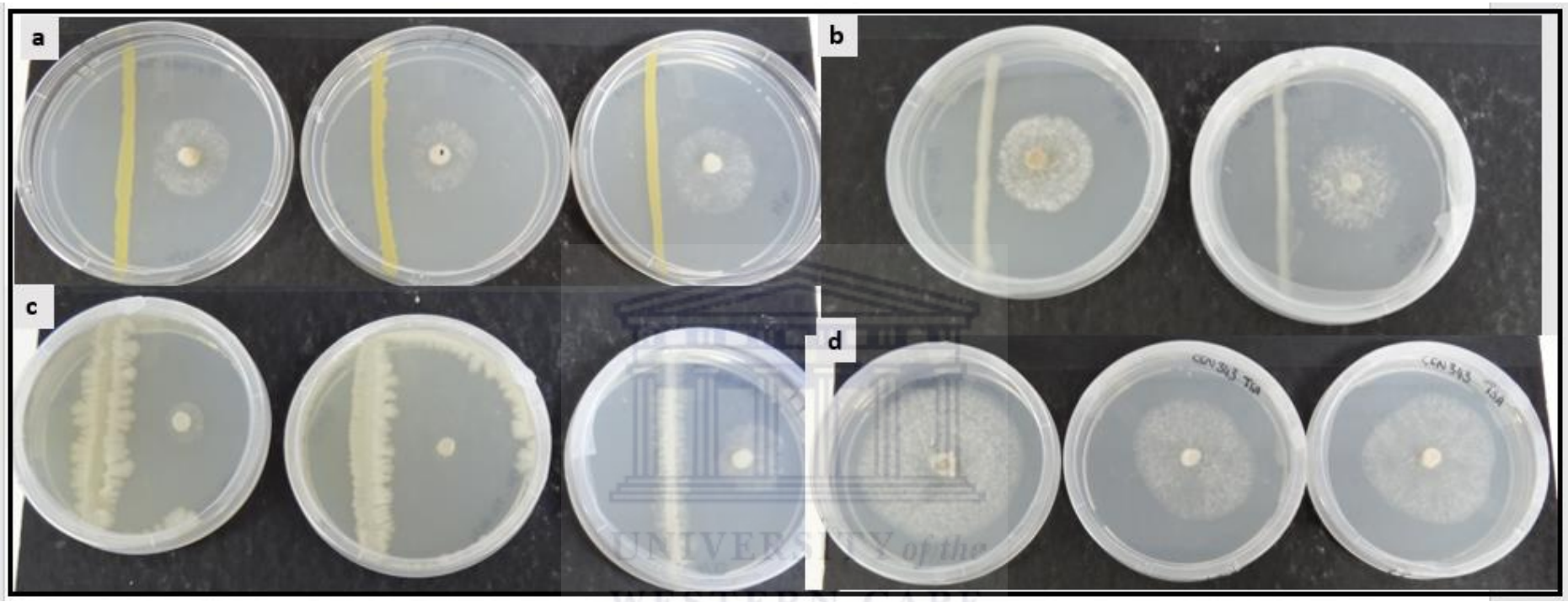

Figure 2.1: Screening results of bacterial isolates cultivated on TSA media against one of the inhibited olive trunk pathogens *D. foeniculina* - CSN343 (a) PE8-149a; (b) PE14-07; (c) PE6-126; (d) CSN343 control growing on TSA media. Distance between the bacterial streaking and the inoculated fungal mycelial plug was 2 cm.

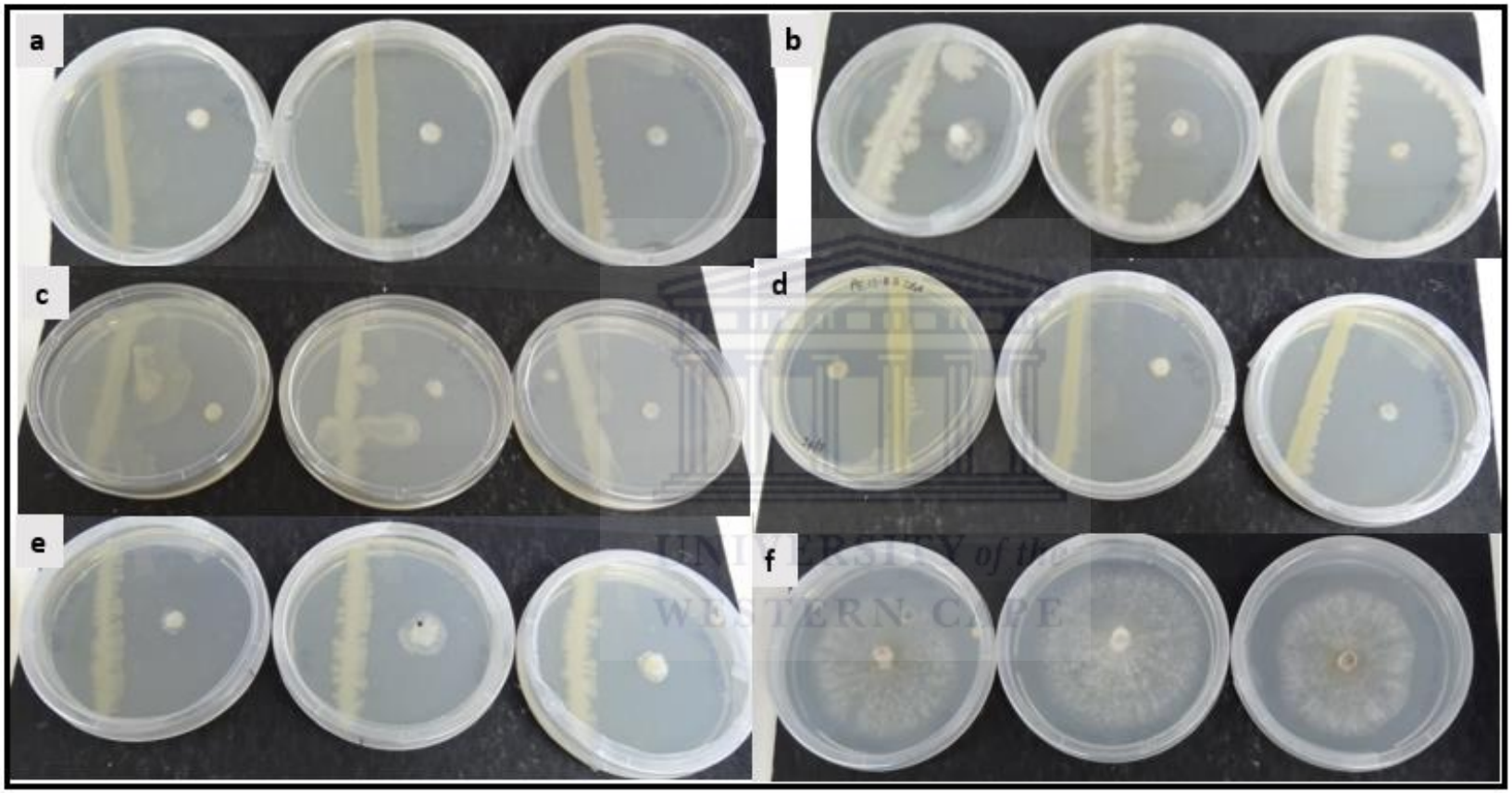

Figure 2.2: Screening results of bacterial isolates cultivated on ZBA media against *D. foeniculina* - CSN343. (a) PE14-104; (b) PE6-126; (c) PE14-07; (d) PE13-163; (e) PE14-12; (f) CSN343 control growing on ZBA media. Distance between the bacterial streaking and the inoculated fungal mycelial plug was 2 cm.

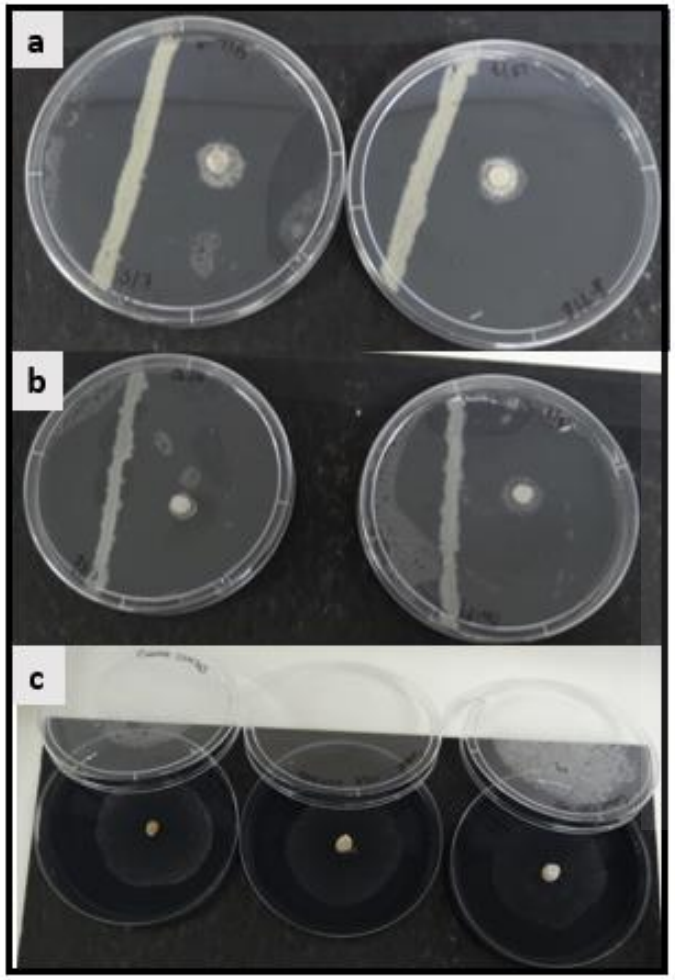

Figure 2.3: Screening results of bacterial isolates cultivated on ACM media against *D. foeniculina* - CSN343. (a) PE6-19; (b) PE8-77B; (c) CSN343 growth control on ACM media. Distance between the bacterial streaking and the inoculated fungal mycelial plug was 2 cm.

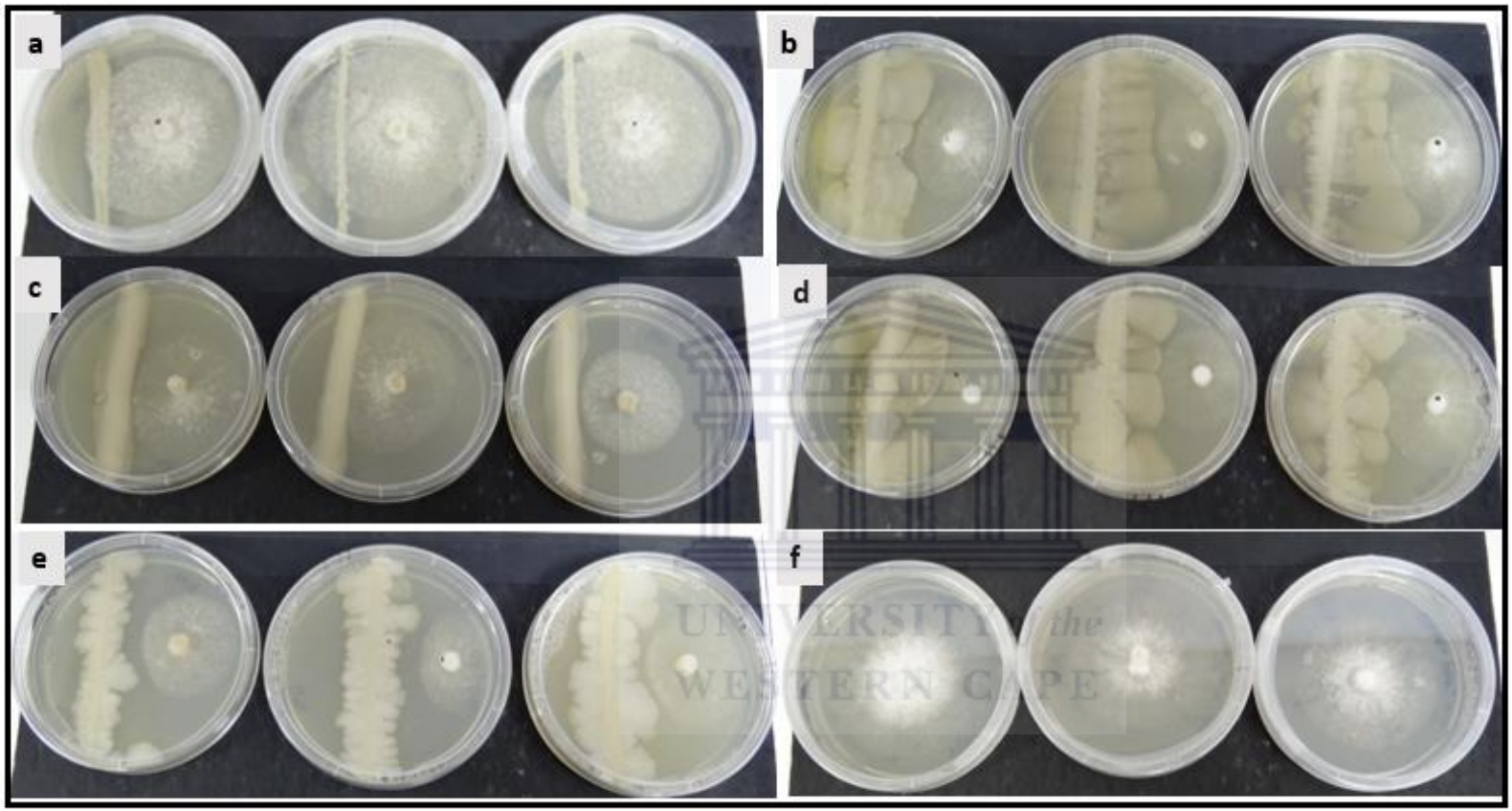

Figure 2.4: Screening results of bacterial isolates cultivated on GYM media against one of the inhibited olive trunk pathogen *D. foeniculina* - CSN343 (a) PE12-95; (b) PE5-34; (c) PE12-106; (d) PE6-34; (e) PE8-10; (f) CSN343 control growing on GYM media. Distance between the bacterial streaking and the inoculated fungal mycelial plug was 2 cm.

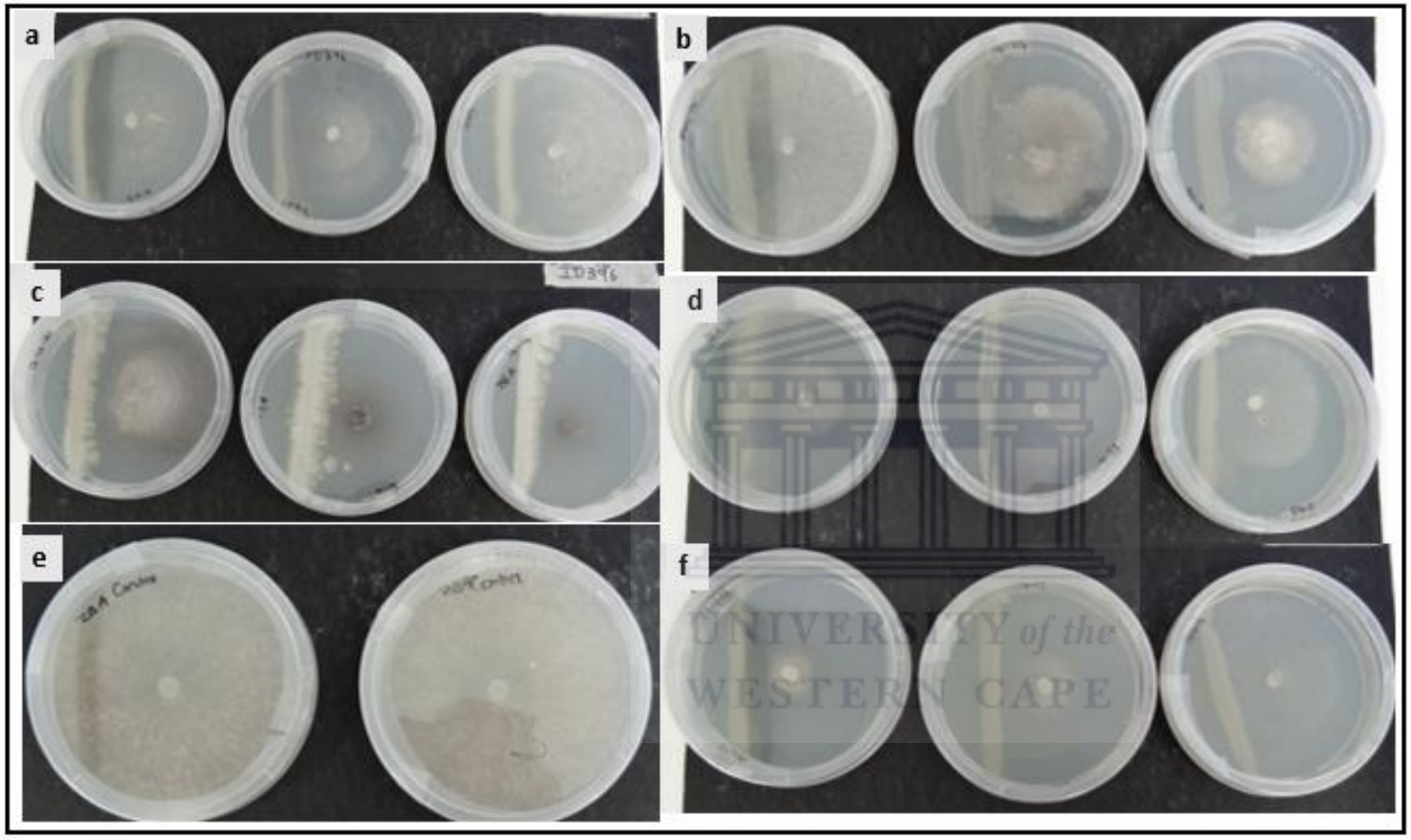

Figure 2.5: Screening results of bacterial isolates cultivated on ZBA media against *N. capensis* - ID396. (a) PE14-07; (b) PE14-104; (c) PE6-126; (d) PE13-163; (e) ID396 control growing on ZBA media; (f) PE14-12. Distance between the bacterial streaking and the inoculated fungal mycelial plug was 2 cm.

It is challenging to find optimal conditions which are required to elicit expression of biosynthetic pathways when screening for antimicrobial activity, however the OSMAC approach has been used by researchers for this purpose. The OSMAC approach follows the principle that under different growth conditions, the strain has the potential to produce multiple compounds as a way to respond to the stress (Bode *et al.*, 2002; Romano *et al.*, 2018). This approach has been developed to activate the expression of silent biosynthetic gene clusters by changing cultivation conditions such as media constituents (carbon, nitrogen source and inorganic salts) and physical parameters (level of aeration, shaking, salinity and temperature) (Romano *et al.*, 2018). For example, in a previous study from IMBM, different antimicrobial activity profiles were observed in relation to the media in which the bacteria were cultured on. The marine bacterium PE7-15 showed antimicrobial activity against the indicator strains *E. coli* 1699 and *Mycobacterium smegmatis* when cultured in GYM medium. However, when cultured on ZBA medium, it displayed antimicrobial activity against *Bacillus cereus* (Matobole, 2015). In another study by Mutawila *et al*. (2016), it was shown that the production of the secondary metabolite 6-pentyl pyrophosphate (6pp) by *Trichoderma* was increased when co-cultured with the pathogen *N. parvum*. Moreover, the study demonstrated that when using different culturing media, there was a difference in 6pp production yields. On one media (GCBM), the secondary metabolite 6pp was produced at a higher concentration compared to when isolates were cultured in Pezet's medium. This is a generally known phenomenon with regards to secondary metabolite production (Muscholl-Silberhorn, Thiel and Imhoff, 2008).

The percentage inhibition of mycelial growth of strains CSN343, CSN182 and PMM 2093 was high (>90%) for the bacterial isolates PE6-126, PE14-12 and PE14-07 (**Figure 2.6**). PMM2090, ID669 and ID318 had percentage inhibition (<70%) by bacterial isolates PE6-19, PE14-104, PE8-77B and PE14-07. Levene's test showed homogeneity of variances for the pathogens CSN343, ID669, PMM2093 and CSN418 and non-homogeneity of variances for the pathogens CSN182, PMM2090, ID318 and CSN946. Analysis of variance of the percentage inhibition mean values for the marine bacteria showed a significant interaction between all the pathogens tested and the bacterial isolates ($P<0.01$), this indicates that these fungal pathogens reacted differently to the marine bacterial isolates used in the assay. Based on the percentage inhibition of the mycelial growth, the marine bacteria PE6-126 had most activity against the fungal pathogens. This result suggested that PE6-126 had a broad range activity. Therefore, this finding led to the selection of this isolate for further evaluation of its activity against fungal strains CSN343, CSN182 and PMM2093.

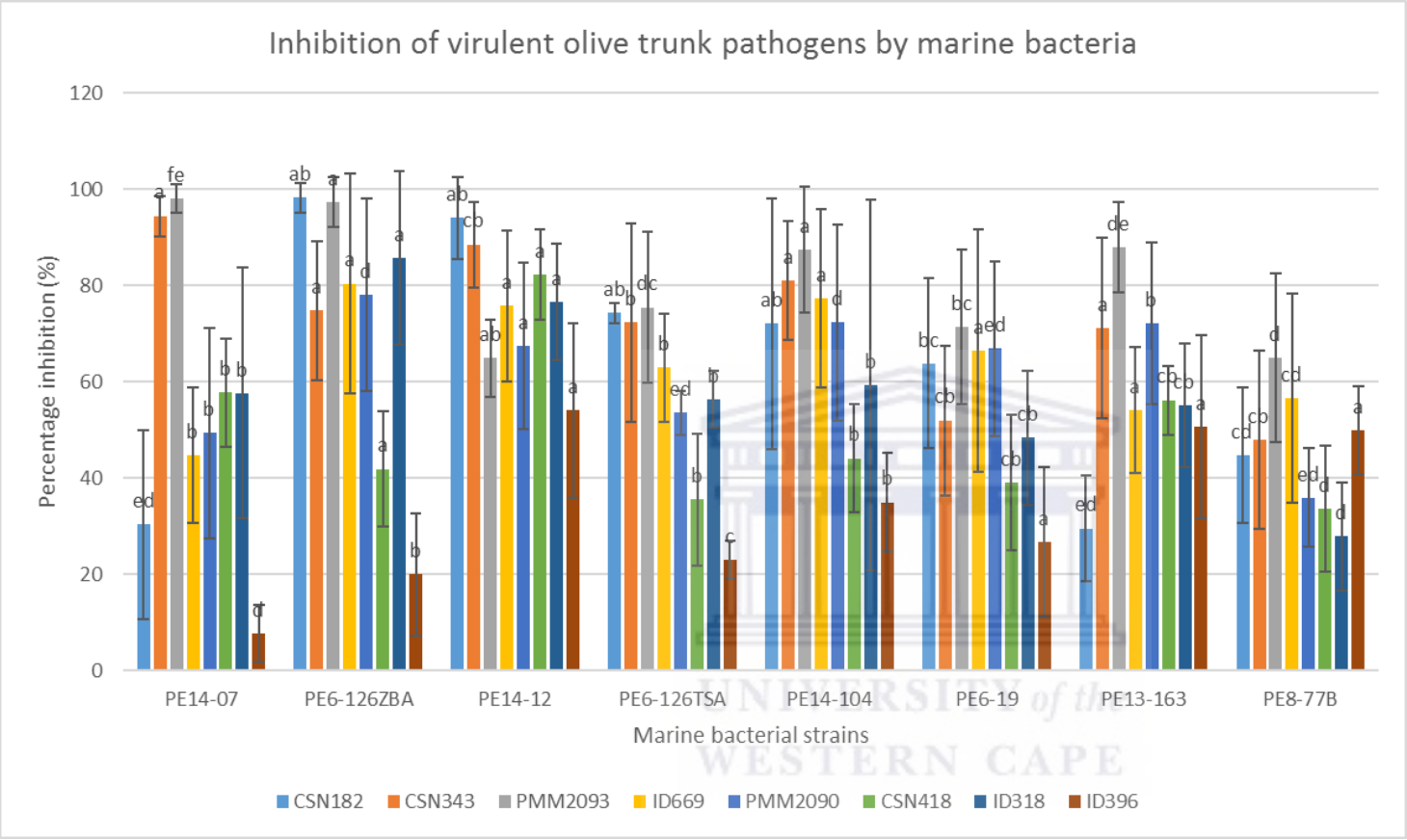

Figure 2.6: Bar graph showing the percentage inhibition of olive fungal trunk pathogens CSN343, CSN182, PMM2093, PMM2090, ID318, ID669 and CSN418 mycelial growth by marine bacterial isolates PE6-19, PE6-126, PE14-07, PE14-12, PE13-163 and PE14-104. The error bar represents the standard deviation of the 3 repeats. Error Bars with the same letter on top show the means which are not significantly different for each pathogen according to the LSD test ($P \leq 0.05$).

2.3.3. Evaluation of antifungal activity of the ethyl acetate extract from PE6-126

Given the fact that antifungal activity was observed mostly on ZBA dual inoculated plates, it was likely that it produces an antifungal compound which diffuses into the culturing media. However, it was also essential to determine whether the observed antifungal activity was a result of nutrient depletion by the bacterium or secretion of the antifungal compound. Secondary metabolites have been produced by fermenting the bacteria in liquid cultures (Robinson, Singh and Nigam, 2001; Anand *et al.*, 2006; Ayuningrum *et al.*, 2019). Considering the fact that extraction of secondary metabolites requires large amounts of biomass, it was feasible to use bacterial liquid fermentation since it would enable the microorganisms to be grown in large volumes in order to scale-up the production of the antimicrobial compound required for further purification and identification (**Chapter 3**). A crude extract was prepared from a liquid culture of PE6-126 in the stationary growth phase and evaluated for antifungal activity using a well diffusion assay. The agar well diffusion assay is a widely used technique to evaluate the antimicrobial activity of microbial extracts (Balouiri, Sadiki and Ibnsouda, 2016). However, no antifungal activity was observed for the PMM2093, CSN182 and CSN343 strains tested and this was indicated by the mycelium growth of the test plates being equal to the control plates (**results not shown**). Thereafter, extraction of the antifungal compound was attempted using ethyl acetate, which has been used in previous studies to isolate various types of antimicrobial compounds from marine bacteria (Schumacher *et al*., 2003; Anand *et al*., 2006; Hayashida-Soiza *et al*., 2008; Mondol and Shin, 2014). The reason why we attempted to extract the antifungal compound using ethyl acetate was because this step would likely reduce the complexity of the extract by eliminating other constituents of the crude extract which could have possibly diluted the compound responsible for the antifungal activity. However, there was no antifungal activity detected.

The common problem associated with natural products studies involves the inability to extract the bioactive compound in adequate concentration in order to be detected in the assay (Bertrand *et al.*, 2013; Pham *et al.*, 2019). The lack of bioactivity in the extracts could be due to the fact that cultivation conditions were changed; for example, from solid to liquid fermentation, which might have impacted on the secondary metabolite production. Changing cultivation conditions may have switched off secondary metabolite pathways which are responsible for the production of the antifungal compound. Therefore, extraction of the antimicrobial compound from dual inoculated ZBA agar plates was attempted; however, no antifungal activity was observed.

2.3.4. Evaluation of antibacterial activity from the ethyl acetate extract of PE6-126

Marine bacteria are known to produce structurally and functionally diverse secondary metabolites with a wide variety of biological activities (Jensen and Fenical, 1994; Duarte *et al.*, 2012; Blunt *et al.*, 2017), and a single strain may be capable of producing several such compounds. Therefore, the antibacterial potential of this strain was also tested in parallel to the antifungal screening. Including multiple test strains would also indicate whether PE6-126 potentially produces a broad-spectrum antimicrobial compound with novel mechanisms of action as compared to previously characterized antimicrobial compounds (Tulp and Bohlin, 2005). As such, 4 indicator strains were used for screening: *B. cereus, E. coli* 1699, *S. epidermidis* and *P. putida* using a well diffusion assay. Out of the 4 bacterial strains tested, the extract from PE6-126 showed antibacterial activity against *B. cereus* and a zone of clearance was observed around the well inoculated with the extract (**Table 2.5**). Similarly, other studies have demonstrated anti-*Bacillus* activity by marine bacteria. For instance, Klein (2015, unpublished data) demonstrated the bioactivity of the marine bacteria PE8-15 against *Bacillus cereus* and other test strains (Klein, 2015). Anand and colleagues (2006) also showed the antimicrobial activity of the marine *Bacillus* sp. (Strain SC3) ethyl acetate extract against *B. subtilis*, *E. coli* and *C. albicans* with zones of inhibition. In this study *E. coli* 1699 was also included as an indicator strain since it is engineered to be a multi-drug resistant strain against 52 known antibiotics (**Appendix C**). Therefore, bioactivity against this strain would indicate that the bioactive compound produced by PE6-126 potentially has a different mode of action (Hentschel *et al.*, 2001). Although PE6-126 showed antibacterial activity against the indicator strain *E. coli* 1699, the antibacterial activity was not consistent, which might be due to the low concentration of the bioactive compound in the extract and therefore it was not detected in the bioassay. It would however be worthwhile to investigate whether a higher concentration of the extract would yield different results. Based on these antimicrobial activity results, *B. cereus* was selected as a test strain for the rest of the study.

Table 2.5: Antimicrobial activity of PE6-126 extract on 4 bacterial indicator strains.

Indicator strain	**Antimicrobial activity**
P. putida	-
B. cereus	+
E. coli 1699	-
S. epidermidis	-

+ represents a positive result, - represents a negative result.

2.4. Conclusions

This study has demonstrated the antimicrobial potential of marine bacteria as possible sources for natural product discovery. From the dual inoculation assay, the marine bacteria showed mycelial growth inhibition of olive trunk pathogens. However, due to little/no antifungal activity observed in the well diffusion assay from fermentation extracts, the obtained result was inconclusive. The possible explanation for the loss of antifungal activity can be attributed to several factors including, switching of cultivation conditions, residual degradation of the bioactive compound after inoculation into the well or it might be due to low concentration of the extracted bioactive compound. Therefore, in an attempt to determine if concentration of the bioactive compound was a factor, scaled up fermentation and extraction of PE6-126 was performed (**Chapter 3**). PE6-126 also demonstrated activity against *B. cereus*, showing that it has the potential of either producing a broad-spectrum antimicrobial agent or several antimicrobial compounds. Therefore, further purification of the antimicrobial compound/s was performed (**Chapter 3**) using a bioassay guided fractionation approach coupled to LC-MS. Genome mining of PE6-126 was also conducted to assist with dereplication and assess the secondary metabolite capacity of the bacterium.

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Chapter 3 - Bioassay and genome guided isolation of an antimicrobial compound from PE6-126

3.1. Introduction

In drug discovery, the utilization of a traditional bioassay guided fractionation of microbial extracts linked to chromatographic separation techniques, has effectively led to the isolation of bioactive compounds (Rakshith *et al.*, 2016). However, frequent rediscovery of known or uninteresting compounds remains a major challenge (Duarte *et al.*, 2012; Rakshith *et al.*, 2016; Naughton *et al.*, 2017). In the last number of years, analytical and bioinformatics-based approaches have improved the rate of microbial natural product drug discovery through de-replication of compounds and/ or bioactive strains (Xiong *et al.*, 2013). The advances and affordability of genome sequencing technologies has increased the number of publicly available bacterial genome datasets (Naughton *et al.*, 2017). This has enabled genome sequence analysis and mining of the biosynthetic gene clusters for the strain we had in our collection. This approach allows identification of the secondary metabolite pathways which may be responsible for producing novel metabolites (Naughton *et al.*, 2017).

In this chapter, whole genome sequencing followed by genome mining was used in an attempt to identify the secondary metabolite pathways responsible for antimicrobial activity against the indicator test strain *B. cereus*. In parallel, bioassay guided fractionation coupled to LC-MS was used to isolate the antimicrobial compound, using genome mining data as a guide.

3.2. Materials and Methods

3.2.1. Siderophore assays

In order to assay the ability of the bacterium to produce a siderophore, the iron binding capacity of the marine bacterial isolate (PE6-126) was determined using Chrome Azurol Sulfonate (CAS) agar (**Appendix A8**) blue solution according to Schyn and Neilands (1987). The bacterial culture was streaked in the centre of a CAS plate and incubated at 23°C until a colour change was observed (after 4-5 days of incubation). A positive reaction was revealed by a colour change from blue to orange in the CAS–iron complex plate.

3.2.2. Whole genome sequencing and mining of PE6-126

3.2.2.1. Genomic DNA extraction

The bacterial isolate PE6-126 was cultured on ZBA agar plates and incubated as mentioned in **section 2.2.1** until growth was observed (48 hours). Genomic DNA was extracted from a loopful of cells scraped from the ZBA agar plate, the extraction was performed using a modified version of the method by Wang and colleagues (1996). The following modifications

were made: lysozyme concentration was increased to 25 mg/mL, 0.2 mg/mL (final concentration) of proteinase K (Thermo Fisher Scientific, SA) was added to the lysis buffer, and cells were incubated overnight at 37°C in the lysis buffer. A loopful of cells were scraped from the plate and re-suspended in 400 µL of lysis buffer (25 mM Tris-HCL pH8, 50 mM glucose, 10 mM EDTA, 25 mg/mL lysozyme, 20 mg/mL proteinase K) and incubated at 37°C overnight. Sodium dodecyl sulphate (SDS) was added to a final concentration of 1% and incubated at 65°C for 30 min. An equal amount of Phenol: Chloroform: Isoamyl alcohol (PCI) (25:24:1) was added and mixed by inverting the tube several times and then centrifuged at 18928 x *g* for 10 min. Following centrifugation, the top aqueous phase was transferred to a new tube and the P: C: I step repeated three times until the solution and the interface was completely clear. Thereafter, the aqueous phase was transferred to another tube and DNA was precipitated through the addition of 0.6 volumes of ice-cold isopropanol and 0.1 volumes of 3.0 M sodium acetate pH 5.2. The tubes were incubated at -20°C overnight and were centrifuged at 18928 x *g* for 10 min. The supernatant was discarded, and the resulting pellet was washed with 1 mL of ice cold 70% ethanol. The ethanol was discarded and the DNA pellet air dried (Wang, Zhang and Ruan, 1996). The pellet was then re-suspended in 100 µL TE buffer containing 100 µg RNaseA/mL (Thermo Fisher scientific, USA).

3.2.2.2. Qualitative and quantitative DNA analysis

The concentration and purity of the extracted DNA was assessed based on the 260/280 nm ratio. This was determined using the NanoDrop® ND-1000 (NanoDrop technologies, Inc., USA). Agarose gel electrophoresis was used to determine the quality of DNA and was prepared at a concentration of 1% (w/v) in 1 x TAE buffer (40 mM Tris, 5 mM Sodium acetate and 0.9 mM EDTA, pH 7.9). Ethidium bromide staining solution (0.5 µg/mL) (Sigma Aldrich Chemical Company, Deissenhof Germany) was added to the agarose gel for visualising DNA. Thereafter, 6 x DNA loading buffer (DNA tracking dye) was added to the DNA samples and electrophoresis performed in 1 x TAE running buffer at 120 Volts for one hour. The size of DNA was estimated using a molecular weight marker (Phage lambda DNA digested with PstI endonuclease). Thereafter, the gel was visualised under UV light using the digital imaging system Alpha Imager® HP 2000 (Alpha Innotech, USA).

3.2.2.3. Whole genome sequencing, assembly and analysis

The extracted amount (0.575 ng) of gDNA from PE06-126 was sequenced at the IMBM sequencing facility using the Illumina Miseq platform. Following sequencing, the raw

sequence reads were provided in FASTQ format and uploaded on CLC Genomics workbench version 11.0.1. The forward and reverse sequence read files were then paired to create paired end read pairs. These were used to perform a *de novo* assembly using default settings to produce 141 contigs. Phylogenetic analysis of the PE6-126 genome sequence was performed against other genomes using the MiGA database. MiGA genome database determines the closest relatives and most likely taxonomic classification and novelty rank (http://microbial-genomes.org/) (Rodriguez-R *et al.*, 2018).

3.2.2.4. Genome mining for secondary metabolite pathways and genome annotation

In order to identify secondary metabolite biosynthetic gene clusters in the genome sequence of PE6-126, the assembled contigs were queried in the antiSMASH Version 4.1.0. Pipeline (Antibiotics and Secondary Metabolite Analysis Shell) and the analysis was performed for bacterial DNA using the parameters; KnownClusterBlast, smCoG analysis, Active SiteFinder, SubCusterBlast. antiSMASH identifies the biosynthetic clusters that cover a wide range of known secondary metabolites classes (Blin *et al.*, 2019). Parallel to this, the NCBI blast web server (www.ncbi.nlm.nih/BLAST) was used for searching homologous classes of known gene clusters involved in the biosynthesis of the secondary metabolites. The genome was then further annotated using the RAST (Rapid Annotation using Subsystem Technology) server (http://rast.nmpdr.org/)

3.2.3. Bioassay guided isolation and characterization of the antimicrobial compound from PE6-126.

For the bioassay guided fractionation, 2 bioassay methods; well diffusion and 96 well plate assay were used to assess bioactivity of the extract and the chromatographic fractions from PE6-126. Both of these methods are suitable for performing antimicrobial tests. The initial evaluation of antimicrobial activity was performed using the well diffusion assay however, since the 96 well plate is a more sensitive method which also uses less extract, it was used to assay the fractions.

3.2.3.1. Size exclusion fractionation of the extract with antimicrobial activity

To separate the antimicrobial molecule in the extract from other contaminants and determine the relative size of the compound, the extract re-suspended in 60% methanol was first filtered using a 0.45 µm membrane filter and further size fractionated using centrifugal filter units (Amicon® Ultra 15 mL, Millipore Corporation) with molecular weight cut-offs of 50 kDa and

3 kDa respectively. One mL of the antimicrobial extract was first added to a 50 kDa cut-off filter and the tube was centrifuged at 4000 x *g* for 10 min. The process was repeated twice and 60% methanol was included between the centrifugation times for elution. The collected filtrate was then applied to the 3 kDa cut-off filter and was centrifuged at 4000 x *g* for 20 min. Fractions were collected, dried down and tested for antibacterial activity against *B. cereus* using a 96 well plate assay. For antimicrobial testing, the fractions were re-suspended in 20% DMSO and control wells were inoculated with 50 µL of 20% DMSO and 50 µL of the bacterial culture.

3.2.3.2. Fractionation of the extract using high performance liquid chromatography (HPLC)

To purify the antimicrobial compound from the ethyl acetate extract, HPLC was performed. The chromatographic separation was performed on a Dionex Ultimate 3000 HPLC machine (Thermofischer Scientific, USA) and a computer which uses Chromeleon software for analysis of the HPLC data obtained. A Phenomex kinetex C18 column (diameter 250 µm x 10 µm; 100 Å pore size) was used for separation at a temperature of 30°C. The mobile phase consisted of Millipore water with 0.01% vol/vol Triflouroacetic Acid (TFA) (Eluent A) and 100% methanol (0.01% TFA vol/vol) (Eluent B). The run conditions for the linear ramp gradient were as follows; 0% - 5% eluent B from 0 - 20 min, 5% - 20% eluent B from 20 min - 40 min, 20% - 70% eluent B from 40 min - 70 min, 70% - 100% eluent B from 70 min - 90 min, then finally back to 5% eluent B until 100 min. The injected volume was 100 µL at a flow rate of 2 mL/ min and as the sample eluted from the separation column it was detected by UV-Vis at wavelengths ranging from 220 nm to 540 nm. The eluting fractions were then collected (10 mL) and dried down to evaporate the methanol. The fractions were re-suspended in 20% DMSO and tested for bioactivity using the assay mentioned in **section 3.2.3.3**.

For re-fractionation, all the fractions that gave bioactivity from the first HPLC run were pooled together and re-injected on the HPLC for further fractionation using the same separation column. However, variations were made in the gradient of the solvent mixtures (water and methanol). A multistep gradient was employed according to the following gradient ratios; 65% of eluent B (methanol) for 15 min, 70% of eluent B for 20 min, 80% of eluent B for 20 min, 90% of eluent B for 20 min, 100% eluent B for 60 min and then finally back to 80% eluent B for 5 min.

3.2.3.3. 96 well plate assay

In order to evaluate the antimicrobial activity of the PE6-126, fractions generated from amicon size exclusion filters and HPLC (**Section 3.2.3.1 and 3.2.3.2**) were tested using this assay. In this assay, the most susceptible indicator strain (*B. cereus*) to the extract was cultivated as indicated in section **2.2.1** and diluted to an OD (600 nm) of 0.02. Fifty microliters (50 µL) of the indicator strain inoculum was incubated with 50 µL of the extract at 10X dilution. Chloramphenicol (Sigma Aldrich) was used as a positive control (at concentrations of 2.125; 4.25; 8.5; 17 and 34 µg/mL) in LB broth. Since the extracts were re-suspended in 20% DMSO, control wells were inoculated with 50 µL of 20% DMSO and 50 µL of the bacterial culture. Plates were wrapped with a breathable film to prevent contamination and incubated on a shaking incubator at 37°C overnight. To determine the amount of microbial growth absorbance measurements at OD (600 nm) were determined on a Spectrostar® Nano plate reader (BMG LABTECH). The absorbance of the culture was measured prior to incubation and again after overnight incubation. The values were subtracted from one another to give the optical density increase following incubation. The relative percent growth of *B. cereus* was calculated according the following formula:

Relative (%) growth = (A/B) x 100

Where A is the OD (600 nm) value of the well inoculated with microbial culture and extract and B is the OD (600 nm) value of the growth control well inoculated with 20% DMSO and the bacterial culture.

3.2.3.4. Sensitivity of extract to enzymatic degradation and heat treatment

To determine the nature of the antimicrobial compound/(s), the ethyl acetate extract obtained from the culture supernatant (**Section 2.2.3**) was subjected to proteolytic digestion and heat treatment. Thereafter, it was assessed through assaying for loss of activity using the well diffusion assay (**Section 2.2.4**). Proteolytic digestion was conducted by adding proteinase K (Sigma Aldrich) to a final concentration of 1 mg/mL to the extract and incubating the mixture at 37°C for 1 hour. Controls included extract on its own incubated at 37°C, extract mixed with proteinase K reaction buffer, reaction buffer mixed with proteinase K and proteinase K mixed with DMSO. The stability of the extract after heat treatment was also investigated. One hundred microliters (100 µL) of the extract was incubated for 15 min each at 37, 60 and 100°C in a water bath. The bioactivity of the heat-treated extracts was then tested against *B. cereus* using the well diffusion assay.

3.2.3.5. LC-MS of the fractions with antimicrobial activity against *B. cereus*

The antimicrobial fraction was analysed using LC-MS at the Central analytical services (CAF) Mass Spectrometry unit, Stellenbosch University. Each fraction was dissolved in 1 mL methanol (ROMIL Pure Chemistry, Cambridge) and diluted 10-fold in methanol. Two µL of the fraction was injected into a stream of methanol at a flow rate of 0.35 mL/min. The chromatographic separation was performed on a Waters BEH C18, 2.1x100 mm Column on Waters Ultra Pressure Liquid Chromatography (UHPLC) (Waters, Midford, USA) which conveyed the sample to a Waters Synapt G2 quadrupole time-of-flight (QTOF) mass spectrometer for high resolution accurate mass analysis. The gradient of the eluent was as follows: 100% A (0, 50 min); 78% (6, 0 min); 56% (11, 0 min); 0% (13-16 min). The mobile phase consisted of 0.1% formic acid in water (A) and in acetonitrile (B). The mass spectrometer instrument was operated with an electrospray ionization (ESI) probe and acquired in positive mode. The data was acquired in scan mode at a cone voltage of 15 V, nitrogen was used as a de-solvation gas at 650 L/hr and de-solvation temperature of 275°C.

3.3. Results and Discussion

3.3.1. Whole genome sequencing, annotation and analysis

Due to the observed antimicrobial activity of PE6-126 against *Bacillus cereus and E. coli* 1699, the genome of PE6-126 was sequenced and analysed in an effort to establish the secondary metabolite biosynthetic capacity of the isolate and to assess its potential to be producing a novel antibacterial compound. *De novo* assembly of the PE6-126 genome resulted in 141 contigs with N50 value of 85249 bp. The size of the genome was 5,652,742 bp, with a GC content of 35.1% and 99 RNA's (**Table 3.1**). Out of the 100 copies of RNA, 92 tRNA and 8 rRNA were present in the genome. The RAST annotation of the assembled contigs predicted a total of 5298 protein coding sequences (CDS) and were assigned to 497 subsystems. Among the subsystem feature counts, there were 8 features involved in the synthesis of secondary metabolites (**Figure 3.1**).

Table 3.1: General features of PE6-126 genome.

Genome Property	**Value**
GC-Content	35.1%
Genome size	5,652,742
Number of contigs	109
Number of coding systems	5298
Number of subsystems	497
Number of RNA's	99
N50	85249
L50	50

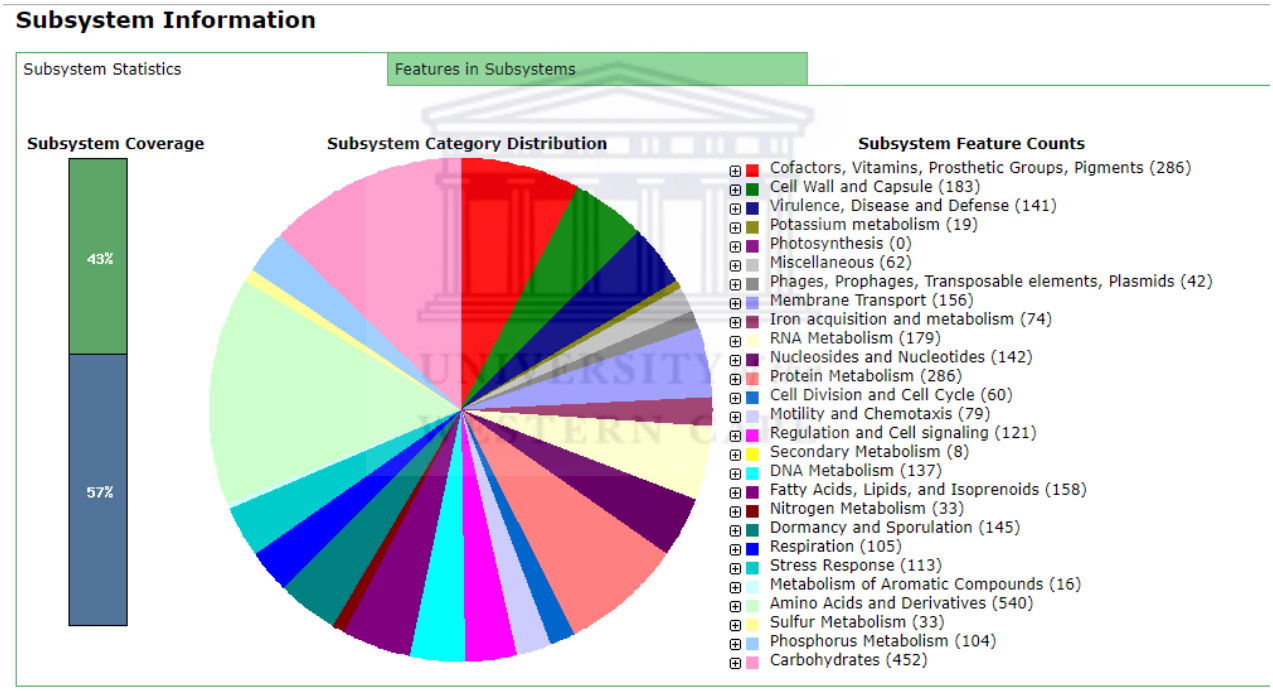

Figure 3.1: RAST annotation of the PE6-126 genome. The pie chart represents the subsystem category distribution according to their colour codes.

The genome sequence of PE6-126 was deposited to the Microbial Genomes Atlas (MiGA) web server for phylogenetic identification of this strain. MiGA offers taxonomical classification of a query genome or assembled contiguous sequences based on their Average Nucleotide Identity (ANI) (Rodriguez-R *et al.*, 2018). It represents the average nucleotide identity of orthologous genes shared between 2 genomes and resolution between closely related species (Arahal, 2014;

Rodriguez-R *et al.,* 2018)**.** The MiGA tool uses a reference database such as NCBI Refseq which consists of 1927 high quality genomes, and also makes use of the NCBI Genome database_Prok (NCBI Prok) which consists of 11487 genomes from 3921 species (Rodriguez-R *et al.*, 2018). The result obtained from MiGA showed that PE6-126 belonged to the genus *Bacillus* (Phylum-Firmicutes) and had the highest homology to *Bacillus cereus,* with a similarity of 99.89% (accession number NZ_CP009605.1). The closest relatives (>95% ANI) of PE6-126 according to ANI are presented in **Table 3.2**.

The presence of a low GC content is a characteristic of some members of the *Bacillus* genus within the Firmicutes phylum (Bohlin *et al.*, 2017). The genomic properties of PE6-126 such as genome size range, GC content and CDS was in accordance with the ones reported for other *Bacillus* strains (Klein, 2015; Zhu *et al.*, 2016; Lopes *et al.*, 2017). The *Bacillus* genus represents a group of Gram-positive rod species found in a variety of environments (Mondol, Shin and Islam, 2013). This study also found that *Bacillus* strains closely related to PE6-126 were widely distributed in different environmental niches (**Table 3.2**). It has been suggested that their diverse physiological properties and ability to form endospores, is the reason why they survive in different environments (Nicholson *et al.*, 2000). The bacterial isolate PE6-126 was isolated from a marine tunicate, other studies have similarly reported the association of *Bacillus* with marine invertebrates (Hentschel *et al.*, 2001; Anand *et al.*, 2006; Rath *et al.*,2011; Ayuningrum *et al.*, 2019).

Table 3.2: Closely related strains to PE6-126 based on ANI and their respective source of isolation.

Bacterial strain	**ANI (%)**	**Fraction of genome shared (%)**	**Accession number**	**Isolation source**
Bacillus cereus S2-8	99.89	94.68	NZ_CP009605	Soil
Bacillus cereus AH820	99.07	89.15	NC_011773	Diseased patient
Bacillus thuringiensis HD1011	99.03	81.47	NZ_CP009335	Unknown
Bacillus thuringiensis HD682	98.97	91.2	NZ_CP009720	Unknown
Bacillus cereus ISSFR-3F	98.73	89.15	CP_018931	Air
Bacillus thuringiensis serovar monterrey BGSC 4AJ1	98.69	75.27	NZ_CM000752	Unknown
Bacillus anthracis str. Ames	98.65	90.76	NC_003997	Dead cow
Bacillus cereus MH19	98.26	87.36	CP_039269	Soil
*Bacillus thuringiensis BM-*BT15426	98.25	86.04	NZ_CP020723	*Lactococcus* culture
Bacillus thuringiensis serovar andalousiensis BGSC 4AW1	98.22	84.91	NZ_CM000754	Unknown
Bacillus thuringiensis 97-27	98.19	86.33	NZ_CP010088	Human tissue
Bacillus sp. *HBCD sjtu*	98.18	86.75	NZ_CP025122	Waste water
Bacillus thuringiensis serovar pulsiensis BGSC 4CC1	98.16	78.9	NZ_CM000757	Grain field
Bacillus anthracis MCCC 1A02161	98.01	88.7	NZ_CP031642	Marine sediment
Bacillus cereus Rock3 42	97.86	88.7	NZ_CM000732	Soil
Bacillus cereus biovar anthracis str. CI	97.82	84.51	NC_014335	Deceased wild chimpanzee
Bacillus thuringiensis str. Al Hakam	97.67	86.55	NC_008600	Bioweapon facility

Bacillus cereus F83776	97.61	86.71	NC_ 016779	Contaminated prostate wound
Bacillus cereus D17	97.57	82.61	NZ_CP009300	Unknown
Bacillus cereus 03BB102	97.57	84.85	NZ_CP009318	Human blood
Bacillus cereus 03BB108	97.32	77.85	NZ_CP009641	Dust
Bacillus cereus BGSC 6E1	97.3	82.57	NZ_CM000716	Unknown
Bacillus subtilis HDZK-BYSB7	97.24	87.07	CP_026608	Bark of cypress
Bacillus cereus E33L	97.16	80.61	NC_006274	Zebra carcass
Bacillus anthracis MCCC 1A01412	97.03	86.31	NZ_CP031643	Marine sediment
Bacillus cereus CC1	95.44	83.98	NZ_CP023179	Marine sediment
Bacillus cereus m1293	95.32	84.68	NZ_CM000714	Cream cheese
Bacillus cereus Q1	95.23	80.32	NC_011969	Deep-subsurface oil reservoir
Bacillus sp. ABP14	95.22	86.54	NZ_CP017016	Compost
Bacillus cereus ATCC 10987	95.09	86.96	CP_026375	River
Bacillus cereus ATCC 4342	95.07	86.3	NZ_CM000721	Unknown
Bacillus sp. FDAARGOS 527	95.07	78.49	NZ_CP033795	Broviac blood
Bacillus cereus BDRD ST26	95.05	80.17	NZ_CM000724	Stock strain

All the strains represented here have >95% similarity to PE6-126.

3.3.2. Genome mining of PE6-126 for secondary metabolite pathways

In order to identify secondary metabolite biosynthetic gene clusters in the genome of PE6-126 and attempt to link the antimicrobial activity to the clusters, antiSMASH was used as a tool to mine the genome. This web-based tool is used for identification of gene clusters encoding biosynthetic pathways for secondary metabolites (Blin *et al.*, 2013). Based on antiSMASH analysis, a total of 9 biosynthetic gene clusters were identified for different types of secondary metabolite pathways (**Table 3.3**). Two bacteriocins, followed by 2 non-ribosomal peptides (NRPs) clusters and one each for siderophores, terpenes, sactipeptides, betalacone and LAP-bacteriocin. According to antiSMASH, most of the secondary metabolite pathways (bacteriocin, NRPS, LAP-bacteriocin) that were identified had very low (< 46% similarity at the nucleotide level) to no similarity to any known or characterized biosynthetic gene clusters, suggesting that these pathways may encode for synthesis of novel natural products or natural products with no characterized BGCs (**Table 3.3**). For two of these BGCs (sactipeptide and siderophore pathways), 100% of their genes showed similarity to the known and characterized biosynthetic gene clusters encoding thuricin CD and petrobactin respectively. In order to further characterize the unidentified bacteriocins that did not have similarity to any previously known BGCs from antiSMASH analysis, BAGEL was used in parallel. BAGEL (http://bagel.molgenrug.nl/) is a genome mining platform that specializes in the detection and annotation of bacteriocins (modified and non-modified) and Ribosomally synthesized and Post-translationally modified Peptides (RiPPs) (van Heel *et al.*, 2013). Similar to antiSMASH, BAGEL uses HMM to detect core RiPPs encoding genes, however, it is independent from ORF calling, and it is extended to cover more "novel" classes of post translationally modified peptides (van Heel *et al.*, 2013). A total of 4 putative biosynthetic gene clusters from PE6-126 were predicted by BAGEL, 3 of these predicted clusters were sactipeptides and 1 LAP (**Table 3.4**). From this analysis, it was observed that the number of clusters identified by antiSMASH as bacteriocins were lower than what BAGEL predicted. Previous studies have observed BAGEL to be more accurate in identifying the number of bacteriocins as compared to antiSMASH. In a study by Machado and colleagues (2015), it was demonstrated that antiSMASH predicted approximately 1-5 bacteriocin clusters in the genomes of the strains analysed, however when the genomes of those strains were analysed in Bagel 4, only a few bacteriocin hits were obtained (Machado *et al.*, 2015). However, these are bioinformatic predictions and still need to be experimentally verified in order to determine if these gene clusters are expressed by the organism.

Table 3.3: Biosynthetic gene clusters coding for secondary metabolite pathways in PE6-126 as identified by antiSMASH.

Region / contig nr	**Type**	**% similarity to known BGC**	**Most similar known cluster**	**From**	**To**	**(Nucleotide NCBI Blast)**
Contig 26	Bacteriocin	NS	NS	34,789	47,032	100% similar to *Bacillus cereus* JEM-2 complete genome accession number CP018935.1
Contig 30	NRPS	NS	NS	4,155	51,165	100% similar to *Bacillus cereus* S2-8 complete genome accession number CP009605.1
Contig 30	Bacteriocin	NS	NS	65,000	75,266	100% similar to *Bacillus cereus* MH19 chromosome, complete genome Accession number CP039269.1
Contig 32	LAP /bacteriocin	NS	NS	32,955	56,462	100% similar to *Bacillus anthracis* strain FDAARGOS_699 chromosome Accession number CP050973.1
Contig 36	Betalactone	40	fengycin	85,753	110,991	100% similar to *Bacillus* sp HBCD-situ chromosome, complete genome Accession number CP025122.1
Contig 42	Siderophore	100	petrobactin	60,102	68,879	100% similar to *Bacillus cereus* strain S2-8 complete genome Accession number

						CP009605.1
Contig 43	NRPS	46	bacillibactin	1	33,186	100% similar to *Bacillus cereus* strain S2-8 complete genome Accession number CP009605.1
Contig 51	Sactipeptide	100	thuricin CD	6,980	26,230	*Bacillus thuringiensis* DPC6431 thuricin operon, partial sequence Accession number HQ446454.1
Contig 79	Terpene	17	molybdenum cofactor	1	17,658	100% similar to *Bacillus cereus* strain S2-8 complete genome Accession number CP009605.1

Type - product type detected by antiSMASH; *NS- no similarity to known BGCs; From/to - location of nucleotide region in base pairs; Most similar known cluster blast - closest compound from the MiBIG database; similarity - a percentage of genes within the closest known compound that have a significant BLAST hit to genes within the current region.

Table 3.4: Proposed bacteriocin gene clusters of PE6-126 from BAGEL analysis.

Contig of interest	**Gene start position**	**Gene end position**	**Class**
Contig 32.12	36149	56149	Linear-azolin containing Peptides (LAP's)
Contig 51.31	4844	26229	thuricin_CD_beta
Contig 82.4	6491	26491	sactipeptides
Contig 4.90	39689	59689	sactipeptides

Contig of interest shows the predicted potential clusters responsible for production of bacteriocins and RiPPs. From/To- region on the genome sequence where the bacteriocin sequence is recognised; Class- represents classes of RiPPs.

Although PE6-126 showed a very high ANI (%) to the *Bacillus cereus* group, the reference strains related to PE6-126 were not studied in the context of secondary metabolite production. Therefore, these strains were analysed further to determine their biosynthetic gene clusters and compare with PE6-126. The genomes obtained from GenBank for species closely related to the bioactive strain PE6-126 were deposited to antiSMASH in order to determine the distribution and abundance of SM pathways. The results from the analysis showed that a total of 14 different classes of biosynthetic gene clusters were found (**Figure 3.2**). From these 14 classes, bacteriocin and NRPS pathways were the most abundant class of SMs, and were present in all the *Bacillus* genomes. In contrast, hybrid pathway LAP-bacteriocin, betalactone, siderophore and terpene pathways were moderately distributed in all species. LAP, lassopeptide and lantipeptides biosynthetic genes were the least abundant classes and were not present in all genomes. Hybrid pathways (NRPS/PKS and NRPS/LAP/bacteriocin), tRNA-dependent cyclodipeptide synthases (CDPS), aryl pyrone and sactipeptide pathways were only detected in *B. subtilis*, *B. anthracis* and *B. cereus* strains, with only 1 per strain (**Figure 3.3**). It was also observed that *B. cereus* contained the most diverse range of biosynthetic gene clusters in comparison to the other *Bacillus* species analysed. Although it is worthwhile to represent all the genomes for each species equally for this analysis, *B. subtilis* and *B. anthracis* were the least represented genomes since there was no GenBank files available, and for some genomes, antiSMASH could not detect any BGCs.

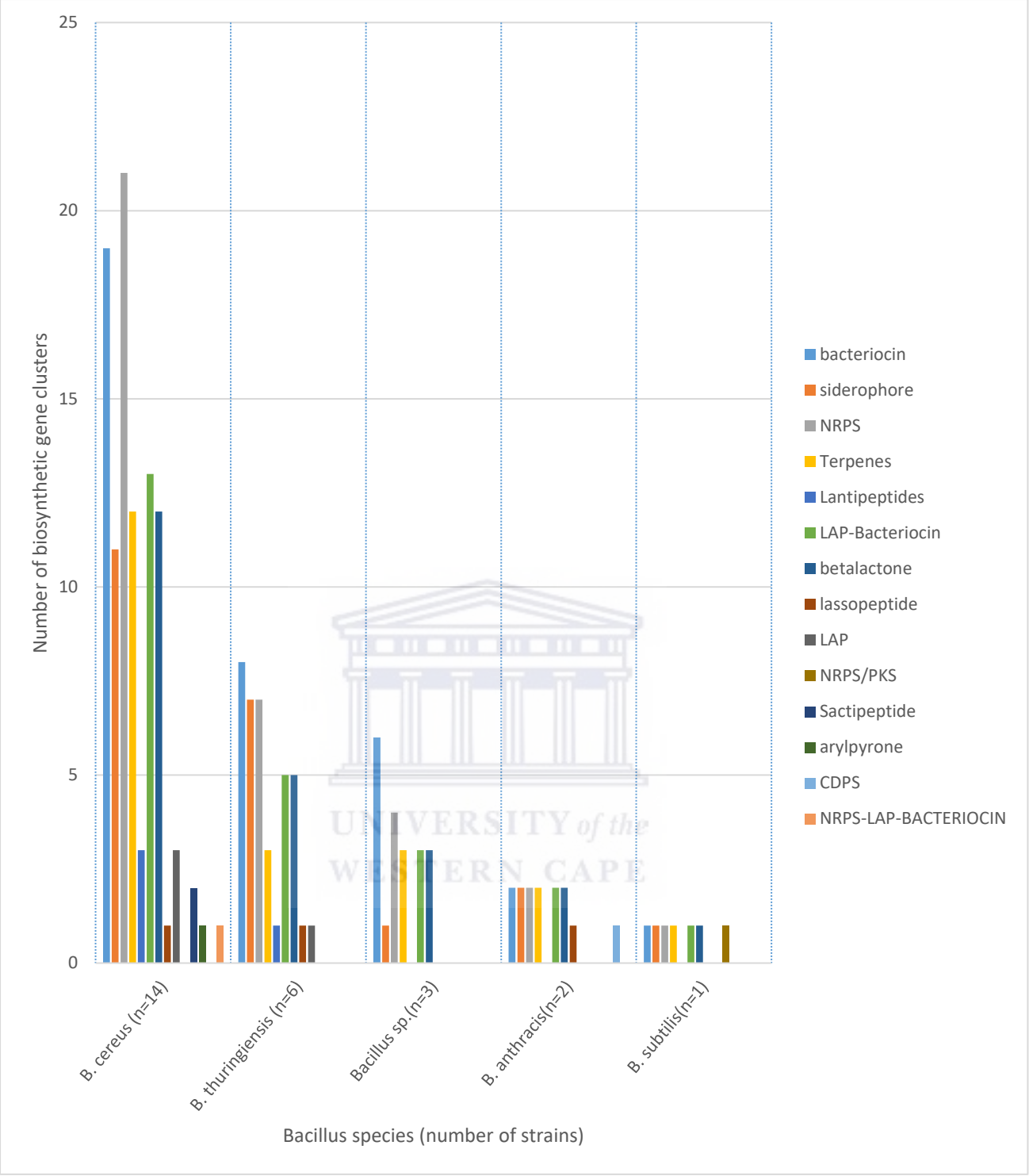

Figure 3.2: Qualitative analysis of the distribution of secondary metabolite classes in *Bacillus* strains closely related to PE6-126 as determined by ANI% (**Table 3.2**). BGCs were identified using antiSMASH 5.0 software. The number of strains analysed for each *Bacillus* species were as follows; *B. cereus*; n = 14, *B. thuringiensis*; n = 6, *Bacillus* sp. n = 3, *B. anthracis*; n = 2, *B. subtilis*; n = 1.

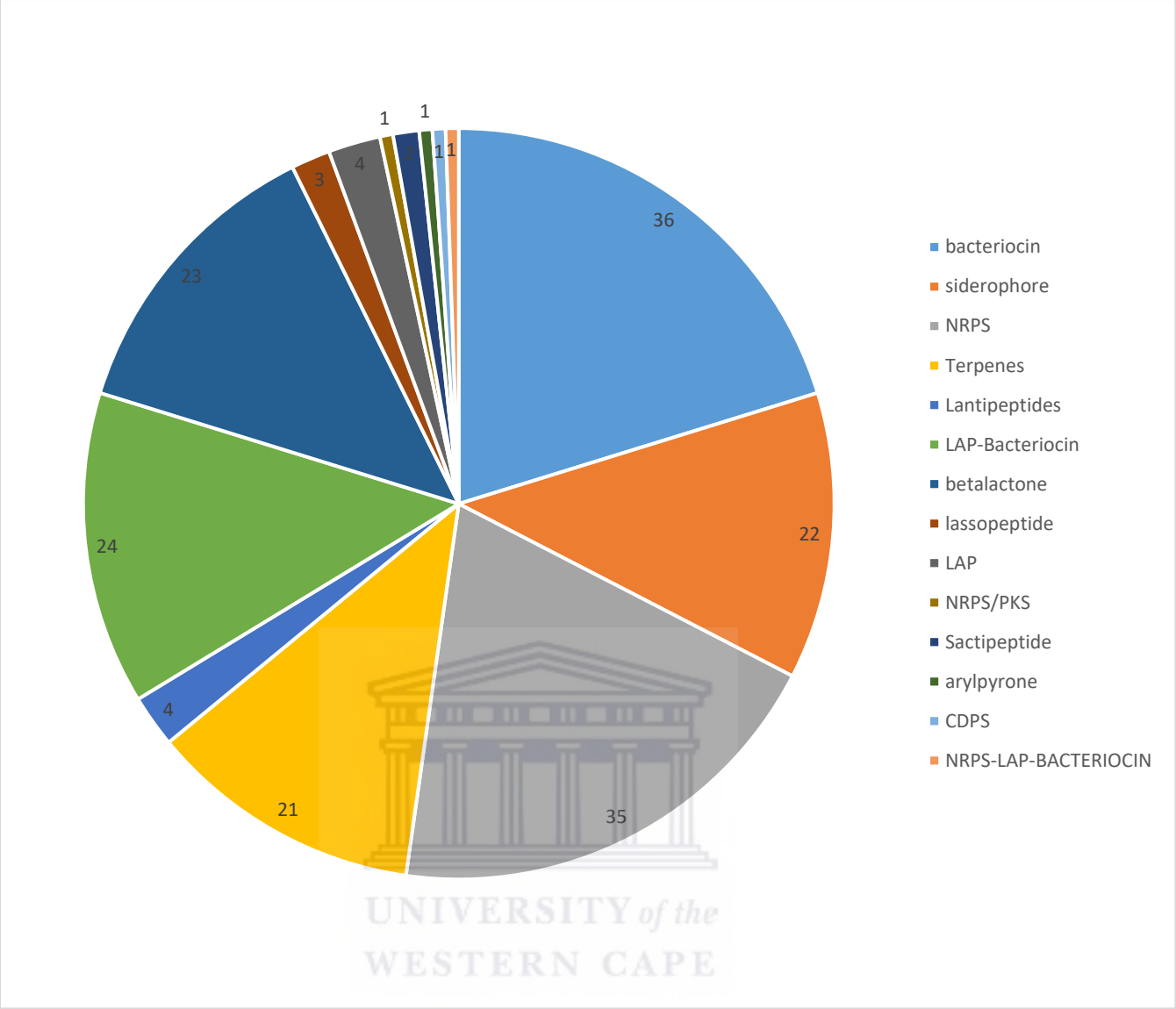

Figure 3.3: Abundance of the biosynthetic gene clusters present in the strains closely related to PE6-126 based on ANI% (**Table 3.2**). BGCs were identified using antiSMASH 5.0 software. The number of strains analysed for each *Bacillus* species were as follows; *B. cereus*; n = 14, *B. thuringiensis*; n = 6, *Bacillus* sp. n = 3, *B. anthracis*; n = 2, *B. subtilis*; n = 1.

A closer analysis of *B. cereus* strains revealed that sactipeptide, arylpyrone and CDPS BGCs were only present in these strains (**Figure 3.4**). Bacteriocins, NRPS, siderophore, terpenes, LAP-bacteriocin and betalactones were present in all *B. cereus* strains except *B. cereus biovar anthracis*. The *in silico* analysis of secondary metabolite abundance in PE6-126 was consistant with this finding. The sactipeptide, arylpyrone and NRPS-LAP-Bacteriocin were present in only *B. cereus* CC1 and *B. cereus* Rock3 respectively. The analysis showed that the majority of secondary metabolite genes within the analysed *B. cereus* genomes belonged to the same classes with similar abundance within the genomes. This could mean that secondary metabolite

genes of the same classes are evenly distributed within related members of the same species. In PE6-126, a sactipeptide gene cluster was predicted and 100% of these genes showed similarity to the thuricin CD gene cluster found in *B. thuringiensis* DPC 6431. From all the closely related strains to PE6-126, only one bacterial strain, *B. cereus* CC1 contained the sactipeptide gene cluster as predicted by antiSMASH (**Table 3.3**). However, this sactipeptide biosynthetic gene cluster in *B. cereus* CC1 showed that only 40% of genes were similar to the BGC of thurincin H. Both thuricin CD and thurincin H differ in their structure, with thurincin H being a single peptide with 4S-to-α-carbon bonds and thuricin CD being a 2-component linear sactipeptide (**Section 1.5.1.1.1**) (Mathur *et al.*, 2015). These sactipeptides are produced by *B. thuringiensis* and have a narrow antimicrobial spectrum against Gram-positive bacteria, including *B. cereus*. Although in this analysis PE6-126 was the only *B. cereus* strain with a sactipeptide gene cluster encoding for the thuricin CD pathway, other studies have also identified thuricin CD pathways in other *B. cereus* strains. This implies that this pathway was probably not recently acquired through horizontal gene transfer events, but rather by vertical gene transmission from the *B. cereus* group. In an *in-silico* study by Murphy and co-workers, fifteen novel thuricin CD-like gene clusters with putative bacteriocin structural gene/(s) located nearby were identified. Interestingly, this study also found that thuricin CD gene clusters were present in isolates from a variety of environments, suggesting that thuricin CD bacteriocin producers are widely distributed in different environmental niches (Murphy *et al.*, 2011).

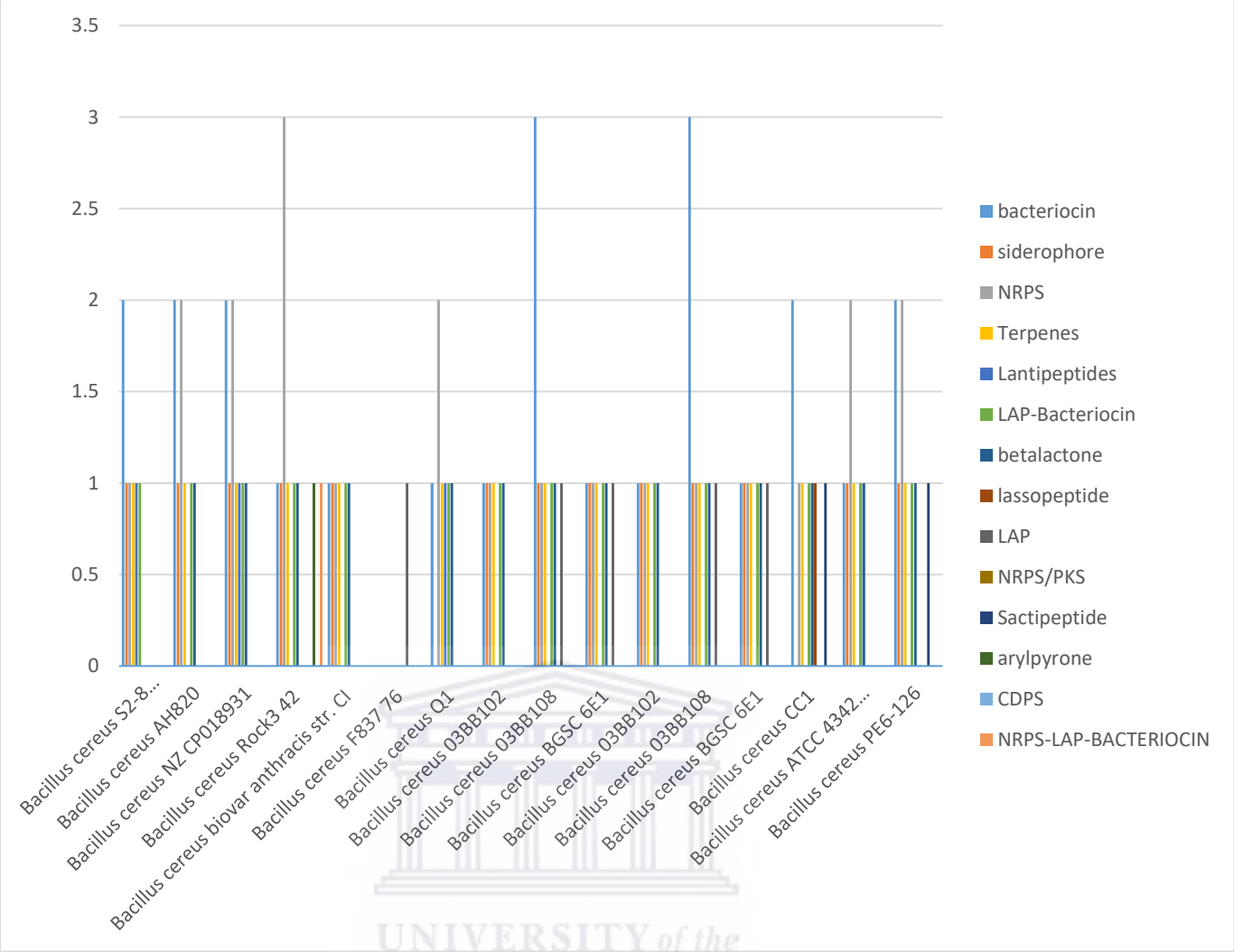

Figure 3.4: Analysis of biosynthetic gene clusters present in the 13 *Bacillus cereus* strains closely related to PE6-126 based on ANI% (**Table 3.2**). BGCs were identified using antiSMASH 5.0 software. The number of strains analysed for *B. cereus* species were n = 14.

In order to establish which gene cluster/(s) may possibly play a role in conferring antimicrobial activity of PE6-126, the clusters were analysed further and complemented with literature on previous antimicrobial activity shown by these clusters.

Bacteriocins are low molecular weight antimicrobial peptides which are produced by bacteria and are active against closely related species (**Section 1.5.1.1**) (Yang *et al.*, 2014). The bacteriocin gene clusters in PE6-126 predicted by antiSMASH were class 1 bacteriocins; sactipeptide and LAP. Thuricin CD is a ribosomally synthesized class 1 bacteriocin that contains post translationally modified peptides trnα and trnβ with unusual sulphur to α carbon linkages (**Section 1.5.1.1.1**) (Rea *et al.*, 2010). This peptide has known antimicrobial activity against bacterial pathogens including *B. cereus*. In this study, 1 sactipeptide gene cluster was

identified from the PE6-126 genome and 100% of their genes showed similarity to the thuricin CD pathway encoded by the *B. thuringiensis* DPC643 strain (**Figure 3.5**). The known antimicrobial properties of thuricin CD against *B. cereus* is the reason this gene cluster was hypothesized to be likely involved or responsible for the observed anti-bacterial activity against *B. cereus* (discussed and analysed further in **section 3.3.2.1**).

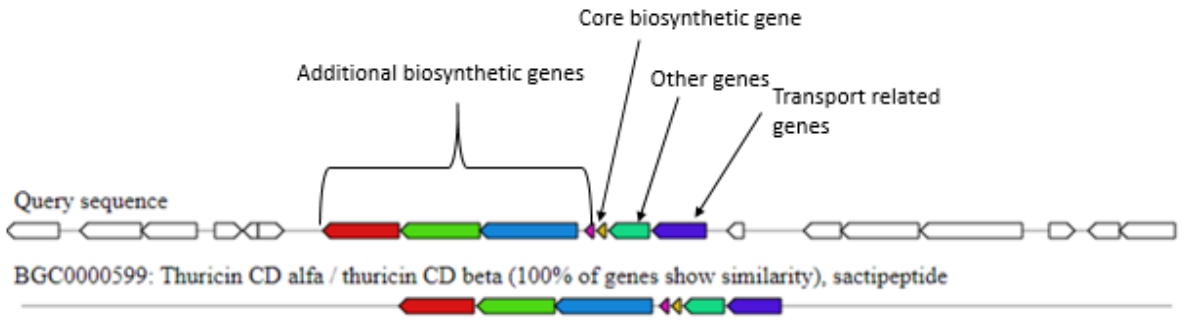

Figure 3.5: Comparison of the homologous sactipeptide gene cluster of PE6-126 (query sequence) and *Bacillus thuringiensis* DPC6431 (BGC0000599).

Linear azole containing peptides possess various combinations of heterocyclic rings of thiazole and methyl oxazole, derived from cysteine, threonine and serine residues (Arnison *et al.*, 2013) (**Section 1.5.1.1.2**). In this study, 1 putative gene cluster of a LAP/bacteriocin hybrid was identified in the genome of PE6-126. Furthermore, there were no genes which showed similarity to known or characterized BGCs, suggesting that the pathway has the potential to produce potentially novel compound/(s).

The lipopeptides in the *Bacillus* genus are grouped into 3 major cyclic compound families; fengycin, iturins and surfactants (Raaijmakers *et al.*, 2010; Meena and Kanwar, 2015). The difference between the 3 lipopeptide families is in the modification of their fatty acid moiety, both surfactins and iturin incorporate a β-hydroxy fatty acid, whereas fengycins incorporate a β-amino fatty acid. In this study, the betalactone gene cluster revealed that 40% of genes showed similarity to the secondary metabolite pathway encoding for fengycin lipopeptide. Fengycins, produced by several strains of *B. subtilis* are known for their antifungal activity against pathogenic filamentous fungi occurring in various agricultural crops (Raaijmakers *et al.*, 2010; Meena and Kanwar, 2015). It was therefore unlikely that this gene cluster was responsible for the observed antimicrobial activity against *B. cereus.* However, due to the

displayed antifungal activity of PE6-126 against olive trunk fungal pathogens in the initial screening, (**Chapter 2**), this cluster may potentially be one of the clusters responsible for the observed antifungal activity. This will need further experimentation to determine whether this gene cluster is expressed under laboratory conditions and tested again for validation of antifungal activity.

Siderophores are low molecular weight compounds which chelate iron with a high affinity (Lee *et al.*, 2007). Two biosynthetic gene clusters; NRPS and siderophore were predicted to encode for siderophores bacillibactin and petrobactin respectively. Petrobactin and bacillibactin are catecholate-type siderophores produced by members of the *Bacillus* genus (Koppisch *et al.*, 2005). Both of these siderophores have been isolated from the closely related species; *B. anthracis*, *B. cereus* and *B. thuringiensis* (Wilson *et al.*, 2006). This observation of siderophores being produced by similar bacteria is likely a result of high genome conservation of siderophore biosynthetic genes within species (Koppisch *et al.*, 2005). Siderophores can either be synthesized via a NRPS-dependant pathway or NRPS-independent mechanism (Miethke, Schmidt and Marahiel, 2008). It is noteworthy that although antiSMASH is capable of distinguishing siderophores based on their biosynthesis machinery, it still classifies NRPS dependant siderophores as a NRP (Schorn *et al.*, 2016). This observation was apparent in PE6-126 whereby the bacillibactin siderophore was predicted to be encoded by a NRPS gene cluster.

To complement the genome mining data obtained from antiSMASH, where 2 siderophore producing pathways were predicted, a CAS assay specific for siderophore detection was performed for PE6-126. From this experiment, the results showed a positive reaction of siderophore activity as indicated by a colour change of the media from blue to orange (**Figure 3.6**). The CAS assay is used for detecting siderophores and is based on their high affinity for ferric iron (Schwyn and Neilands, 1987; Louden, Haarmann and Lynne, 2011). In this assay, the CAS dye complexes with ferric iron and after inoculation and incubation of the bacterial strain on the plate, the iron which is bound to the CAS can be chelated by the siderophores produced by the bacterium (Louden, Haarmann and Lynne, 2011). This result was expected since PE6-126 is a marine bacteria and seawater has extremely low levels of iron (1-3 ppb) (Carvalho and Fernandes, 2010). As a result, marine bacteria are well-known for producing siderophores to chelate, solubilize and transport iron from the marine environment into the cell (Carvalho and Fernandes, 2010). Although the two putative siderophores (bacillibactin and petrobactin) were predicted, it was not possible to show whether only one or both were expressed and responsible for the detected siderophore activity. For this reason, other

biosynthetic gene clusters were analysed to determine if they could potentially be responsible for the observed antimicrobial activity against *B. cereus*.

Figure 3.6: Siderophore production by PE6-126 bacterial colony on a CAS plate. A colour change from blue to yellow indicates chelation of iron by the bacterium.

3.3.2.1. Sensitivity of extracts to enzyme degradation and heat treatment

As previously discussed, the antiSMASH results predicted that 100% of genes of the sactipeptide BGC were similar to the secondary metabolite pathway for thuricin CD (**Section 3.3.2**). Due to the known antimicrobial activity of thuricin CD against *B. cereus*, it was hypothesized that this gene cluster could be responsible for conferring the antibacterial activity against *B. cereus*. According to literature the biochemical nature of thuricin CD is as follows; heat stable up until 85°C, loses bioactivity at 100°C and susceptible to proteolytic digestion by proteinase K (Rea *et al.*, 2010). Therefore, as a first step to determine whether thuricin CD was expressed and responsible for the antimicrobial activity of PE6-126 against *B. cereus*, the extract from PE6-126 was tested for heat stability (up to 100°C) and susceptibility to proteolytic digestion by proteinase K. The results obtained from these treatments revealed that the antimicrobial activity of PE6-126 extract against *B. cereus* was retained (**Figure 3.7A and B**). This suggested that a different metabolite with anti-*B. cereus* activity was produced. Since the assay was only semi-quantitative, it would be difficult to conclude that thuricin CD is not expressed. Moreover, these results need to be interpreted with caution, because another factor

to consider is that DMSO (used to re-suspend the extract) might have inactivated the proteinase K, hence the antimicrobial activity was retained. Therefore, this result is inconclusive and further studies with more focus on proteolytic susceptibility of the extract should be performed to confirm the results. The strain *B. thuringiensis* DPC 6431, which is responsible for production of thuricin CD was cultured in brain heart infusion broth and thuricin CD was purified from the cell fee supernatant using XAD beads (Rea *et al.*, 2010). These cultivation and extraction conditions were not used in this study; therefore, it is necessary to determine whether the gene cluster is expressed in our experimental conditions.

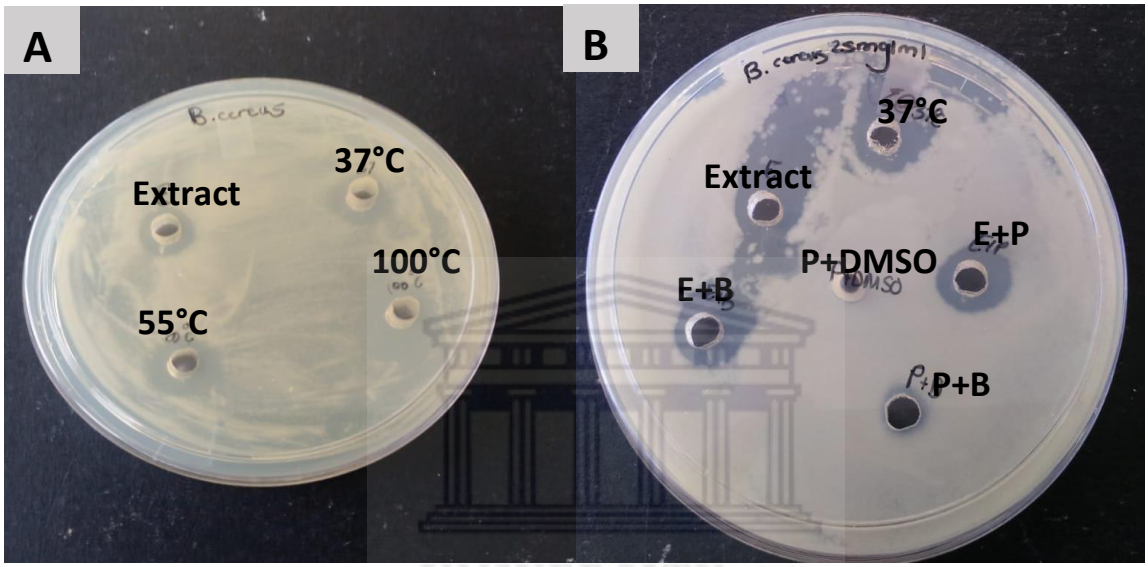

Figure 3.7: Antimicrobial activity of PE6-126 extract against *B. cereus* after treatment with (A) heat and (B) proteinase K. Zone of inhibition indicates the antimicrobial activity retained after treatment. In B, P+DMSO represents proteinase K and DMSO, E+B represents extract and buffer, E+P represents extract and proteinase K, P+B represents proteinase K and buffer.

Genome mining led to an increased understanding of the secondary metabolism of PE6-126, although at this point, it is not possible to evidently show the gene clusters responsible for conferring the antimicrobial properties of PE6-126. It was interesting to note that although the genome sequence analysis of PE6-126 revealed that it has 99% ANI to *B. cereus* (**Section 3.3.1**), which is a well-studied organism, antiSMASH predicted some biosynthetic gene clusters with no similarity to characterized or known gene clusters. This means that PE6-126 has the potential to synthesize novel secondary metabolites. Due to the antimicrobial activity of PE6-126 against *B. cereus* and antiSMASH analysis, the data suggested that the antimicrobial compound being produced is possibly a thuricin CD because it has well known activity against *B. cereus*. Since thuricin CD has no known activity against Gram-negative

bacteria (Rea *et al.*, 2010), it would suggest that the activity PE6-126 also displayed against the multi-drug resistant Gram-negative *E. coli* 1699 strain was conferred by another antimicrobial compound. The only other secondary metabolite predicted with high confidence to be encoded by this strain is petrobactin (100% sequence similarity). However, as far it is known, petrobactin has no antimicrobial activity against *E. coli*. Therefore, in this study it would not have been rational to dereplicate and discard this strain for further chemical analysis based only on the 16S rRNA identification and genome data. It is important to keep in mind that antiSMASH does not identify all secondary metabolite genes and pathways (Blin *et al.*, 2019). For example, rhamnolipids are biosurfactants with known antibacterial activity, yet they are not detected by antiSMASH. Other examples of gene clusters not detected by antiSMASH include prodigiosin, alkaloids, streptides and oligosaccharides (Medema *et al.*, 2011; Cimermancic *et al.*, 2014; Tedesco *et al.*, 2016; Blin *et al.*, 2019; Timmermans *et al.*, 2019). Whether the anti-*E. coli* activity is encoded by one of the novel pathways predicted by antiSMASH has yet to be determined. Therefore bioactivity-guided fractionation was pursued in the next section to identify whether PE6-126 produced thuricin CD and/or other potentially novel secondary metabolites.

3.3.3. Bioassay guided isolation and characterization of the antimicrobial compound

3.3.3.1. Size exclusion fractionation of the antimicrobial extract

To establish whether the anti-*B. cereus* compound was a small molecule, the antimicrobial extract was fractionated using filters with different molecular weight cut-off ranges. After fractionation of the crude extract according to size and testing for antimicrobial activity, the results showed that the antimicrobial activity was retained in MWF3 (fractions with molecular weight <3 kDa), containing compounds smaller than 3 kDa. MWF1(fractions with molecular weight >50 kDa) did not have any antimicrobial activity (100% growth) and MWF2 (fractions with molecular weight between 3<50 kDa) had very low antimicrobial activity (85% growth) with a high error rate to be considered as an active fraction (**Figure 3.8**). This result suggested that the antimicrobial compound is probably a secondary metabolite since they are low molecular weight compounds less than 3 kDa in size (Bérdy, 2005; Ruiz *et al.*, 2010).

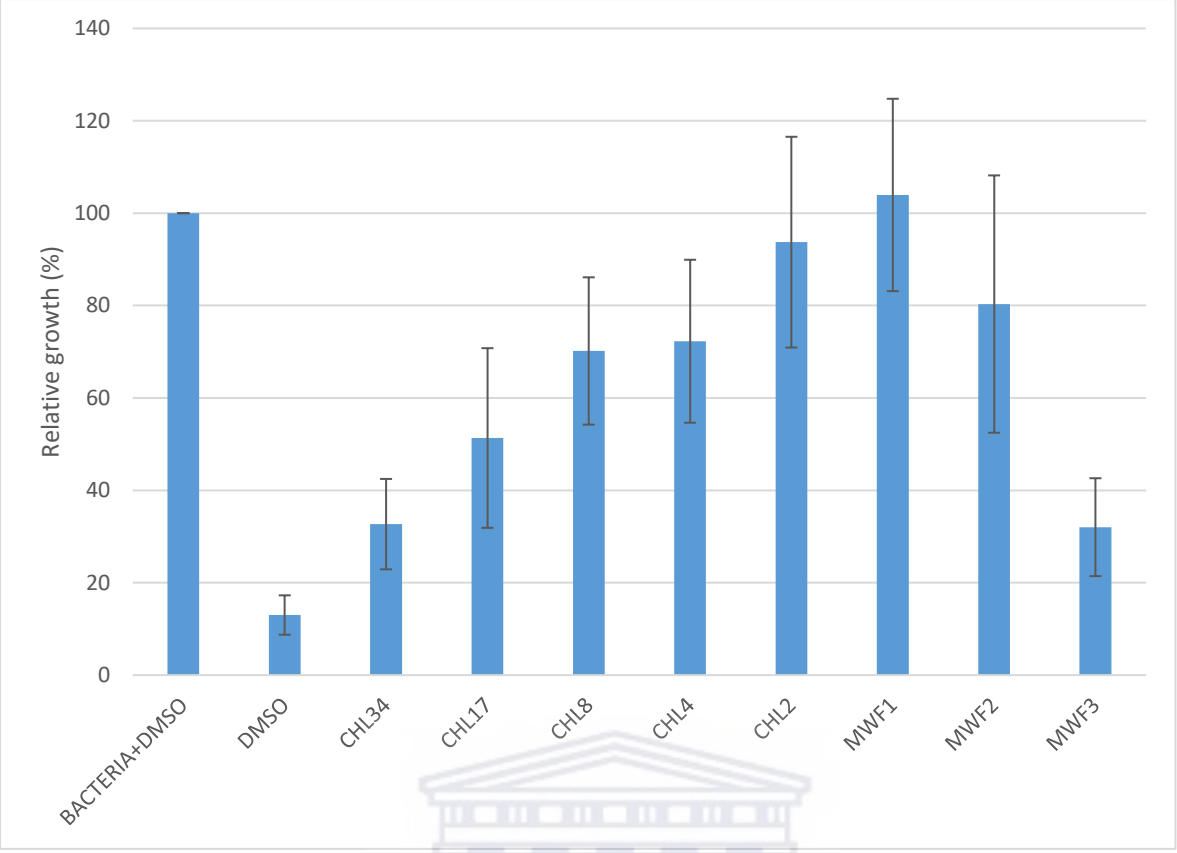

Figure 3.8: Histogram showing anti-*B. cereus* activity of PE6-126 fractions based on molecular weight. MWF1 represents fraction containing compounds greater than 50 kDa, MWF2 represents fractions containing compounds between 3 kDa and 50 kDa, MWF3 represents fraction containing compounds less than 3 kDa. Error bars represent the standard deviation of the mean from triplicate reads. Controls included 20% DMSO, bacteria inoculated with 20% DMSO and chloramphenicol (CHL) as an antibiotic control, CHL34 µg/mL, CHL17 µg/mL, CHL8.5 µg/mL, CHL4.2 µg/mL, and CHL2.1 µg/mL represents the concentrations of chloramphenicol used. Bacteria inoculated with DMSO served as a negative control (100% relative growth).

3.3.3.2. HPLC fractionation of the antimicrobial extract

The fraction which retained antimicrobial activity (MWF3) was subjected to HPLC in an attempt to isolate the antimicrobial compound. Fractionation of this extract yielded 19 fractions with several peaks of varying intensities (**Figure 3.9**).

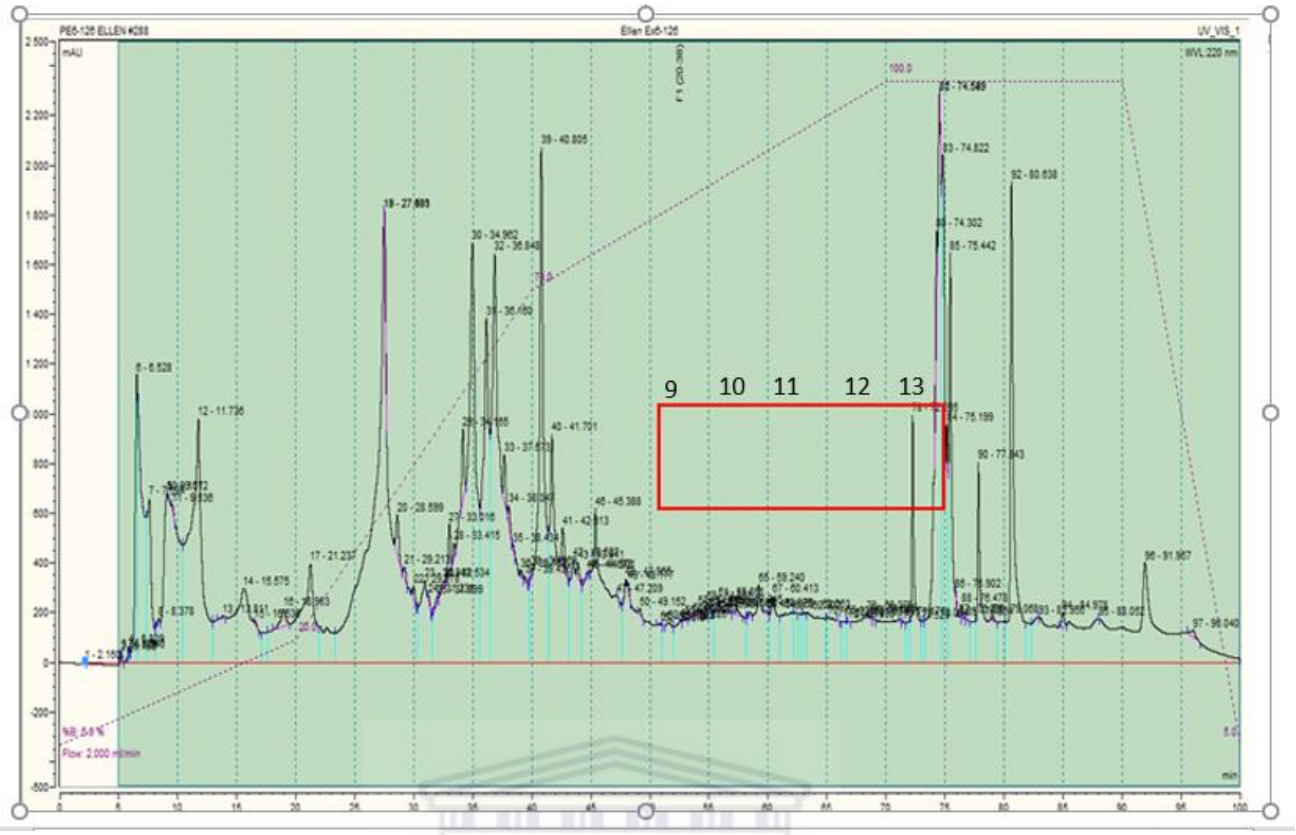

Figure 3.9: HPLC chromatogram of an ethyl acetate extract of PE6-126 on a C18 column detected at UV absorbance of 220 nm: Gradient elution: 0-20 min, 5% methanol-95% water; 20-40 min 20% methanol-80% water; 40-70 min, 70% methanol-30% water; 70-90 min, 100% methanol - 0% water; 95-100 min, 0% methanol-100% Water.19 fractions were obtained and collected every 5 minutes. All the fractions were tested for antimicrobial activity and in the red box are fractions (fraction 9 - fraction 13) which demonstrated antimicrobial activity against *B. cereus*.

Out of the 19 fractions collected and tested for antimicrobial activity using a well diffusion assay, 5 antimicrobial fractions conferring activity against *B. cereus* were identified. The 5 antimicrobial fractions eluted at a retention time (Rt) of 50 min until approximately 75 min. In addition, these compounds eluted when the methanol gradient was increased from 70% to 100%. Due to methanol being a polar solvent, this suggested that the compound/(s) in the fractions with antimicrobial activity could potentially have similar polarity properties, implying that they may be moderately hydrophilic in nature.

After confirmation of antimicrobial activity using the well diffusion assay (**Figure 3.10A**), the 96 well plate assay was used to assay the potency of the extract (**Figure 3.10B**). This method has the advantage of using less extract (Balouiri, Sadiki and Ibnsouda, 2016), therefore it was used throughout the study for the bioassay guided fractionation technique. The positive result

was observed on the ethyl acetate extract and the active fractions F10-F13 with relative bacterial growth of 33.92% and 17.61%, 20.81%, 18.57%, 33.97% respectively. Although fractions 8 and 9 initially showed activity in the well diffusion assay (**Figure 3.10A**), consistent activity was observed from fractions 10-13 in the 96 well plate assay. Since the 96 well plate assay is more sensitive than the well diffusion assay, these fractions (10-13) were therefore considered as positive for antimicrobial activity and prioritised. The low relative growth of the bacterial cultures inoculated with the fractions indicated that the fractions contained compounds that inhibited the bacterial culture from growing. The fractions which were inactive (F1, F2, F8, F9 and F14) were represented by wells that had relatively high bacterial growth (>50% growth). Therefore, they were disregarded and not analysed further. All the fractions that showed activity (F10-F13) were further fractionated using HPLC in an attempt to isolate the antimicrobial compound (**Figure 3.11A, B and C**). Prior to the second round of HPLC fractionation, the previous fractionation method was modified in order to find optimal conditions for separating each of the fractions with antimicrobial activity. According to literature, optimization of HPLC fractionation can be achieved by changing the column type, temperature or changing the percentage of mobile phases (Snyder, 1997). In this study, in order to improve fractionation resolution, the active fractions were further separated using a methanol multistep gradient elution from 65% to 100% methanol.

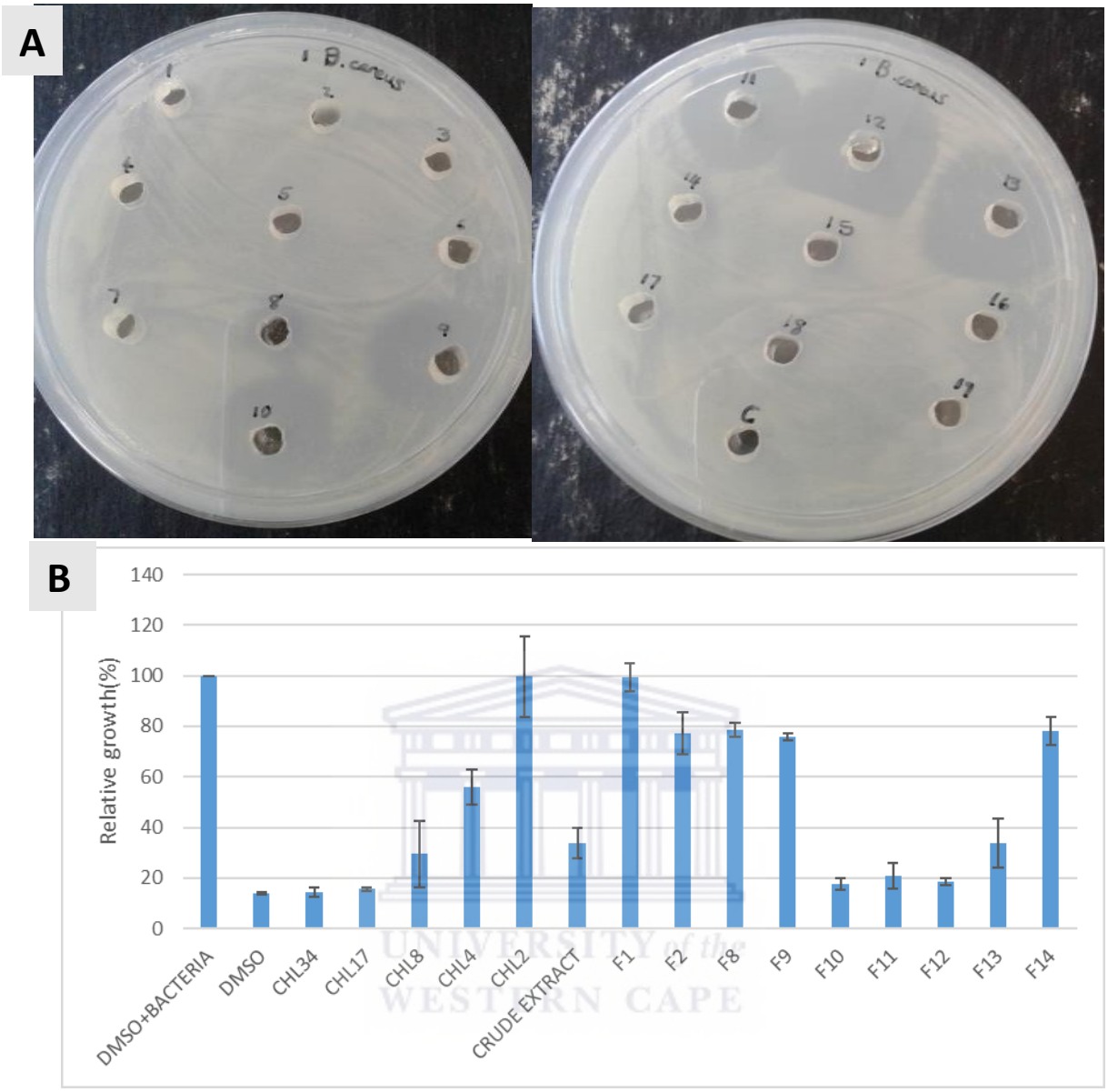

Figure 3.10: Bioassay results of PE6-126 HPLC fractions. (A) Well diffusion assay- zones of clearance around the well indicate fractions with anti-*B. cereus* activity. C- represents the control well inoculated with 20% DMSO. (B) Relative growth of *B. cereus* determined from the 96 well plate assay experiment. Out of 19 fractions tested, fraction 10-13 showed inhibition of *B. cereus* as indicated by relative bacterial growth below 50%. Controls included 20% DMSO inoculated with the bacterial culture and 20% DMSO only. Chloramphenicol (CHL) was also included as an antibiotic control. The error bars represent the standard deviation of the mean obtained from the triplicate reading. Bacteria inoculated with DMSO served as a negative control (100% relative growth). The fractions with relative growth below 50% are considered as showing inhibition.

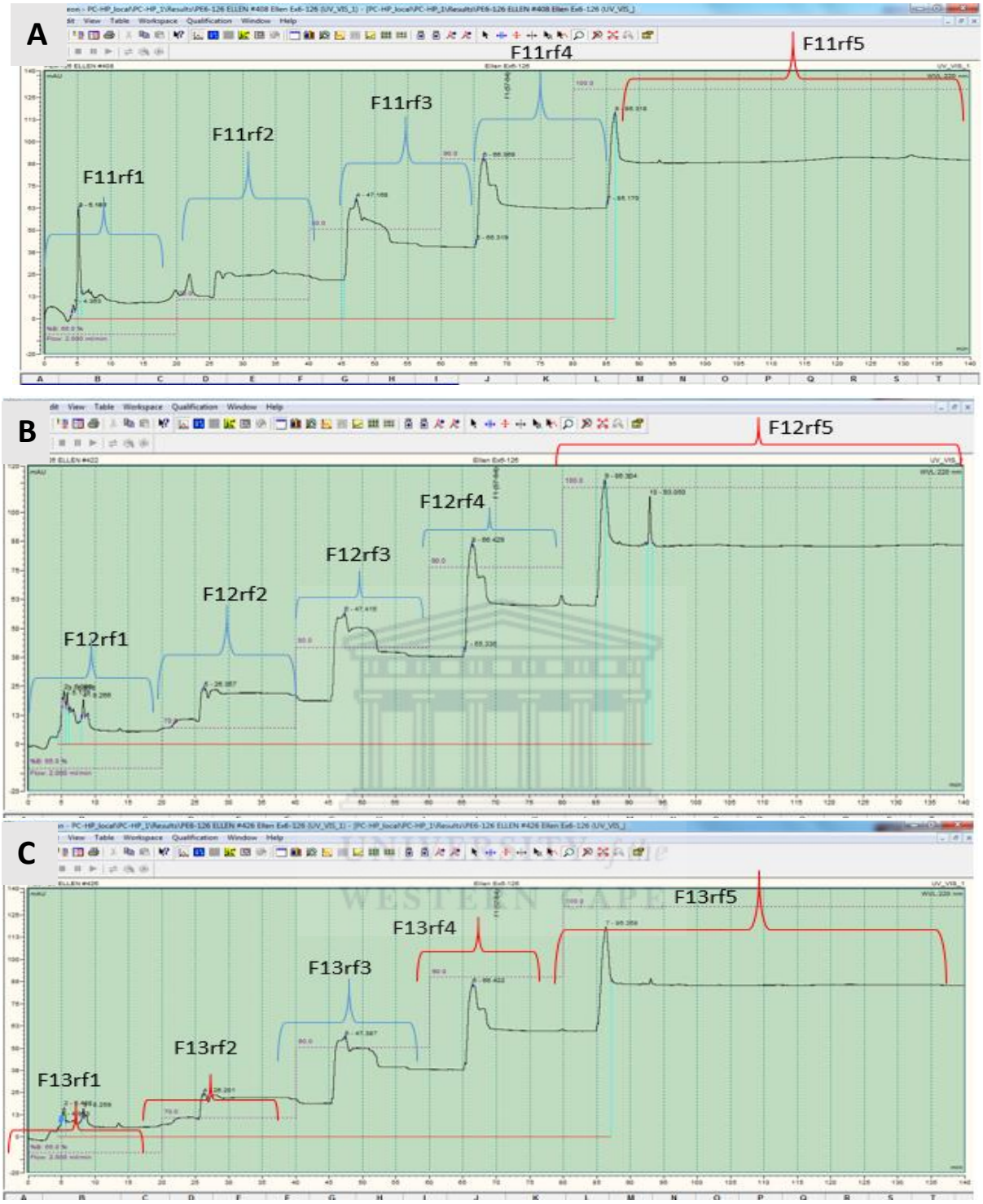

Figure 3.11: HPLC chromatogram showing re-fractionated fractions with anti-*B. cereus* activity. F represents the fraction number, rf- represents the re-fractionated fraction number. A) Fraction 11 chromatogram, B) Fraction 12 chromatogram, C) Fraction 13 chromatogram. Out of all the fractions tested for antimicrobial activity, fractions in the red bracket showed antimicrobial activity. Multistep gradient elution- 0-20min, 65% methanol; 20-40 min, 70% methanol; 40-60 min, 80% methanol; 60-80 min, 90% methanol and 80-140 min, 100% methanol.

The results of the assay for the active re-fractionated fractions are shown in **Figure 3.12**. The results from this assay showed that the sub-fraction of F11 (F11rf5), F12 (F12rf5), F13 (F13rf1, F13rf2 and F13rf5) had antimicrobial activity against *B. cereus*, whereas the re-fractionated F10 (not shown in the graph) had no antimicrobial activity.

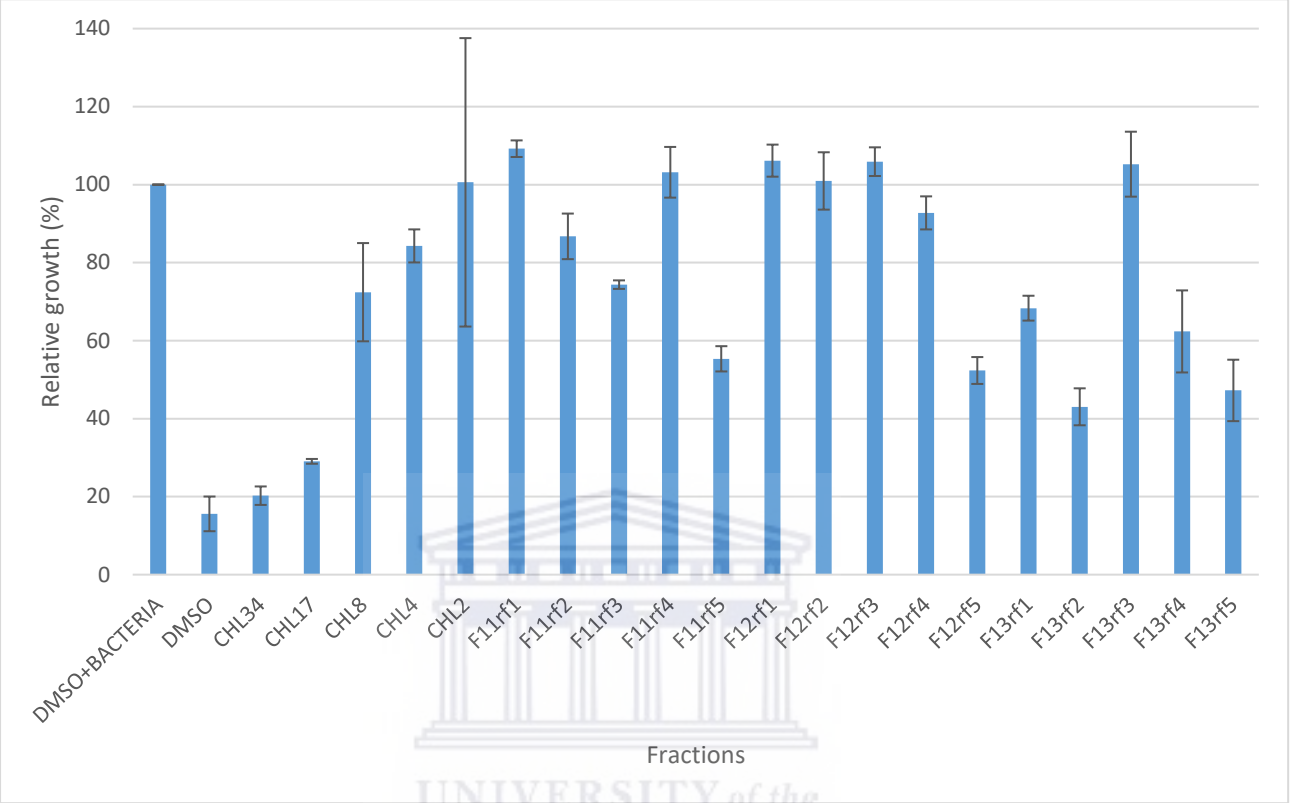

Figure 3.12: Graph showing re-fractionated fractions tested against *B. cereus*. F represents the fraction number, rf- represents the re-fractionated fraction number. Error bars represent the standard deviation of the mean obtained from the triplicate readings.

3.3.3.3. LC–MS Analysis of the antimicrobial fraction active against *B. cereus*

Liquid Chromatography-Mass Spectrometry (LC-MS) involves coupling of separation of liquid chromatography with mass analysis to detect and confirm molecular identities based on mass spectral data. The obtained mass data can then be used to confirm the size of different natural compounds, and their subsequent molecular weights can be compared against online informative databases of natural products such as Massbank, Marinlit, Pubchem, and Chemspider (Gerwick and Moore, 2012). LC-MS can therefore rapidly identify whether the metabolite produced by the microorganism is novel. All the fractions which showed antimicrobial activity (F11rf5, F12rf5, F13rf2 and F13rf5) were submitted for LC-MS analysis. However, there was low to no signal detected on LC-MS for fractions; F12rf5, F13rf2 and F13rf5, as a result F11rf5 was selected and analysed further in an attempt to identify the

antimicrobial compound which had activity against *B. cereus*. **Figure 3.13A** and **Figure 3.13B** show the LC-MS chromatogram of the antimicrobial fraction with 2 abundant peaks recorded at 280 nm. The peak which eluted at Rt= 12.66 min corresponded to ESI-MS molecular ion peak [M +H] with m/z of 399.2514 in ESI positive mode (**Figure 3.13C**). The second major peak which eluted at Rt= 13.50 revealed a molecular ion peak [M + H] with m/z = 193 in ESI-MS positive mode (**Figure 3.13D**).

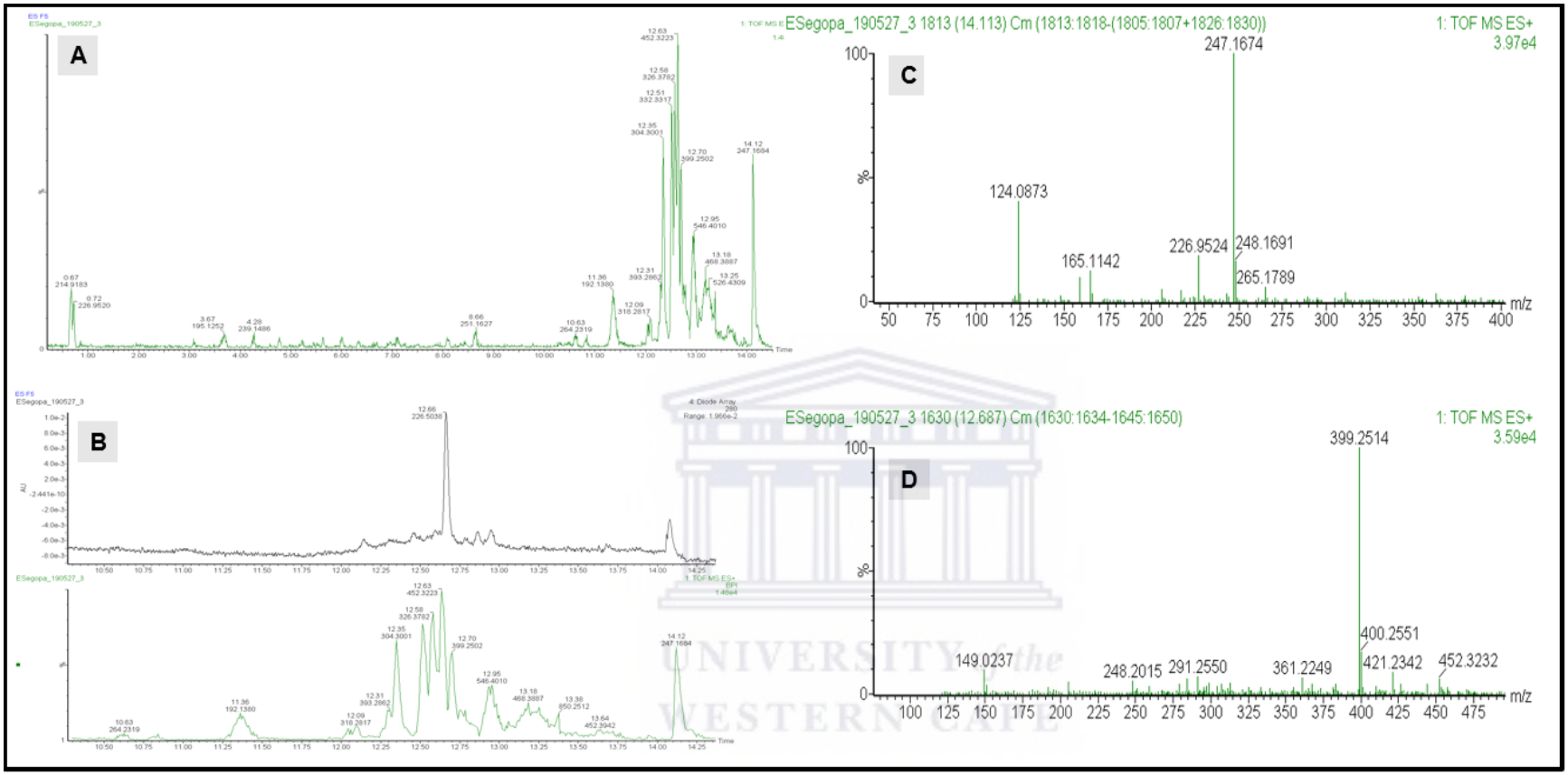

Figure 3.13: LC-MS analysis of F11rf5 fraction. (A) Chromatogram showing peaks corresponded with the retention times (min) and their respective mass (m/z). (B) Zoomed-in chromatogram of fraction F11rf5 showing the two main peaks in the UV chromatogram (280nm) at 12.66 and 14.08 minutes and their corresponding peaks in the ESI positive BPI (base peak ion) chromatogram (m/z 399.2592 and 247.1684 respectively). (C) Mass spectrum for the main peak 247.1684. (D) Mass spectrum for the main peak 399.2514.

The 2 main peaks were analysed for accurate elemental composition and the molecular formulae using Q-TOF-MS (**Table 3.5**). Based on the accurate elemental composition, the molecular formula of these peaks was obtained. The mass errors of one of the peaks with m/z 399.2514 and a predicted molecular formula of C20H35N206 was below 5ppm, which was within the error tolerance range. Based on this proposed elemental composition, it was tentatively identified as an alkaloid. Alkaloids are structurally diverse compounds which are generally classified based on their molecule and the presence of nitrogen atoms (Zotchev, 2013). According to literature, alkaloids from marine bacteria are predominantly produced by actinomycete and cyanobacteria (Zotchev, 2013), only a few alkaloids have been isolated from *Bacillus* species. Furthermore, as far as it is known, there are no studies which have reported alkaloids from *Bacillus* species with antibacterial activity against *B. cereus*. As mentioned earlier (**section 3.3.2.1**), it is noteworthy that the antiSMASH prediction tool does not detect genes responsible for certain secondary metabolite classes such as alkaloids (Blin *et al*., 2019).

Table 3.5: Q-TOF-MS accurate mass elemental composition analysis of the 2 main peaks.

	Experimental m/z	**Theoretical m/z**	**Mass error**		**DBE**	**Molecular formula**	**i-FIT**	**i-FIT (Norm)**
			mDa	**ppm**				
1	399.2514	399.2495	1.9	4.8	4.5	C20H35N206	27.6	3.233
2	247.1672	247.1658	1.4	5.7	1.5	C11H23N2O4	24.6	4.232

mDa - millidaltons of error between the mass found and the accurate mass of each peak, ppm-parts per million, DBE-double bond equivalent.

In order to identify whether the antimicrobial compound/(s) from all the peaks in fraction F11rf5 are novel or known, informative databases containing analytical information of natural compounds, such as MassBank, Pubchem and MarinLit were used. Masses of each peak were searched on these databases and the results showed that there were no hits matching known compounds (**Table 3.6**). All the searches had a high error rate above 5 ppm, except for 1 peak mass with m/z 195.1252. This result could mean that the compound/(s) present in F11rf5 which showed antimicrobial activity against *B. cereus* could possibly be novel or it has not been previously described.

Table 3.6: LC-MS tentative identification of peaks found on the fraction F11-rf5 chromatogram (**Figure 3.13A**).

Peak Number	Rt (min)	Experimental m/z	Theoretical m/z	Error (PPM)	Compound
1	0.67	214.9183	215.0807	755	Kinetin
2	0.72	226.9520	227.09061	610	2-Deoxycitidine
3	3.67	195.1252	195.12593	3.74	2,5 dimothy amphetamine
4	4.28	239.1486	239.15215	14.84	Salbutamol
5	8.66	251.1627	251.14224	81.461	N-Desmethylmirtazapine
6	10.63	264.2319	264.18378	182	Oxymatrine
7	11.36	192.1380	192.0699	354.432	DL Isocitric acid
8	12.09	318.2817	318.15552	318.15552	Fluvoxamine
9	12.31	393.2862	NH	NH	NH
10	12.35	304.3001	304.16745	435	1,7,7-trimethylbicyclo [2.2.1] hept-2-yl 4-hydroxy-3-methoxybenzoate;
11	12.51	332.3317	332.33890	21.574	Sanguinarine
12	12.58	326.3782	326.41919	125.5904	N-methylisothebainium
13	12.63	452.3223	NH	NH	NH
14	12.70	399.2502	399.16818	208.44	Colchicine
15	12.75	335.2805	335.40369	59284	Perforine
16	12.78	440.3594	440.26752	209	Norverapamil
15	12.95	546.4010	NH	NH	NH
16	13.18	468.3887	468.42099	68	Glyrophoric acid
17	13.25	526.4309	NH	NH	NH
18	14.12	247.1684	247.1523	45.19	Pethidine

Rt - retention time; *NH: No hits found; PPM (Parts per Million) - error rate of 5ppm and below regarded as a positive hit.

3.4. Conclusion

This study demonstrated the potential of PE6-126 to produce antimicrobial compounds with antibacterial activity against *B. cereus*. Genome mining by antiSMASH revealed that multiple gene clusters involved in secondary metabolite biosynthesis were present in PE6-126, including novel gene clusters and known clusters which were previously characterized in other bacteria. In this study, the predicted siderophore pathway from genome mining was expressed and produced through experimental work, however, there is no evidence that this pathway is responsible for the observed antimicrobial activity. A limitation in this study was failure to conclusively align the observed antimicrobial activity to a specific compound produced by PE6-126. This means that in this study, the use of the bioassay guided fractionation coupled to LC-MS could not lead to identification of the antimicrobial compound/(s) as predicted by antiSMASH, however, there is no evidence to exclude thuricin CD and/or other pathways as possible clusters responsible for conferring antimicrobial activity of PE6-126 against *B. cereus*. This study has also shown that, although the *Bacillus* genus are well known for producing secondary metabolites, there is still potential to discovering novel natural products from this genus.

3.5. References

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Chapter 4 - General discussion, conclusion and recommendations

4.1. General discussion, conclusion and recommendations.

Natural products discovered from terrestrial microorganisms have been successfully used as antibiotics to combat pathogenic microbes. However, the discovery of novel compounds from terrestrial microbes has decreased due to the rediscovery of already known compounds (Jensen and Fenical, 1994; Sekurova, Schneider and Zotchev, 2019). This has led research to explore other environments such as the marine for discovering novel bioactive compounds (Naughton *et al.*, 2017). Marine bacteria have been increasingly studied as sources of novel bioactive compounds because of their potential to produce immense structurally diverse and bioactive compounds suitable for a range of biotechnological applications (Jensen and Fenical, 1994; Gerwick and Moore, 2012). The current study aimed to screen, isolate and identify novel antimicrobial compounds for pathogen control in the agricultural and pharmaceutical industries. The approaches used in this study included bioassay guided fractionation and genome sequencing coupled with genome mining for secondary metabolite biosynthetic gene clusters.

4.1.1. Antimicrobial activity of the marine bacterium PE6-126

In order to determine the antimicrobial activity of these marine bacterial strains against recently identified olive trunk fungal pathogens and a panel of bacterial indicator strains, bioassay screening techniques such as well diffusion and dual inoculation assays were used (**Chapter 2**). PE6-126 displayed inhibitory activity against multiple bacterial and fungal strains. This is consistent with what other studies have shown with regards to the ability of marine bacteria associated with invertebrates to produce secondary metabolites with diverse bioactive properties (Anand *et al.*, 2006; Matobole *et al.*, 2017; Ayuningrum *et al.*, 2019). Although the current study demonstrated the potential of marine bacteria to inhibit olive trunk fungal pathogens that affect olive production in South Africa, further studies focusing on the isolation and characterization of the antifungal compound/(s) from PE6-126 will have to be conducted. The initial antimicrobial activity of PE6-126 against a multi-drug resistant strain *E. coli* 1699 was of particular importance because the observed activity suggested that the antimicrobial compound/(s) produced by PE6-126 was likely to be a novel compound. In this regard, PE6-126 can be regarded as a priority antibacterial strain with the capacity to synthesize novel bioactive compound/(s) even though it is a well-known species (**Section 4.1.2**). This in turn also highlights the importance of dereplication based on the metabolic capacity of a strain to synthesize chemically diverse novel compounds, and not only focusing on a single factor to select strains for further compound isolation. The results obtained have important implications

for the discovery of novel antimicrobial compounds from marine bacteria in order to combat drug resistant pathogens which are a major concern worldwide. The novel activity of PE6-126 against a multi-drug resistant strain pathogen shows that the South African marine coast is worthy for further exploration.

4.1.2. Dereplication as a tool to discover novel natural products

Dereplication aims to eliminate isolates or compounds that are already known, with the primary goal being to identify potential novelty of these strains/compounds. This step is critically important to avoid wasting time, costs and resources on already known compounds, which is a major bottleneck in the drug discovery process (Rocha-Martin *et al.*, 2014). Initially, 16S rRNA gene sequence-based taxonomy was used as a way to prioritize bacterial strains with low sequence identity to well-known reference strains (Brandão *et al.*, 2002). Previously, it was hypothesized that bacteria with >99% sequence identity are likely to produce similar secondary metabolites. However, advances in genomics demonstrated that this was not entirely the case, because it may sometimes lead to elimination of valuable strains with potential to synthesize novel compounds (Inui *et al.*, 2012; Vynne, Mansson and Gram, 2012; Sekurova, Schneider and Zotchev, 2019). In this study, WGS was used to assess whether this isolate, belonging to a well-known and studied species (*B. cereus*), had the potential to produce novel compounds by assessing the secondary metabolite BGCs through query in antiSMASH. A high similarity to known pathways generally implies that the pathway from the query sequence is possibly involved in producing the same compound as predicted by antiSMASH. Therefore, the high similarity to the sactipeptide BGC that encodes thuricin CD led immediately to the hypothesis that PE6-126 may also produce this compound; which is known to confer antimicrobial activity against *B. cereus*. Based on the principle of genomic dereplication, this strain could have been disregarded for further pursual due to this known compound, however this strain had also initially displayed antimicrobial activity against a multiple drug resistant strain *E. coli* 1699. This meant that it could be producing a potentially novel compound with a different mechanism of action (Hentschel *et al*., 2001). Moreover, none of the biosynthetic pathways from PE6-126 were predicted to encode a known compound with anti- *E. coli* activity. Therefore, genome guided dereplication served as an effective method for the prioritization of PE6-126 for further compound isolation. The reason being that it dereplicates based on the secondary metabolite gene clusters and the ability of the strain to produce potentially novel compounds irrespective of its phylogenetic affiliation. Genome-guided dereplication may therefore prove to be an efficient approach for reducing redundancy in natural product discovery programs. The

genome guided methodology is what directed the study to the bioassay guided fractionation approach coupled with LC-MS in an attempt to isolate and identify this antimicrobial compound/(s) (**Chapter 3**). Although this approach would lead to identification of the compound, it is highly dependent on the expression of these secondary metabolites, which may sometimes be a challenge under standard lab conditions. Furthermore, this approach requires highly purified extract/(s) for accurate identification of the compound/(s), which may take considerable amount of time to achieve (Lewis, 2013). In this study, HPLC was used as a separation technique; fractions which showed anti-*Bacillus* activity were analysed further in an attempt to identify the compound/(s) using LC-MS. However, tentative identification (based on mass) performed through analytical information databases showed that there were no hits to other known and characterized natural products. The study could not fully identify the compound/(s) responsible for the anti-*Bacillus* activity due to lack of purity. Therefore, it was not possible to link the activity to one of the BGCs predicted by antiSMASH. However, this study does highlight the benefit of employing integrated methods for assessing the potential of an isolate to produce novel antimicrobial compounds.

4.1.3. Marine environment as a source for novel bioactive compounds

The literature review highlighted marine natural products with specific interest in marine bacteria associated with invertebrates as an untapped source of novel secondary metabolites Hentschel *et al*., 2001; Sekurova, Schneider and Zotchev, 2019). As previously mentioned, PE6-126 belongs to the *Bacillus* genus, which is known to be distributed in different environments and represent a rich source for diverse secondary metabolite gene clusters (**Chapter 3**). When analysing the closely related strains to PE6-126, isolated from different environmental niches, only 3 (out of 33) were isolated from the marine environment. Although none were isolated from a marine invertebrate/tunicate consortium, they had similar secondary metabolite biosynthetic gene cluster distribution. Furthermore, PE6-126 had the biosynthetic capacity to produce potentially novel compounds, this shows that *Bacillus* associated with marine invertebrates should still be investigated further as profitable sources of novel secondary metabolites.

Other studies conducted from the South African marine environment have showed promise in the drug discovery process (Davies-Coleman and Veale, 2015). For instance, *Tsitsikama favus;* a latrunculid sponge endemic to the South African coast, is known for the production of unique pyrroloiminoquinone alkaloids, known as tsitsikammamine A and B. These alkaloids have demonstrated interesting bioactivities including antimicrobial and cytotoxic activity (Antunes

et al., 2004). Studies from the IMBM research group also identified marine invertebrate associated bacteria which have shown diverse biological activity (Klein, 2015; Hoosen, 2016; Matobole *et al.*, 2017). In addition to previous studies, this study confirms that the South African coastline, with its unique biodiversity, remains a hotspot to discover potentially novel bioactive compounds from marine bacteria associated with endemic invertebrate species.

4.1.4. Future studies

Future studies would require large scale fermentation of PE6-126 to increase the antimicrobial compound titre and further purification for downstream applications such as structural elucidation using NMR (Montaser and Luesch, 2011). However, large scale fermentation requires optimization of fermentation conditions, which may impose the risk of genes responsible for secondary metabolite production to become differentially expressed. In some cases, this can be resolved using mixed fermentation (Montaser and Luesch, 2011). Cryptic secondary metabolite pathways may encode potentially novel compounds; therefore, they are important in natural product discovery (Corre and Challis, 2007; Romano *et al.*, 2018). OSMAC can be included as one of the techniques in future studies since it is a useful tool to detect secondary metabolites predicted to be final products of secondary metabolite gene clusters in various microorganisms (Bode *et al.*, 2002). This technique has shown that shifting minor culture conditions results in substantial changes in metabolic profiles of microorganisms. These cultivation changes can be altered systematically in order to identify suitable conditions which stimulate expression of cryptic biosynthetic pathways from a single microbe (Bode *et al.*, 2002). OSMAC may also lead to enhanced production of compounds which were previously produced at a low concentration under standard lab conditions, furthermore it can unlock activity from isolates which previously had no activity. It should be noted that although this technique may be essential to improve discovery of novel secondary metabolites from microbes, it is laborious and very time consuming.

Another option which can be used to discover novel compounds from uncharacterized BGC's includes heterologous expression (Zhang *et al.*, 2019). This technique requires cloning of the BGC to suitable expression vectors and a suitable heterologous host. Comparative metabolic profiling using HPLC-MS may then be applied to assist in identification of the novel products. An appropriate method would be TAR cloning which is the most robust method and is not limited by the large size of the BGC (Zhang *et al.*, 2019). The ideal host for the expression of the sactipeptide cluster would be *E. coli*, which has become a popular heterologous host for sactipeptides and other RiPPs families (Zhang *et al.*, 2018). After successful expression of the

gene cluster, the compound can be purified and tested again for antimicrobial activity against the indicator strains. Using one of the pathways identified in the PE6-126, this approach could be employed as follows: to assess the antifungal activity of PE6-126 against olive fungal trunk pathogens, heterologous expression of the betalactone BGC could be conducted to determine whether a fengycin-like compound is produced. Fengycins are known for their antagonistic activities towards a wide range of phytopathogens. These lipopeptides are of particular importance since they can be applied as potential environmentally friendly biocontrol agents against fungal pathogens (Meena and Kanwar, 2015). Upon successful expression of this cluster, the purified compound encoded by this cluster can then be tested against olive trunk fungal pathogens to determine whether it confers the antifungal activity of PE6-126.

Structural elucidation of the compound would then be conducted to confirm whether it is novel, as postulated based on the sequence analysis. This heterologous expression technique, however, is a trial-and-error approach and has a lot of challenges. There may be problems in the pathway being expressed due to the reliability of other metabolic products from other metabolic functions in order to produce the final product, this may interfere with genes not expressing in the host and therefore failure to produce the compound from the cloned pathway (Gabor, Alkema and Janssen, 2004). Another challenge is that heterologous hosts often produce their own secondary metabolites, thus eliminating the precursors required for the biosynthesis of an exogenous compound, leading to a low yield and challenge in purification of the produced compound (Sekurova, Schneider and Zotchev, 2019). Therefore, while a heterologous approach can assist in linking novel compounds to the corresponding biosynthetic pathways and contribute to natural product discovery, it is not the most efficient approach given the many limitations associated with it.

Based on the potential of marine bacteria to produce bioactive compounds, continuous research using interdisciplinary approaches mentioned above can pave a way for novel natural product discovery.

4.2. References

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Appendices

Appendix A - General cultivation media used in this study

Culture media used in this study are listed in **appendix A1-A8**. All media used were autoclaved at 121°C for 20 min. Agar was added at a concentration of 15 g/L for the preparation of solid media and pH adjusted using 32% HCl and/ or 10 M NaOH (pH adjustments were made for marine bacteria media only). PDA media (**Appendix A5**) was amended with chloromycetin (0.25 g/L)

Appendix A 1: Composition of Zobell Agar media (ZBA)

Ingredient	Mass (g/L)
Yeast Extract	1.25
Peptone	3.75
NaCl	18
MgCl2	2
KCL	0.525
CaCl2	0.075
Agar (Optional)	15
0.1% Crystal Violate Solution (Optional)	1

Appendix A 2: Composition of Trypticase Soy Agar (TSA)

Ingredient	Mass (g/L)
TSB	3
NaCl	18
$MgCl_2$	2
KCL	0.525
$CaCl_2$	0.075
Agar (Optional)	15

Appendix A 3: Composition of Activated Charcoal Media (ACM)

Ingredient	Mass (g/L)
HEPES	2.38
Sodium pyruvate	3
Yeast Extract	0.1
Soybean peptone	3
$NaNO_3$	0.34
$KHPO_4$	0.1
$MgSO_4 * 7H_2O$	0.15
Activated charcoal	3
Agar (Optional)	15

Appendix A 4: Composition of Glucose Yeast Malt Media (GYM)

Ingredient	Mass (g/L)
D-Glucose	4
Yeast Extract	4
Malt Extract	10
CaCo3	2
NaCl	24
MgCl2	5,3
KCl	0.7
CaCl	0.1
Agar (Optional)	15

Appendix A 5: Composition of PDA Media

Ingredient	Mass (g/L)
Potato dextrose agar	39
Agar (Optional)	15

Appendix A 6: Composition of LB Media

Ingredient	Mass (g/L)
Tryptone	10
Yeast extract	5
NaCl	10

Appendix A 7: Composition of LB agar media

Ingredient	Mass (g/L)
Tryptone	10
Yeast extract	5
NaCl	10
Agar	15

Appendix A 8: Composition of CAS media

Solution 1	Weight / mL
1mM $FeCl_3 \cdot 6H_2O$ in 10mM HCl	2.70 mg $FeCl_3 \cdot 6H_2O$ / 10 mL
CAS	60.5 mg / 50 mL
CTAB	72.80 mg / 40 mL

Solution 2	Weight / 800 mL
KH2PO4	0.3 g
NaCl	0.5 g
NH_4Cl	1 g
PIPES	30.24 g
agar	15 g
Add KOH crystals to adjust pH to 6.8 and adjust volume to 800 mL	

Solution 3	Weight / 70 mL
glucose	2 g
mannitol	2 g
MgSO4·7H2O	493 mg
CaCl2	11 mg
MnSO4·H2O	1.17 mg
H3BO3	1.4 mg
CuSO4·5H2O	0.04 mg
ZnSO4·7H2O	1.2 mg
Na2MoO4·2H2O	1 mg

Solution 4	Weight / 30 mL
10% filter sterilized casamino acids	3g /30 mL

Solutions (1, 2 and 3) were autoclaved and cooled to 50°C. Solutions 1, 3 and 4 were added to solution 2 and mixed altogether prior to pouring the agar plates.

Appendix B - Buffers and stock solutions used in this study

Appendix B 1: Stocks and final concentrations of antibiotics used in the study

Antibiotic	Preparation
Chloramphenicol	34 mg/mL in 100% Ethanol stock 34 µg/mL final concentration

Appendix C - Antibiotics which *E. coli* 1699 is resistant against

Appendix C 1: List of antibiotics to which the multi-drug resistant *E. coli* 1699 exhibits resistance.

Antibiotic	MIC (µg/mL)	Target	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
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 Tetracycline	256 >256	protein synthesis	macrolide
Thiostrepton	>512	protein synthesis	tetracycline
Tylosin	512	protein synthesis	oligopeptide
Virginamycin	64	protein synthesis	macrolide
Rifampin	>256	RNA polymerase	rifamycin
Rifamycin SV	>64	RNA polymerase	rifamycin
Albomycin	high	Iron metabolism	Cyclic polypeptide
Trimethoprim 64	>400	DHFR	diaminopyramidine