Bioactivity-and-genome guided isolation of a novel antimicrobial protein from *Thalassomonas viridans*

UNIVERSITY *of the*
WESTERN CAPE

Shanice Raquel Adams

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MAGISTER SCIENTIAE (M.Sc.)

Department of Biotechnology
University of the Western Cape

Bellville

Supervisor: Prof. Marla Trindade (Tuffin)

Date:

Declaration

I, Shanice Raquel Adams, hereby declare that “Bioactivity-and-genome guided isolation of a novel antimicrobial protein from *Thalassomonas viridans*” is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used have been indicated and acknowledged**.**

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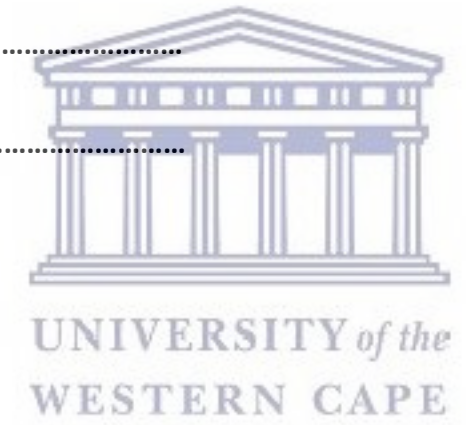

Abstract

The continued emergence of bacterial resistance to the antibiotics currently employed to treat several diseases has added to the urgency to discover and develop novel antibiotics. It is well established that natural products have been the source of the most effective antibiotics that are currently being used to treat infectious diseases and they remain a major source for drug production. Natural products derived from marine microorganisms have received much attention in recent years due to their applications in human health. One of the biggest bottlenecks in the drug discovery pipeline is the rediscovery of known compounds. Hence, dereplication strategies such as genome sequencing, genome mining and LCMS/MS among others, are essential for unlocking novel chemistry as it directs compound discovery away from previously described compounds. In this study, the genome of a marine microorganism, *Thalassomonas viridans* XOM25^{T} was mined and its antimicrobial activity was assessed against a range of microorganisms. Genome sequencing data revealed that *T. viridans* is a novel bacterium with an average nucleotide identity of 81% to its closest relative *T. actiniarum*. Furthermore, genome mining data revealed that 20% of the genome was committed to secondary metabolisms and that the pathways were highly novel at a sequence level. To our knowledge, this species has not previously been exploited for its antimicrobial activity. Hence, the aim of this study was to screen for bioactivity and identify the biosynthetic gene/s responsible for the observed bioactivity in *T. viridans* using a bioassay-and-genome- guided isolation approach to assess the bioactive agent. The bioassay-guided fractionation approach coupled to LCMS/MS led to the identification of a novel antimicrobial protein, TVP1. Bioinformatic analyses showed that TVP1 is a novel antimicrobial protein that is found in the tail region of a prophage in the *T. viridans* genome. Phage-derived proteins have previously been

shown to induce larval settlement in some marine invertebrates. Since the mechanism of action of TVP1 remains unknown, it remains a speculation whether it may offer a similar function. More research is required to determine the biotechnological application and the role of TVP1 in its host and natural environment.

Key words: *antibiotic resistance, marine natural products, non- ribosomal peptides, polyketides, Thalassomonas viridans, marine invertebrate larvae, OSMAC, antimicrobial screening, genome mining, transcriptional profiling, bioassay-guided fractionation, liquid chromatography mass spectrometry, bacteriophages, antimicrobial proteins.*

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Lastly, I would like to thank my husband for his patience, constant support, encouragement and his unconditional love.

Dedication

This work is dedicated to my family, especially my mother (Georgina Adams), grandmother (Rose Adams), grandfather (George Adams) and my aunty Patty for their love, patience, kindness and sacrifices. To my uncles Rodger, Julian and husband for the inspiration.

Abbreviations

°C	Degree Celsius
µg/ml	Microgram per millilitre
µl	microlitres
µm	micrometre
A domain	Adenylation domain
ACN	Acetonitrile
ACP	Acyl carrier protein
AMPs	Antimicrobial proteins
antiSMASH	Antibiotics and secondary metabolites analysis shell
APS	Ammonium persulfate
AT	Acyltransferase
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCWH's	Bacterial cell wall hydrolases
BGC	Biosynthetic gene cluster
BLAST	Basic Local Alignment Search Tool
bp	base pair
C domain	Condensation domain
cDNA	Complementary DNA
CLB's	Colicin-like bacteriocins
CLF	Chain length factor
cm	Centimetre

cm	centimeter
CoA	Co-Enzyme A
contig	Contiguous
DAS	Distributed Annotation System
DEBS	6-deoxyerythronolide B synthase
DH	Dehydratase
DH domain	Dehydratase domain
dH2O	Distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribose nucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphates
dNTPs	Deoxynucleotide triphosphates
DoBISCUIT	Database of Biosynthesis Clusters Curated and InTegrated
EDTA	Ethylenediaminetetracitic acid
ER	Enoylreductase
ER	Enoyl reductase
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, and Enterobacter*
EtBr	Ethidium Bromide
FA	Formic acid
FACS	Fluoresence assisted cell sorting
FPLC	Fast performance liquid chromatography
g	Gravitational force
g	grams

g/L	Gram per litre
GPC	Gel Permeation chromatography
HPLC	High performance liquid chromatography
ID	Identity
IEC	Ion Exchange chromatography
IMBM	Institute for Microbial Biotechnology and Metagenomics
IMG/VR	Integrated Microbial Genome/Virus
kDa	Kilo Dalton
KR	Ketoreductase
KS	Ketosynthase
KSα	Ketosynthase alpha subunit
KSβ	Ketosynthase beta subunit
L	litre
LB	Lysogeny broth
LC	Liquid chromatography
LCMS	Liquid chromatography mass spectrometry
MA	Marine agar
MB	Marine broth
MCO	Molecular weight cut-off
MDR	Multi-drug resistant
mg/ml	Milligram per millilitre
MIC's	Minimum inhibitory concentrations
min	minutes
ml	Millilitres
mM	millimolar

mm	millimetre
MMTS	S-Methyl methanethiosulfonate
NaAc	Sodium Acetate
NaPDoS	Natural Product Domain Seeker
NCBI	National Centre for Biotechnology Information
NDM	New Dehli metallo beta-lactamase
ng	Nanogram
NMR	Nuclear magnetic resonance
NRPs	Non-ribosomal peptides
NRPS	Non-ribosomal peptide synthase
OD	Optical density
OSMAC	One strain many compounds
PCP	Peptidyl carrier protein
PCR	Polymerase chain reaction
PKs	Polyketides
PKS	Polyketide synthase
Ppant	Phosphopantentheine
PRISM	Prediction Informatics for Secondary metabolomes
RAST	Rapid Annotation using Subsystem Technology
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl Sulphate

SDS-PAGE	Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis
sec	seconds
T domain	Thiolation domain
TAE	Tris-Acetate-EDTA buffer
TCEP	Tris (2-carboxyethyl) phosphine
TE	Thioesterase
TE	Tris-EDTA buffer
TEAB	Tetraethylammonium bromide
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
TFA	Trichloroflouric acid
UTI's	Urinary tract infection
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
WGS	Whole genome sequencing
WHO	World Health Organisation

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Chapter 1 Literature review

1.1 Introduction

Since the development of penicillin in 1940, antibacterial drugs (antibiotics) have been widely and effectively used for eliminating pathogenic bacteria without harming the somatic cells of the treated individual (Levy, 1998; Levy, 2002). Antibiotic activity is achieved by either preventing the disease causing bacteria (pathogen) from maintaining the integrity of their cell walls or by targeting a specific protein that is needed for the pathogen to survive or replicate (Kohanski et al., 2010). Essentially, antibiotics block a life sustaining function of the invading pathogen. During the initial stages of drug development, a total of 15-20 antibiotics were developed every 10 years, however, in the last 10-12 years, only six antibiotics have been released (Who.int, 2015).

Paralleled to antibiotic development has been the evolution of antibiotic resistant bacterial strains that evolved shortly after the introduction of the first antibiotic. For example, in 1953, resistance to streptomycin, chloramphenicol, tetracycline and sulfonamides was noted in the *Shigella* dysentery outbreak in Japan, only a decade after these antibiotics were introduced (Davies, 2007). Antimicrobial resistance is caused by rapid evolution of the bacterial genome under selective antibiotic pressure and by the selective pressure of the environment (Kolář et al., 2001; Levy & Marshall, 2004). The overuse and misuse of antibiotics has been one of the main factors accelerating the process of antimicrobial resistance as the incorrect use of antibiotics leads to a greater opportunity for bacteria to mutate into resistant forms (Williams et al., 2007; Williams et al., 2008). Today, treating the infected has become a daunting challenge to the public health sector as bacterial strains causing life threatening diseases have

acquired resistance to more than 100 antibiotics used in the medical industry (Levy, 1998; Saha *et al*., 2014).

There are several mechanisms that bacteria have evolved to become resistant to antibiotics (Figure 1.1). One method is the targeted removal of the antibiotic from the cytoplasm by coding for efflux pumps to expel the antibiotic from inside the bacterial cell. This mechanism is employed by pathogenic bacteria to mediate resistance to tetracycline, chloramphenicol, and fluroroquinolones. Another resistance mechanism is to target the antibiotic itself by encoding antibiotic degrading enzymes such as β-lactamases that destroys penicillin and cephalosporins (Levy, 1998; Levy & Marshall, 2004). Bacteria can also gain resistance by producing enzymes to modify the chemistry and inactivate the antibiotic such as streptomycin, gentamycin and tetracycline (Levy & Marshall, 2004). Certain bacterial species modify their intracellular components, such as the ribosome, metabolic enzymes or proteins involved in DNA replication or cell wall synthesis, making the antibiotic unable to inhibit a vital function in the bacterial cell (Levy & Marshall, 2004). Bacteria can also laterally inherit their resistance genes from other bacteria through horizontal gene transfer (Levy, 1998). An example of horizontal gene transfer that hit the "headlines" in 2010 was the transfer of genes coding for the enzyme NDM-1 (New Delhi metallo beta-lactamase). NDM-1 is an enzyme that destroys antibiotics, even the very strong antibiotics that are used to fight multi-drug resistant (MDR) bacteria such as carbapenum (Escobar Pérez et al., 2013).

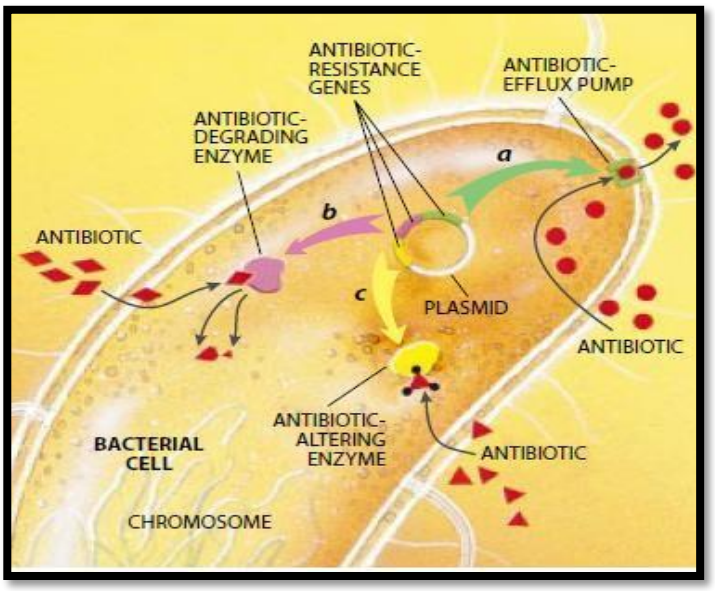


Figure 1.1: Mechanisms of antibiotic resistance. A) Bacterial genes coding for efflux pumps that eject the antibiotic. B) Genes coding for proteins that degrade antibiotics. C) Genes giving rise to enzymes that chemically alter or inactivates the drug. There are also resistance genes that have been acquired that are either located on the plasmid or chromosomal DNA (*Adapted from* Levy, 1998).

New resistant mechanisms acquired by pathogens are continuously emerging, threatening the clinician's ability to treat common infectious disease. Antibiotic resistance is increasing to dangerously high levels and has become an alarming global concern due to the rate at which bacteria are developing resistance to antibiotic therapies (Figure 1.2). Currently a list of resistant infections include pneumonia, tuberculosis, septicemia and gonorrhea that are becoming more challenging to treat as antibiotics become less effective. Without effective antimicrobials for the prevention and treatment of infections, medical procedures such as organ transplantation, chemotherapy, diabetes management and major surgeries become very dangerous (Brüssow, 2017). According to the World Health Organisation (WHO), a post-antibiotic era is approaching where common infections and minor injuries may result in death and the reason for this is because the rate at which antibiotic resistance is evolving, outcompetes the discovery and development of new antibiotics

(Who.int, 2015). Evidently, there is a need to discover and develop new antibiotics, preferably antimicrobial compounds with different modes of action that are less susceptible to microbial resistance.

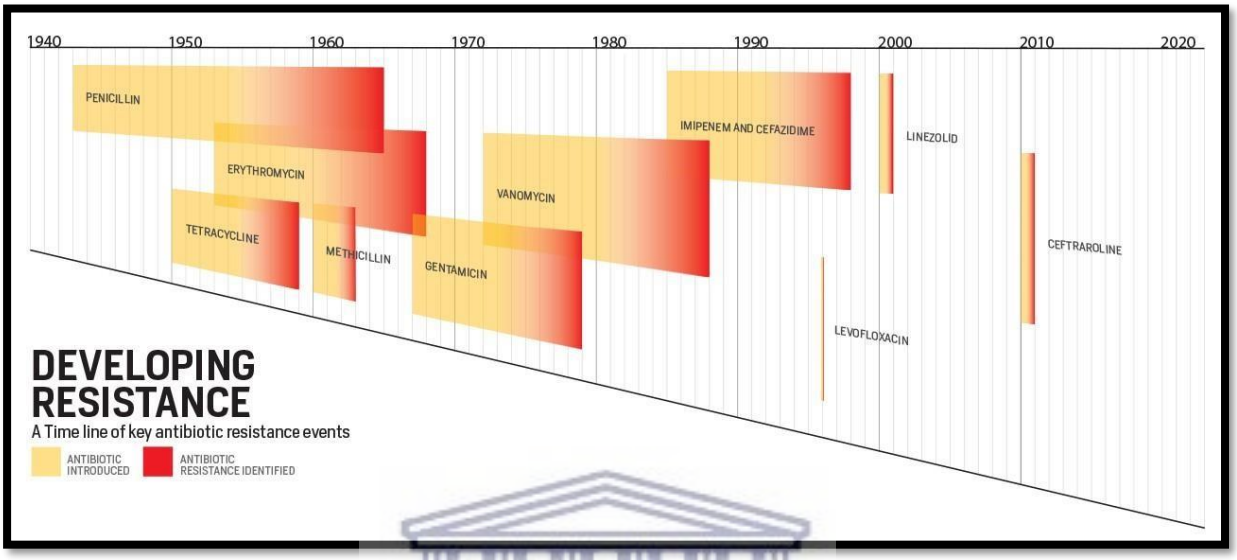


Figure 1.2: The rate at which bacteria acquires resistance to antibiotics. (*Adapted from* Medium, 2013*)*

1.2 Natural products in drug discovery

Natural products have served as the source of origin for a large fraction of commercially available antimicrobials, including antibacterial, anti-cancer, anti-fungal, antiviral and antiparasitic compounds (Villa & Gerwick, 2010; Bérdy, 2012). Historically, natural products were considered as plant-derived extracts that were used to treat a range of diseases and illnesses. For example, in 2600 B.C, the Mesopotamians documented the use of *Cupressus semperivens* (Cypress) and *Commiphora* (myrr) oils for the treatment of coughs, colds and inflammation (Dias et al., 2012). Natural products include secondary metabolites that are produced by diverse forms of life from both terrestrial and marine environments, such as plants, algae, invertebrates and microorganisms (Villa & Gerwick, 2010). Secondary

metabolites are the by-products of an organism's metabolism which are usually produced for the organisms' survival during unfavourable environmental conditions (Yung et al., 2011). Unlike primary metabolites, the function of these secondary metabolites is not associated with the growth, development or propagation of a species, as they are not generated by central metabolic pathways (Lopanik, 2014). Secondary metabolites are an important contributor to an organism's interaction with, and response to, its surroundings (Lopanik, 2014). Natural products are usually relatively small molecules with a molecular weight below 3,000 Daltons and exhibit considerable structural diversity (Zahner, 1979). These metabolites have been mined for many years and have become of great importance to researchers for the development of new antimicrobials due to the unique range of structures and unusual functional groups they possess (Villa & Gerwick, 2010). Several reviews have highlighted the contribution of natural products to drug discovery and how the identified lead compounds have contributed to the improvement of human health (Mizuno et al., 2013; Nass et al., 2017; Rizzo et al., 2018). For example, chloramphenicol and tetracycline are commonly used, effective antibiotics, derived from natural products produced by *Streptomyces aureofaciens* and *Streptomyces venezuelae*, respectively (Figure 1.3). It has been reported that tetracycline has several therapeutic applications dealing with infections in food production and is active against *Mycoplasma*, *Chlamydia*, *Pasteurella*, *Clostridium* and some protozoa (Granados-Chinchilla *et al*., 2017) while chloramphenicol is used to treat ocular, pulmonary, urinary tract and bloodstream infections (Ray *et al*., 2017).

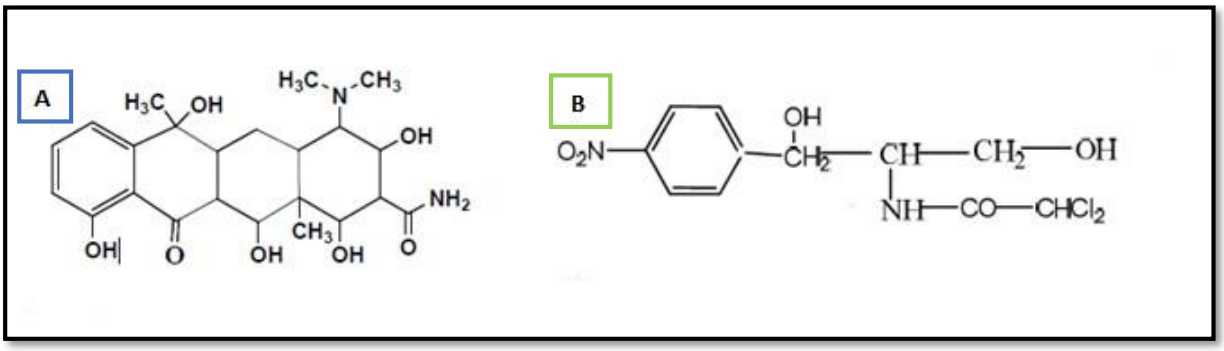


Figure 1.3: Antibiotics isolated from terrestrial microbes. The structures of tetracycline (Figure 2A above) isolated from *Streptomyces aureofaciens* and the chemical structure of chloramphenicol (2B above) isolated from *Streptomyces venezuelae* (*Adapted from* Siddiqui et al., 2014).

1.3 Classes of Natural Products

Natural products with pharmaceutical relevance originate from different chemical classes that are both structurally and functionally diverse. Important classes of pharmaceutically relevant natural products include terpenes or terpenoids, alkaloids, bacteriocins and siderophores among others (Ahmed & Holmström, 2014; Wang et al., 2014). Terpenes are compounds that are derived from isoprene units and play a variety of roles in mediating antagonistic and beneficial interactions among organisms and physiological functions including membrane stabilization, anti-oxidant properties, signalling and protection (De Carvalho & Fernandes, 2010). Alkaloids are naturally-occurring, nitrogen-containing, biologically active compounds and the alkaloids produced by marine microorganisms are known to have anti-malarial, anti-inflammatory and anticancer activities (Chakraborty, 2009). Bacteriocins are antimicrobial peptides that are ribosomally synthesized by bacteria to kill or inhibit other bacterial strains when competing for resources such as space and nutrients (Wang et al., 2014; Yang et al., 2014). Natural products belonging to the non-ribosomal peptide, polyketide and non-ribosomal peptide-polyketide hybrid classes have become a

focus for scientists because of their potential roles in the pharmaceutical industry and will therefore be one of the main focuses of this review.

1.3.1 Non-ribosomal peptides

In nature, non-ribosomal peptides are amongst the most widespread and structurally diverse secondary metabolites. They possess a broad range of biological activities which have been exploited in the development of a variety of important therapeutic agents such as immunosuppressant's, antibiotics and anti-cancer agents (Figure 1.4; Winn *et al*., 2015). Today, many peptides produced via the non-ribosomal pathway are being used in clinics. These include penicillin, cephalosporin, bacitracin, rifamycin, vancomycin and more recently daptomycin which is used in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections (Mishra, 2014). Other than antibiotics, drugs such as bleomycin (anti-cancer agent) and cyclosporine A or rifamycin (an immunosuppressant) are produced by non-ribosomal peptide synthases, and have vastly benefited the pharmaceutical industry (Mootz & Marahiel, 1997; Winn et al., 2015).

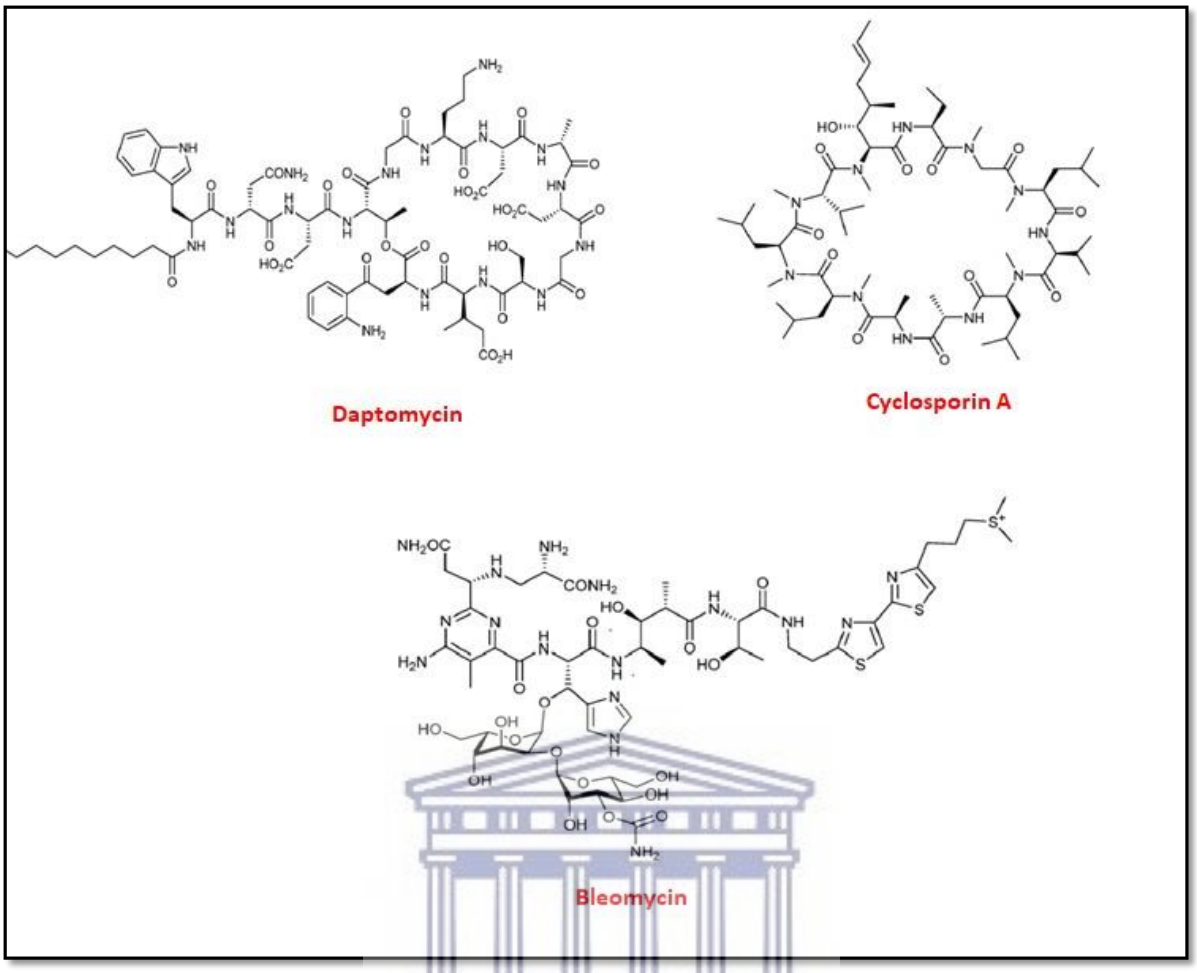


Figure 1.4: Non-ribosomal peptides with clinical applications. Daptomycin is used to treat methicillin resistant *Staphylococcus aureus* infections, cyclosporin A is a potent immunosuppressant and bleomycin is an anti-cancer agent.

1.3.1.1 Biosynthesis of non-ribosomal peptides

Non-ribosomal peptides (NRPs) are synthesized in bacteria and fungi by a family of enzymes referred to as non-ribosomal peptide synthases or NRPS's (Mootz & Marahiel, 1997; Strieker et al., 2010). NRPS assemble into exceptionally large enzyme complexes to form an assembly line (Figure 5; Sieber & Marahiel, 2005). These megaenzymes are made up of multifunctional modules that assemble small molecules from monomeric building blocks (Figure 1.5; Challis et al., 2000). In contrast to proteins produced by ribosomal synthesis, NRPS are not dependent on proteinogenic amino acids for protein synthesis as they are capable of using

both proteinogenic and non-proteinogenic amino acids to build peptide chains. They are capable of processing hundreds of monomers (approximately 500), including α-keto and α-hydroxy acids, heterocyclic and fatty acids among many other moieties (Marahiel, 2016).

Each module is made up of several catalytic domains that are responsible for a particular function. To synthesise a non-ribosomal peptide, the NRPS enzyme needs to contain at least three core domains which include an adenylation domain (A), peptidyl carrier protein (PCP) or thiolation domain (T) and a condensation domain (C) (Martínez-Núñez & López López, 2016).

The A domain is responsible for recognizing and selecting the amino acid substrate that comprises the end metabolic product and activates it as an aminoacyl-adenylate by catalysing hydrolysis of ATP. The activated amino acid is then transferred to the PCP domain, the transport unit that accepts the activated amino acid, which is covalently linked to its 4 phosphopantetheine (Ppant) cofactor as thioester (Nikolouli & Mossialos, 2012). At this stage the substrate can undergo modifications such as epimerization and N-methylation (Martínez-Núñez & López López, 2016). The condensation domain is the central entity of non-ribosomal peptide synthesis because they are responsible for the formation of the peptide bond by catalysing the transesterification of the chosen amino acid onto the amino end of the growing peptide chain (Figure 1.5). In addition to these three domains, a module may contain a methylation domain, which will add a methyl group to the amine in the backbone, or epimerisation, oxidation and reduction domains (Mootz & Marahiel, 1997; Kyung Kim & Fuerst, 2006; Wang et al., 2014). These domains create additional diversity through modifying the backbone of the peptide. At the end of the synthase complex will be a transesterification

domain that releases the peptide (Wang et al., 2014). The wide range of possible subunits and subunit combinations give rise to greatly diverse NRPs and thus chemical scaffolds.

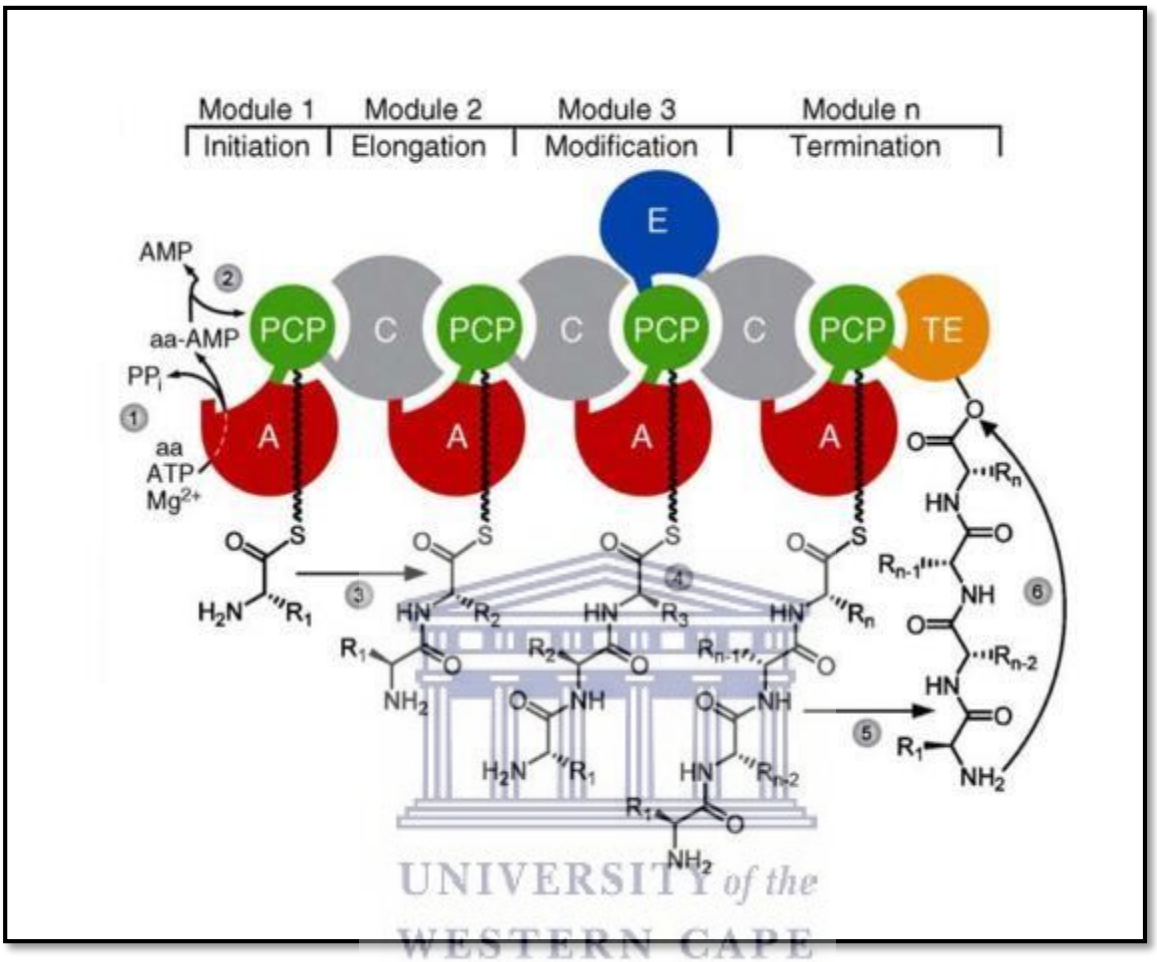


Figure 1.5: Schematic of a NRPSs biosynthesis assembly line. (*Adapted from* Strieker et al., 2010). 1) A specific amino acid is activated and 2) transferred to the PCP (peptidyl carrier protein) which holds onto the growing peptide thioester. 3) A peptide bond is then formed by the C (condensation) domain between the next amino acyl and the peptidyl unit. 4) Amino acids are modified by an integrated epimerisation domain. 5 and 6) The final product is released from the NRP which is the assembled peptide that is usually catalysed through hydrolysis and macrocyclization by the TE (thioesterase) domain (Wang, 2014).

1.3.2 Polyketides

Polyketides (PKs) are a large and highly diverse group of natural products produced by bacteria, fungi and plants (Shen, 2003; Trindade-Silva et al., 2013; Ray & Moore, 2002). PKs are structurally diverse, possessing a wide range of biological activities and include several clinically important drugs such as actinorhodins, erythromycin, rapamycin and lovastatin (Figure 1.6; Shen, 2003; Ridley et al., 2008; Trindade-Silva et al., 2013). Due to the structural diversity, remarkable bioactivity and enormous commercial value of these natural products, it remains the most successful candidates for novel drug discovery (Shen, 2003).

PKs are biosynthesized by a large family of mega-enzymes termed, polyketide synthases (PKSs) (Staunton & Weissman, 2001; Nakano et al., 2009). These enzymes are categorised into three distinct groups based on their structures and biochemistry, namely; Type I, Type II and Type III PKSs (Seow et al., 1997; Shen, 2003).

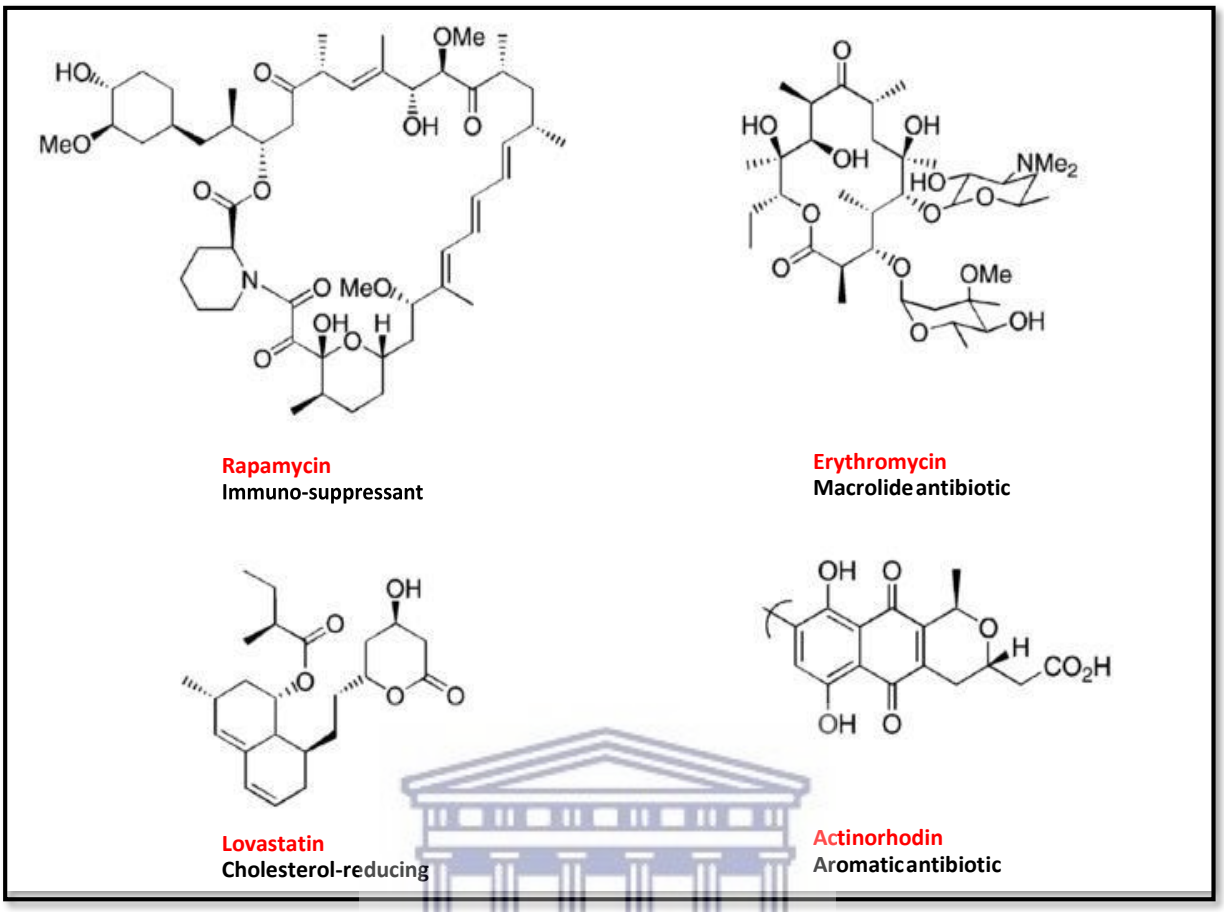


Figure 1.6: Clinically relevant polyketide secondary metabolites. These include the antibiotics; amphotericin B which is an antifungal medicine used to treat serious fungal infections (Ridley et al., 2008), erythromycin, a macrolide antibiotic used to treat a number of bacterial infections such as respiratory tract infections, skin infections, chlamydia infections, pelvic inflammatory disease and syphilis, lovastatin used to fight cholesterol and actinorhodin which is an aromatic antibiotic that exhibits bacteriacidal activity against *Staphylococcol sp.* and enterococci (Nass et al., 2017) (*adapted from* Staunton & Weissman, 2001).

1.3.2.1 Type I PKSs

Type I PKSs are organised into functional modules or catalytic units that assembles molecules by the incorporation of coenzyme A (CoA) derivatives (acetyl-CoA, malonyl-CoA, propionyl CoA, hexanoyl-CoA and pentanoyl-CoA amongst others) into the growing peptide chain (Seow et al., 1997; Dutta et al., 2014). These modules are further divided into domains, each of which

have a distinct set of non-iteratively acting activities responsible for a specific step in the condensation and modification of each added monomer (Ray & Moore, 2002; Cheng et al., 2003; Shen, 2003). Type I PKSs consist of a minimum of three catalytic domains which include a ketosynthase (KS) domain, acyltransferase (AT) domain and an acyl carrier protein (ACP) domain (Seow et al., 1997). The KS domain is responsible for selecting a CoA derivative and attaching it to the ACP domain by catalyzing the condensation reaction between the growing peptide chain and the chosen acyl derivative. The AT domain incorporates and transfers the CoA substrate to the ACP domain. The ACP activates the chosen CoA starter unit and passes the growing peptide intermediate to the next module (Lorente et al., 2014). In the biosynthetic assembly lines of some type I polyketides, additional modification domains that add to the complexity and diversity of the polyketide chain might be present in one or more modules. These include: a keto reductase (KR) domain which is responsible for deciding which stereo isomer is produced, the dehydratase (DH) which eliminates water from the reduced alcohol to an alkene, and the enoyl reductase (ER) domain that further reduces the alkene. Once the synthesis is complete, the peptide chain is released by a thioesterase (TE) domain typically as a free acid with water or as a reduced ketone. The product can then be further modified by post- tailoring enzymes to enhance the functionality (Lorente et al., 2014).

Many clinically important bacterial natural products are synthesized by type I PKSs such as; rapamycin (an immunosuppressant), epothilone (an anticancer agent) and lovastatin (an anti-hypercholesterolemic). Here we present the archetypal example of 6-deoxyerythronolide synthase which is responsible for the biosynthesis of the macrolide antibiotic, erythromycin (Figure 1.7) (Cheng et al., 2003; Ray et al., 2017).

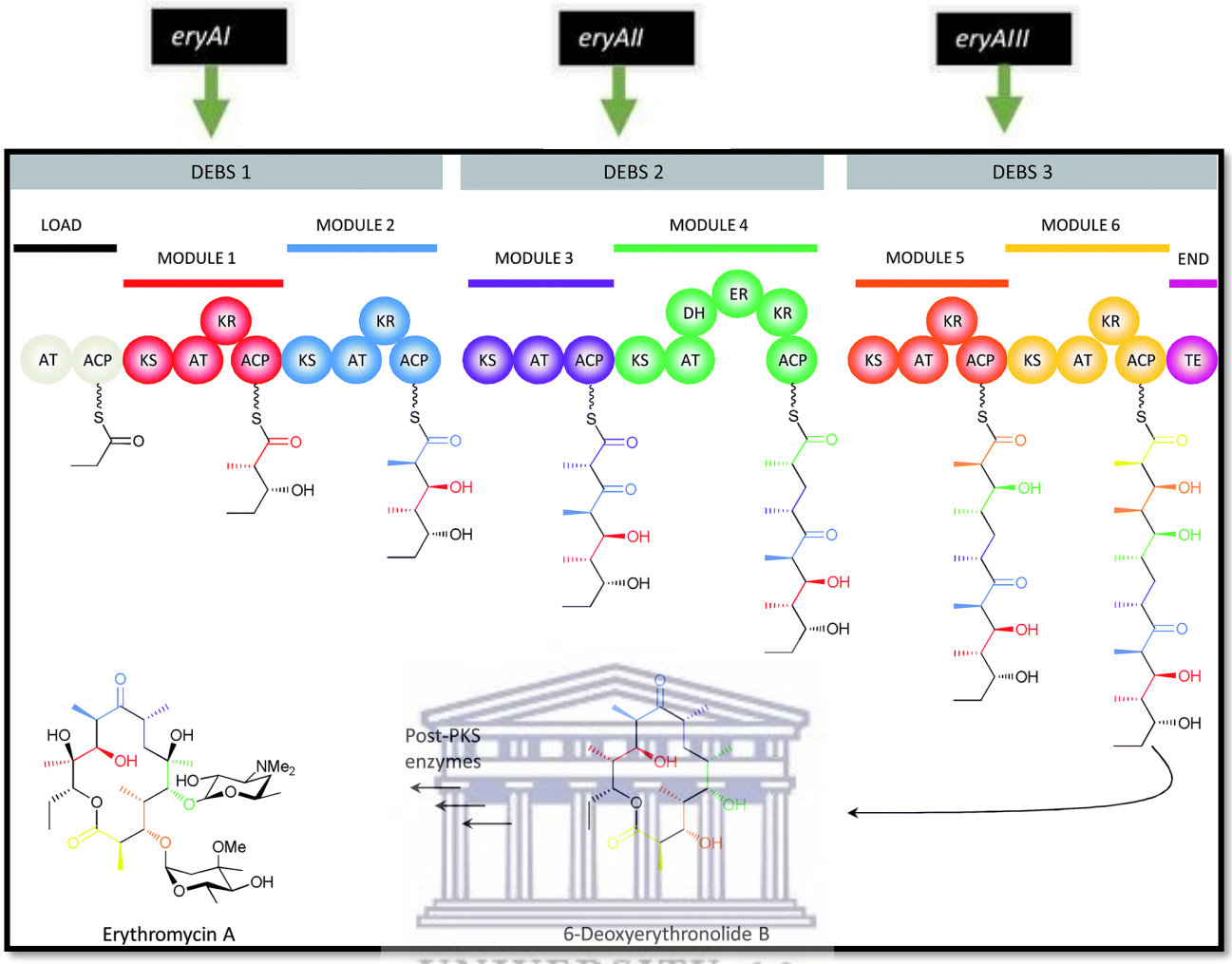


Figure 1.7: Biosynthetic assembly line of the type I polyketide erythromycin gene cluster. The 6-deoxyerythronolide synthase involved in the synthesis of erythromycin is encoded by three genes (*eryAI, eryAII, eryAIII*). These genes codes for the proteins DEBS 1 to 3. Each DEBS protein consists of 2 modules containing three core domains to catalyse one cycle of chain extension (A KS, AT and ACP as well as a variable set of modification domains, KR, DH and ER). In this representation, each module starts with a KS domain and ends with an ACP domain. This assembly line is initiated by DEBS 1 that is fronted with a loading didomain AT and ACP which accepts the starter unit propionate from propionyl-coA. Once the chain is fully formed, it is terminated by the TE domain in module 6 of DEBS 3 which catalyses the offloading and cyclisation of the fully formed heptaketide intermediate to produce 6-DEBS. Once 6-DEBS is released, it undergoes further modifications by post-PKS enzymes to form erythromycin A (Staunton & Weissman, 2001) (*adapted from* Weissman, 2015).

1.3.2.2 Type II PKS

Type II PKS's are involved in the biosynthesis of aromatic polyketides such as actinorhodin, tetracenomycin and doxorubicin (Khosla, 2009). In Type II PKSs, each domain is located on a single gene which expresses the enzymes that carries out the biosynthesis in an iterative manner (Seow et al., 1997). Similar to modular type I bacterial PKSs, Type II PKSs also requires a minimum of three domains for the biosynthesis of the product. These include two ketosynthase units (KS_α and KS_β) and an acyl carrier protein (ACP) (Hertweck et al., 2007). The KS_α domain contains the active site cysteine, that incorporates activated acyl and malonyl-CoA starter units and catalyzes the C-C bond formations through a Claisen type condensation reaction (Hertweck et al., 2007; Helfrich et al., 2014). The KS_β domain is responsible for determining the length of the chain and is therefore also known as the chain length factor (CLF) (Hertweck et al., 2007). Type II PKSs are also dependent on the ACP domain to successfully synthesize aromatic polyketides. The ACP domain is said to be the anchor of bacterial type II PKS as they function as checkpoints during various biochemical manipulations of the synthesis (Shen, 2003).

1.3.2.3 Type III PKS

The Type III PKS is a class of self-contained enzymes that forms homodimers and is widely distributed in fungi and bacteria (Nakano et al., 2009; Shen, 2003). Type III PKS enzymes are different from Type I and II PKS classes as they are very simple in structure and they are not reliant on an ACP domain to successfully complete the biosynthetic process (Cheng et al., 2003; Ray et al., 2017). Since Type III PKS enzymes lack an ACP domain, they have the ability to directly use the free acetyl-CoA- linked thioester substrates in an acyl independent carrier fashion (Yu et al., 2012; Ray et al., 2017). Thus for successful biosynthesis of the polyketide

products, the KS domain is responsible for carrying out all of the required reactions such as the priming, extension and cyclisation reactions (Figure 1.8). This process takes place in an iterative manner, producing a range of polyketide compounds such as chalcones, acricones, pyrones and more (Yu et al., 2012).

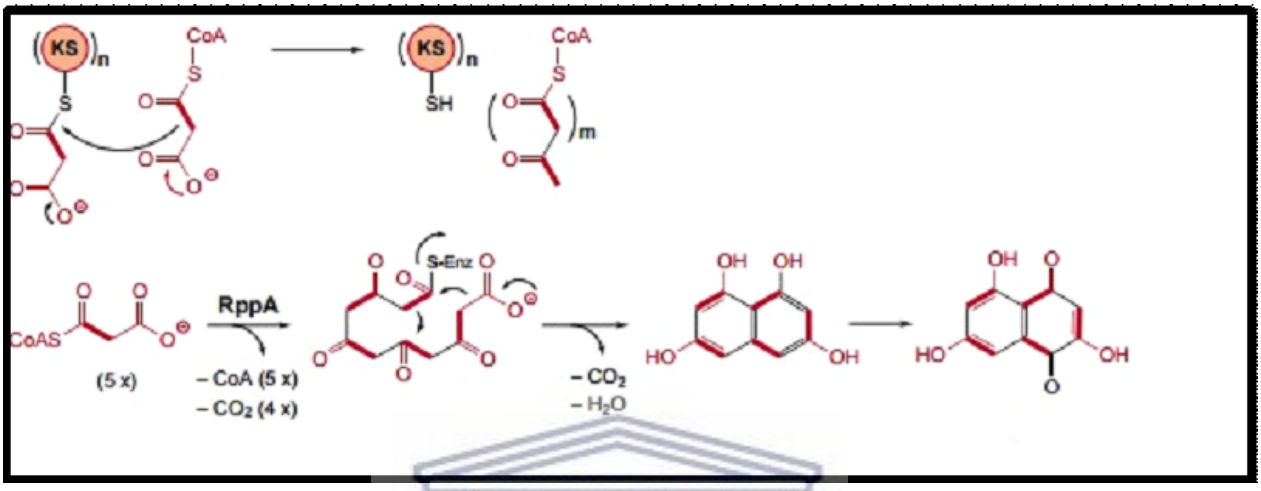


Figure 1.8: Type III PKS structure and mechanism. The figure above depicts the action of the RppA synthase, iteratively acting as the condensing enzyme for the biosynthesis of aromatic polyketides (often monocyclic or bicyclic), such as flavolin (*Adapted from* Shen, 2003).

1.3.1 NRPS-PKS hybrids

This is an intriguing class of natural products that consist of compounds synthesized by a combination of NRPS and PKS biosynthetic machinery. NRPS and PKS might differ in the compounds that they produce, however, their biosynthetic mechanisms share striking similarities. By way of their functional similarities, they synergize to form hybrid NRPS-PKS compounds (Mizuno et al., 2013). The compounds produced by PKS and NRPS are complex and diverse in structure, effective in a wide range of therapeutic applications and very valuable to the pharmaceutical industry (Singh et al., 2017). To date, many of these hybrid compounds have been isolated from bacteria such as the antibiotic andrimide, the immune-suppressant rapamycin and the anti-tumour agent epithilone (Mizuno et al., 2013).

1.4 The marine environment as a rich source for novel natural products

Although drug molecules with a terrestrial plant and microbe origin have been the mainstay of the pharmaceutical industry over many years, researchers have moved away from mining for novel antibiotics from terrestrial origin due to the frequent rediscovery of known compounds (Molinski et al., 2009). For this reason, the biodiscovery focus has now shifted to mining natural products from the marine environment as a novel source (Montaser and Leusch, 2011).

The marine environment constitutes 70% of the earth's surface and represents a large unexplored resource (Bérdy, 2012). It contains many different ecological niches and thousands of microbial species that differ both functionally and phylogenetically (Kennedy et al., 2007; Bérdy, 2012; Rizzo & Giudice, 2018). Organisms inhabiting marine environments encounter diverse conditions where light level, temperature, nutrient availability, hydrostatic pressure, oxygen concentration and salinity, all vary widely (Ferreira et al., 2014). The dynamic conditions associated with this environment as well as the presence of competing microorganisms, drive the evolution of certain biological chemical defence mechanisms for their survival, and result in the production of a variety of natural products with unique structural features. These compounds often exhibit diverse biological activities with possible applications for the treatment of diseases. Previously isolated marine natural compounds have been used to treat diseases such as cancer, inflammation, viral-bacterial-and fungal infections (Figure 1.9) (Anand et al., 2006; Egan et al., 2008; Villa & Gerwick, 2010; Ngo et al., 2012; Cragg & Newman, 2013).

Based on the pharmaceutical significance of marine natural products most research groups have focused on the preparation and analysis of extracts from easily accessible macro-

organisms such as sponges, tunicates, molluscs, echinoderms and others (Leal et al., 2012; Trindade et al., 2015). These marine animals have proved to be a rich source of novel pharmaceutically active compounds, with more than 200 bioactive compounds isolated from marine sponges in the last decade (Yung et al., 2011).

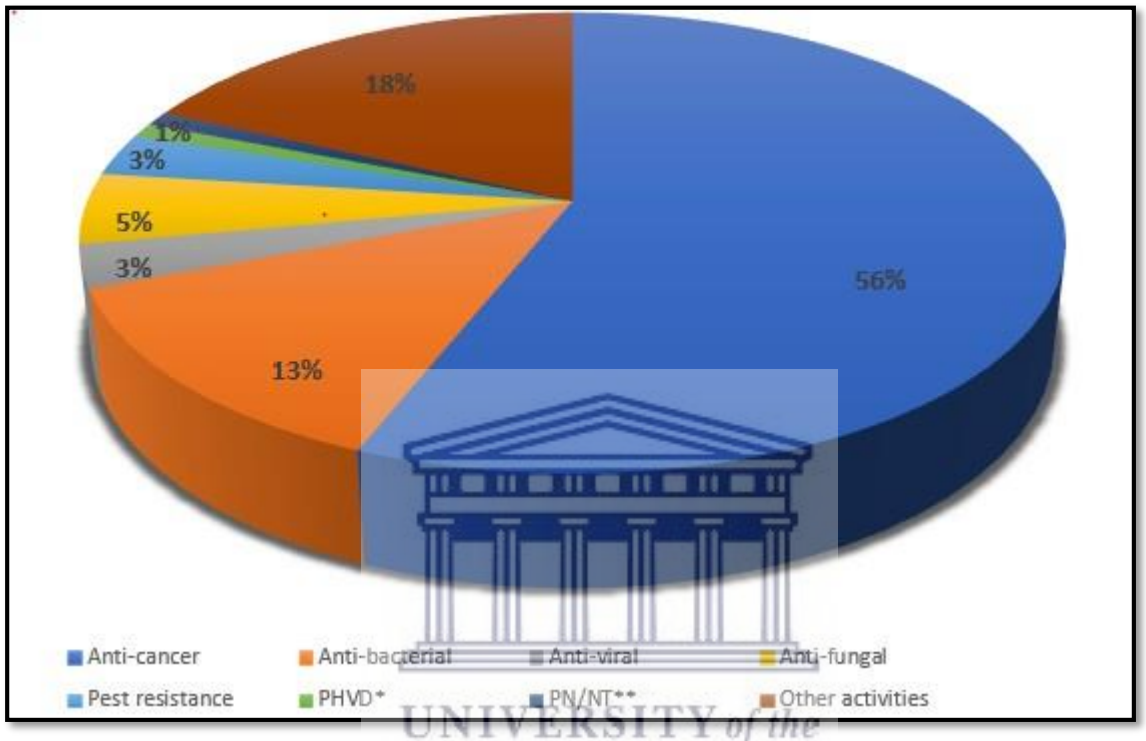


Figure 1.9: Diverse application of marine natural products. The above image represents the estimated bioactivity percentages (anti-cancer, anti-bacterial, anti-fungal, anti-viral and others) of marine natural products between the years 1985-2012 (* PHVD: Prevention of heart and vascular disease, ** PN/NT: Protection of neurons/neurotoxicity) *(Adapted from* Hu et al., 2015).

According to the marine pharmacology website (Marine pharmacology. midwestern. edu.n. d), 11 drugs are being clinically developed, and many other ocean-derived compounds have potential pharmacological activities: 592 marine compounds showed cytotoxic and anti-tumour activity, and 666 additional chemicals demonstrated other pharmacological activities such as anti-bacterial, anti-inflammatory, anti-fungal, anti-helminthic, anti-protozoal and anti-viral activities; with actions on the cardiovascular, endocrine, immune and nervous systems (Marine

pharmacology. midwestern. edu.n. d; Mayer et al., 2009).

Scientists have discovered that bioactive compounds thought to be produced by marine invertebrates shared structural similarity to compounds of microbial origin, and bacterial symbionts are now thought to be the true producers of many of these compounds (Bérdy, 2012). Two examples of this are the discovery of the anti-cancer agents Ecteinascidia 743 (ET-743) and Bryostatin I. ET-743 is a tetrahydroisoquinoline natural product that has been clinically approved in Europe and is used for ovarian neoplasms and sarcoma. ET-743, commercialised as Yondelis, was initially identified in the extract from a marine tunicate, *Ecteinascidia turbinata* (Figure 1.10B) (Bewley and Faulkner, 1998; Rinehart, 2000). Based on the structural similarity of ET-743 to bacterial derived natural products, (such as saframycin A from *Streptomyces lavendulae*, saframycin Mx1 from *Mycoccus xanthus* and safracin B from *Pseudomonas fluorescence*) Rath and co-workers hypothesized that ET-743 is a product of a marine bacterial symbiont (Rath et al., 2011). To test their hypothesis, they sequenced the metagenome of the microbial consortia in *E. turbinata,* and after rigorously analysing the metagenomic sequences, they identified *Candidatus Endoecteinascidia frumentensis* as the true producer of ET-743 (Rath et al., 2011; Trindade et al., 2015).

Bryostatin I, a cytotoxic polyketide agent currently undergoing phase I clinical trials, was initially detected in the extract from the marine Bryozoan, *Bugula neritina*. Researchers suspected that the compound was produced by a bacterial symbiont because there was a stronger correlation in the genetic composition between the Bryostatins and microbial symbionts than the Bryostatin and *B. neritina* (Davidson, 1999). Metagenomic screening was used to identify the PKS genes followed by in situ hybridization to identify the true producer. The results revealed that the origin of Bryostatin I is the bacterial symbiont, *Endobugula sertula*, found in the larvae of the Bryozoan (Davidson, 1999).

A more recent example is the discovery of the cyclic peptide polytheonamide. Although this compound has not been commercialized, it is composed of unusual amino acids and posesses high cytotoxic properties at an IC_{50} value of 78 pg/ml against P388 leukemia cell lines (Hamada et al., 2005). The polytheonamide was identified in the extract of the marine sponge, *Theonella swinhoei*. Microscopic analysis of the *T. swinhoei* extract revealed a highly enriched population of large filamentous bacteria that fluoresce when excited by ultraviolet light. The bacteria were morphologically similar to the symbiont *Candidatus Entotheonella palauensis*, previously reported from a Palauan *Theonella swinhoei* chemotype and suspected as the producer of antifungal peptides (Hamade et al., 2005). Fluorescence assisted cell sorting (FACS) coupled with gene specific PCR analysis indicated that the producer of the polytheonamide was an uncultured *Enthoteonella* sp (Wilson et al., 2014).

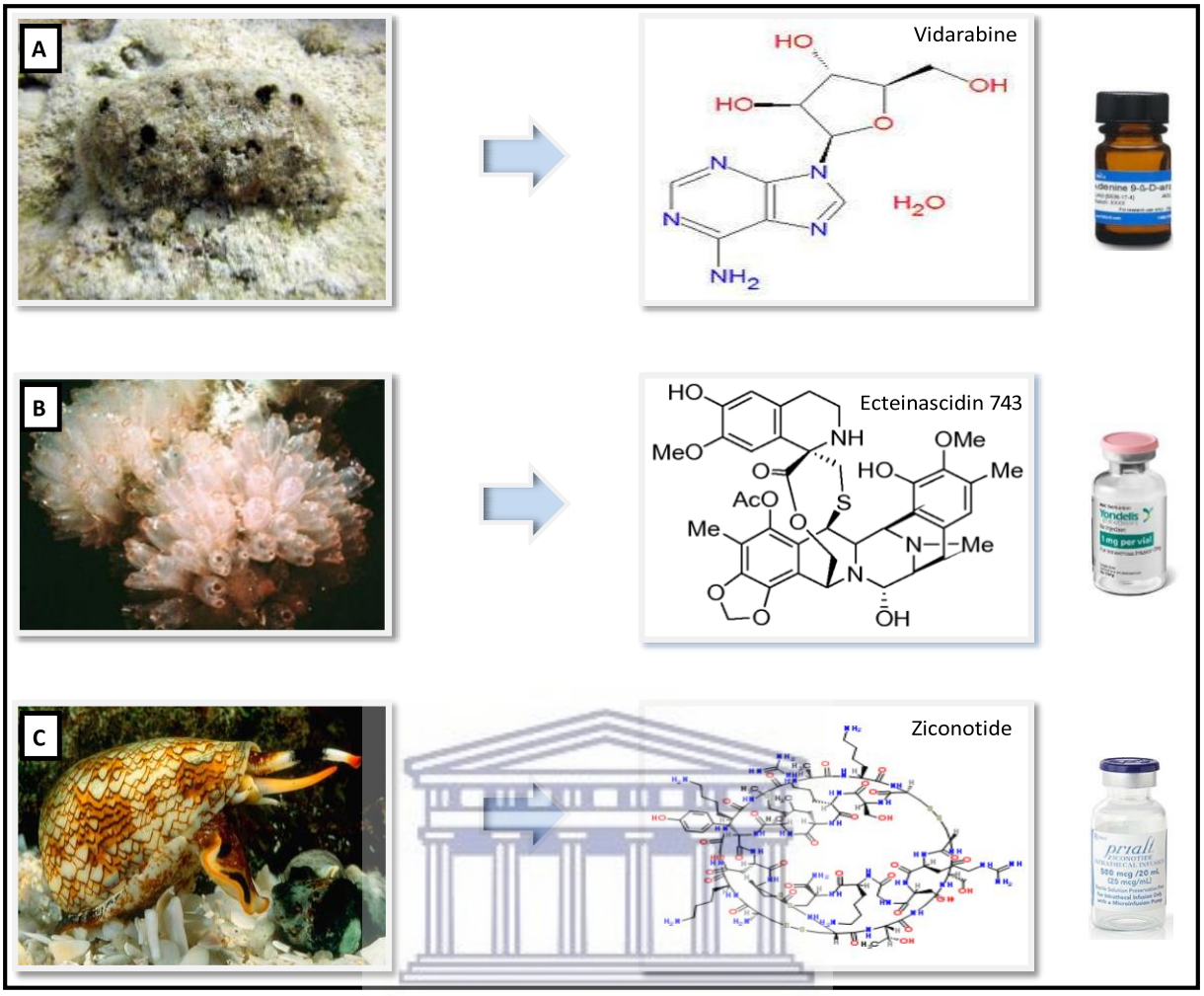


Figure 1.10: Marine bacterial products with clinical application. A) The anti-viral drug, Vidarabine (*adapted from* En.citizandium.org, 2009) that was isolated from the marine sponge, *Tethya crypta (adapted from* Abdullah, 2016). B) The anti-cancer agent, Ecsteinascidin 743 (ET-743) also known as Yondelis (*adapted from* New drug approvals, n.d) is used in the treatment of soft tissue sarcomas and relapsed ovarian cancer (Gerwick, 2012) and was isolated from a marine tunicate, *Ecsteinascidia turbinate (adapted from* Whatischemistry.unina.it, n.d). C) Ziconotide, also known Prialt (*adapted from* Chemspider.com, n.d) was semi-synthesized from a ώ-conotoxin molecule, isolated from the marine cone snail, *Conus magnus* (*adapted from* Biology.unm.edu, 2013) and is now used in the treatment of chronic pain (Gerwick & Moore, 2012).

These findings have led to the realization that many compounds isolated from macro- organisms are actually the metabolic products of their associated microbes (Hochmuth & Piel, 2009). For this reason, marine microorganisms have become a new source for the discovery of novel pharmaceutically active compounds which are now an important source of both existing and future drugs. Although marine sponge endosymbionts have been a reliable source of clinically useful compounds, there are many other marine phyla housing novel compounds with pharmaceutical relevance that requires exploitation (Yung et al., 2011). However, for the purpose of this study the phylum *Mollusca* and class *Bivalvia* is of particular interest.

1.5. Molluscs as a natural product drug cabinet

Mollusca constitutes an enormously diverse phylum and is considered the second largest animal phylum on earth. The majority of the Molluscan diversity occurs in the sea and is generally found on hard substrates such as rocky shores and coral reefs (Sharma et al., 2013). Currently, Mollusca is comprised of approximately 52,000 marine molluscs with an estimated diversity of 100,000-200,000 species (Benkendorff, 2010). Not only are molluscs diverse in their species richness but they also possess a wide range of morphologies and inhabit many different ecological niches (Beckendorff, 2010).

All molluscs are soft bodied organisms which are often found in microbial rich habitats in the sea, where the water column is estimated to have 10^5-10^6 microbial cells ml^{-1} (Periyasamy et al., 2012). Their bodies are often covered by hard exoskeletons as in the shells of snails, clams, oysters and chitons. However, the shell does not serve as a true physical barrier to microbial infection, making the molluscs vulnerable to pathogens and predators (Blunt et al., 2016). In order for Molluscs to protect themselves from pathogens, they rely on their innate immune

system effector molecules (Zannella et al., 2017). An example of their innate immunity is represented by the production of antimicrobial substances, primarily peptides or polypeptides which are produced by different kinds of cells and secretions, and are either constitutively synthesized or induced at the time of infection (Mayer et al., 2009). In some instances, they are also protected by secondary metabolites produced by their symbiotic bacteria (Macintyre et al., 2014). According to literature, molluscs lacking shells are sometimes defended by the secondary metabolites that they produce, and molluscs with shells produce peptide toxins in their defence (Liu et al., 2013). These compounds are thought to be produced by the symbiotic bacteria as a defence against other pathogenic microbes, leading researchers to exploit the microbial potential of the symbiotic bacteria, instead of exploiting the animal as a source of new natural products (Piel et al., 2004). Recent observations have demonstrated that extracts from marine molluscs have potent antimicrobial activity against several human pathogens which include; *Klebsiella pneumonia*, *Proteus mirabilis, Aeromonas hydrophila, Candida albicans*, *Vibrio cholera* as well as *Micrococcus* sp (Kumaran et al., 2011).

1.5.1 Marine Bivalves

Marine bivalves are the second largest class of molluscs that currently consist of 10,000-20,000 species (Sharma et al., 2013). These are primarily filter feeding organisms that pump large volumes of sea water and suspended microorganisms through their gills, making them susceptible to microbial infections (Beckendorff, 2010). Some bivalve families have specifically adapted to house microbial symbionts within their gills or mantle tissue to allow autotrophic nutrition, and in turn the symbionts assists their hosts in food digestion by degrading cellulose and agar from phytoplankton, fixing nitrogen and degrading algae, which

is an important resource for bivalves such as oysters (Li et al., 2017). In addition to their supportive role in oysters, nutritional value and digestion, oyster microbiota also provides protection against pathogenic bacteria by producing antimicrobial peptides or proteins as a defence mechanism to combat the pathogens (Li et al., 2017).

Bivalves have become an important group of organisms for drug discovery. A review written by Li and co-workers (2011) stated that several bivalves (*Mytilus galloprovincialis*, *M. edulis, M. trossulus, Crassostrea virginica* and *Rutitapes phillipinarum*) have been shown to be important sources of antimicrobial peptides. In total, 61 different antimicrobial peptides have thus far been isolated from the class of *Bivalvia* (Li et al., 2011). It is assumed that microorganisms inhabiting bivalves are the true producers of most, if not all of the antimicrobial peptides that are produced (Sharma et al., 2013).

1.6. Alternatives to antibiotics

As bacteria continue to develop resistance to currently used antimicrobials, new antimicrobial agents with different mechanisms of action are needed to fight resistant pathogenic bacteria (Galdiero et al., 2015; Yusof et al., 2017). Many attempts have been made to minimize antibiotic resistance. Advances in microbial genomics have provided tools identifying many new targets (such as those controlling bacterial metabolism through the interruption or diversion of riboswitches and those inhibiting microbial efflux pump activity) for antimicrobial drugs (Lloyd, 2012). However, the concern that bacteria, in time, will develop resistance to any new drug that becomes available, still remains. Therefore, instead of using drugs that target individual genes in disease causing bacteria, there is now a renewed interest in discovering a therapy which is able to eliminate the whole cell and is less likely to promote

resistance. A suitable therapy which is by no means new, is the use of bacteriophages and their lysins. This is because of their specific or broad spectrum of biological activities and their different action mechanisms which involves the degradation of the cell wall (a crucial bacterial component needed for survival), making them less susceptible to antimicrobial resistance (Galdiero et al., 2015).

1.6.1 Bacteriophages and their lysins as alternatives to antibiotics

Bacteriophages (or phages) are viruses that infect bacteria (Parisien et al., 2008). Bacteriophages were discovered many years ago and phage therapy was widely implemented to treat human and animal illnesses with varying degrees of success (Goodridge, 2010; Elbreki et al., 2014). However, the use of phage therapy has declined, chiefly due to the development of antibiotics (Drulis-Kawa et al., 2015). Other contributing challenges included a lack of understanding of phage biology, inadequate phage preparations and experimental techniques, underpowered clinical trials, documented clinical treatment failures (not understanding the cause of the ailments that are treated) and overzealous claims for efficacy (Negash & Ejo, 2016). In Eastern Europe, however, research into phage therapy continued and phages were used to treat a range of pathogenic bacterial infections caused by *Staphylococcus, Pseudomonas*, *Proteus* and enteric bacterial infections (Negash & Ejo, 2016). Recently, due to the alarming increase in the global rate of antibiotic resistance in pathogenic bacteria, phage therapy has re-emerged as a viable alternative to antibiotic therapy (Courchesne et al., 2009; Kiros, 2016; Negash & Ejo, 2016).

Phages are the most abundant, self-replicating organisms on earth and are found in every environment, wherever their hosts survive (Drulis-Kawa et al., 2015; Kiros, 2016). The genetic

material of bacteriophages is either single stranded (ss) DNA, double stranded (ds) DNA, ssRNA or dsRNA (Drulis-Kawa et al., 2015; Negash & Ejo, 2016) with the most abundant group of phages being tailed dsDNA (order *Caudovirales*). These phages are categorized into three families; *Myoviridae* (viruses with nonenveloped heads and contractile tails), *Siphoviridae* (nonenveloped, long non contractile tailed viruses) and *Podoviridae* (viruses with nonenveloped heads and short non-contractile tails) (Matsuzaki et al., 2005; Housby & Mann, 2009; Elbreki et al., 2014; Kiros, 2016). Morphologically, phages are composed of a protein capsid (or head) that stores all of the genetic information (Elbreki et al., 2014). The head is attached to a tail through a hetero-oligomer connector that is composed of head-to-tail (H-T) proteins. The tail functions as a passage way to allow the genetic material to be passed from the head to the bacterial host during infection. At the end of the connector is a baseplate to which the tail fibres are attached (Kiros, 2016). The baseplate and tail fibres are involved in the irreversible attachment of the phage to the outer membrane of its bacterial host (São-José et al., 2006). Surrounding the tail fibres and baseplate is a tail sheath that contracts upon infection (Figure 1.11; Lurz et al., 2001).

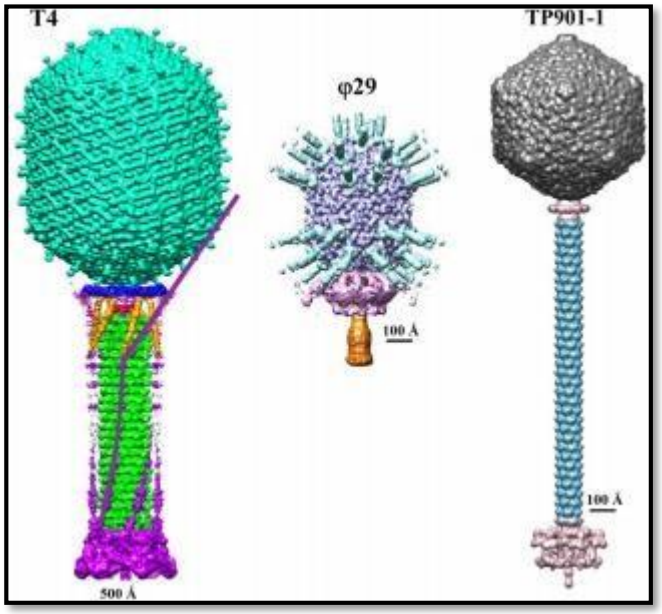


Figure 1.11: **Structure of different bacteriophage families**. Structure of the *Myoviridae* phage T4 (left), *Podoviridae* phage ϕ29 (middle), and *Siphoviridae* phage TP901-1 (right) (*Adapted from* Fokine & Rossmann, 2014).

Bacteriophages are obligate intracellular parasites, since, although they carry their own genetic information required for their reproduction, they cannot replicate without using the energy and protein biosynthetic machinery of a bacterial host (Summers, 2001; Parisien et al., 2008; Negash & Ejo, 2016). Depending on the phage, their lifestyles are divided into two categories; virulent phages that propagate through the lytic life cycle and temperate phages that propagate through the lysogenic life cycle (Inal, 2003; Courchesne et al., 2009). In the lysogenic life cycle, bacteriophages recognize and attach to the receptors on the host cell before injecting its nucleic acids into the host. Once inside the host cell, the viral DNA is integrated into a specific site on the host chromosome to form a provirus. When the cell divides both the host genome and provirus genes are inherited by the daughter cells. Once conditions become unfavourable such as an increase in phage titre and proteins, the provirus excises itself from the host chromosome and enters a lytic cycle. When this occurs, the host

cell's DNA is degraded, new virus particles are produced and assembled, resulting in the rupturing of the host and the release of new virions (Summers, 2001; Courchesne et al., 2009; Elbreki et al., 2014; Drulis-Kawa et al., 2015).

In the lytic cycle, once the virus has attached to the cell, viral DNA is injected into the bacterial host, beginning a cascade of events that results in the degradation of the hosts DNA. Subsequently, the virus totally commandeers the host metabolic activities and propagates using the hosts biosynthetic machinery. The viral DNA directs the production of new viral parts (head, tail, connector, tail spike, baseplate, tail fibre and tape measure protein) as well as DNA replication and DNA packaging into the phage head and the newly produced viral proteins are assembled into new virions. The combined action of lytic enzymes (holins and endolysins), ruptures the host cell and releases mature virions capable of infecting other bacterial hosts (Wommack & Colwell, 2000; Summers, 2001; Doss et al., 2017; Roach & Debarbieux, 2017). The two dominant bacteriophage life cycles (lytic and lysogenic life cycles) are depicted in Figure 1.12.

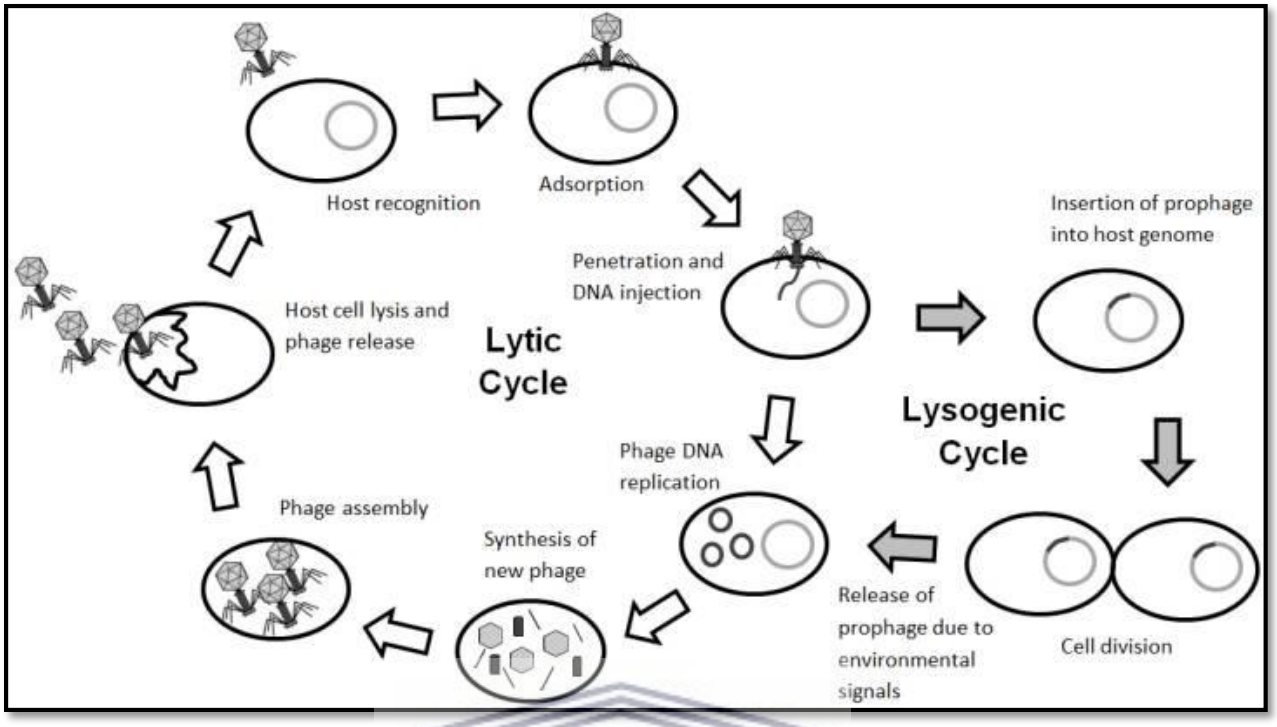


Figure 1.12: Illustration of the lytic and lysogenic cycles that temperate and virulent bacteriophages undergo to propagate. Before host cell rupturing in the lytic cycle, virulent phages destroy the bacterial cell wall by encoding lysin enzymes. During the lysogenic cycle, temperate phages are archived in the host genome and replicate with the host cell machinery without causing cell lysis. At a certain point in the lysogenic cycle, temperate bacteriophages can initiate the lytic cycle, eventually resulting in bacteriolysis (*Adapted from* Doss et al., 2017).

The narrow host specificity of bacteriophages suggest that they would be a useful tool against pathogenic bacteria when trying to specifically target certain species if given as oral, intravenous or topical phage therapy (Negash & Ejo, 2016). In situations where the broad spectrum and intrusive activities of antibiotics fail to clear bacterial infection, phages have been successfully therapeutically employed to cull the pathogenic species (Abedon et al., 2011). By way of their specific lytic activity, phages kill pathogenic hosts with a low risk of resistance evolution and with little to no observed adverse effects as the normal microflora remains unaffected when administered (Courchesne et al., 2009; Elbreki et al., 2014; Kiros, 2016). Table 1.1 summarises additional advantages of using bacteriophages in therapeutics

as opposed to antibiotics to combat antimicrobial resistance.

Table 1.1: Comparison of the therapeutic use of bacteriophages and antibiotics (Sulakvelidze *et al*., 2001).

Bacteriophages	Antibiotics
Very specific (i.e., usually affect only the targeted bacterial species); therefore, dysbiosis and chances of developing secondary infections are avoided	Antibiotics target both pathogenic microorganisms and normal microflora. This affects the microbial balance in the patient, which may lead to serious secondary infections
Replicate at the site of infection and are thus available where they are most needed	They are metabolized and eliminated from the body and do not necessarily concentrate at the site of infection
No serious side effects have been described	Multiple side effects, including intestinal disorders, allergies, and secondary infections (e.g., yeast infections) have been reported
Phage-resistant bacteria remain susceptible to other phages having a similar target range	Resistance to antibiotics is not limited to targeted bacteria
Selecting new phages (e.g., against phage-resistant bacteria) is a relatively rapid process that can frequently be accomplished in days or weeks	Developing a new antibiotic (e.g., against antibiotic-resistant bacteria) is a time-consuming process and may take several years

Phages, however, alone cannot entirely overcome bacterial resistance as bacteria can evolve resistance to phages. The most common type of resistant development is shielding, down-regulation and/or modification of bacterial cell surface receptors that are required for the phage to attach to the host (Roach & Debarbieux, 2017). This evolutionary process is termed “phage resistance” and is however not always detrimental to treatment because the required resistant mechanism could be a disadvantage to the bacteria. For example, should the bacteria lose their pathogenic genes through mutation, they lose their virulence to infect their host, they exhibit a lowered growth rate, a decreased life-span and they lose their ability to invade mammalian cells (Roach et al., 2013; Loc-Carrillo & Abedon, 2011). In certain cases, the evolution of phage resistance could yield pathogenic bacteria more sensitive to antibiotics (Roach & Debarbieux,

2017). Overall, bacteria are less resistant to phages than to antibiotics because phages have a higher mutation rate (Courchesne et al., 2009). According to several studies, phage therapy shows the potential to be used as a viable strategy to cure infectious disease caused by major pathogens (Table 1.2) (Abedon et al., 2011; Zhang et al., 2015).

Table 1.2: The ability of bacteriophages to control pathogenic bacteria. (*Adapted from* Elbreki et al., 2014).

Infection hosts	Bacteria	Phage	Main outcome
Mice	*E. coli*	Anti-K1	Better mice survival rates with phage
BALB/c mice	*Klebsiella*	*Klebsiella pneumoniae* bacteriophage	Rescue of generalized *Klebsiella* infection
Guinea pigs	*P. aeruginosa*	BS24	Skin graft protection from bacteria by phage
Mice	*E. coli, S. typhimurium*	λ and P22	Identification, isolation, and subsequent use of long circulating phage
Chickens and calves	*E. coli H247 (O18 : K1 : H7)*	ΦR	Protection against morbidity and mortality
Hamsters	*C. difficile*	CD140	5/6 hamster survived in the phage-treated group compared with none in the control
BALB/c mice	*Helicobacter pylori*	M13	Reduction of stomach colonization by *Helicobacter*
Mice	*E. faecium*	ENB6	100% survival 45 min after phage administration
Mice	*V. vulnificus*	CK-2, 153A-5, and 153A-7	Different results of mice protection depending on the phage used. CK-2 and 153A-5 protected mice, whereas 153A-7 did not
Mice	*E. coli*	LW and LH	Mortality rates in mice varied depending on the phage used
Mice	*S. aureus*	MR11	Better mouse survival rates with phage administration (MOI40.1) straight after bacterial administration
Chicken skin	*Salmonella enterica* and *Campylobacter jejuni*	P125589 and P22	Reduction by 2 log units in bacterial abundance over 48 hours
BALB/c mice	*Pseudomonas aeruginosa*	Phage Pf3R	Higher survival rate and reduced inflammatory response after 12–24 hours
Rabbits	*S. aureus*	LS2a	Reduction in abscess size in phage-treated animals and no difference when phage administration was delayed
Mice	*E. coli* O157 : H7	SP15, SP21, and SP22	Successive daily phage administration was required to reduce cell numbers from the gastrointestinal tract

Phage derived lytic proteins are called lysins or endolysins and are currently being explored as viable alternative antimicrobials to fight infectious disease (Loeffler, 2001; Negash & Ejo 2016).

Endolysins are lytic enzymes encoded by bacteriophages at the end of the lytic life cycle with the express purpose of lysing the bacterial host from inside. These enzymes target the peptidoglycan bonds in the cell wall and in that way cause cell lysis (Courchesne et al., 2009). The peptidoglycan of microorganisms is an essential component of the bacterial cell wall, conferring strength and rigidity to the cell, allowing growth and division, maintaining cell morphology, and protecting against osmotic lysis (Donovan et al., 2009). For endolysins to access the peptidoglycan layer in the cell wall, they need to cross the inner cell membrane with the help of a second lysis enzyme called a holin. A holin is a small membrane protein that facilitates hole formation in the cell membrane (Young, 1992; Young et al., 2000; Fischetti, 2005). Endolysins and holins are accumulated inside the host cell at the end of the lytic cycle, when the holins form pores in the cell membrane allowing endolysins to pass and break down the cell wall peptidoglycan (Elbreki et al., 2014). This process is depicted in Figure 1.13a.

Endolysins are generally composed of two structural domains: A C-terminal cell-wall binding domain and one or two N-terminal peptidoglycan-catalytic domains (Figure 1.13b) (Fischetti, 2005; Courchesne et al., 2009). The catalytic domain expresses atleast one of the five major types of lytic activity including: N-acetylmurimidase, lytic transglycosylase, endo-b-N-acetylglucosiminidase, endopeptidase or N-acetylmuramoyl-L-alanine amidase (Elbreki et al., 2014; Drulis-Kawa et al., 2015). The first three classes of lytic enzymes target the sugar moiety in the peptidoglycan while endopeptidases target the peptide moiety of the peptidoglycan and the amidases, hydrolyzing the amide bonds connecting the peptide moieties and glycan strand (Fischetti, 2005; Courchesne et al., 2009). Each C-domain is specific to a substrate in the bacterial cell wall. This binding domain is often but not always required for the action of endolysins (Elbreki et al., 2014; Courchesne et al., 2009).

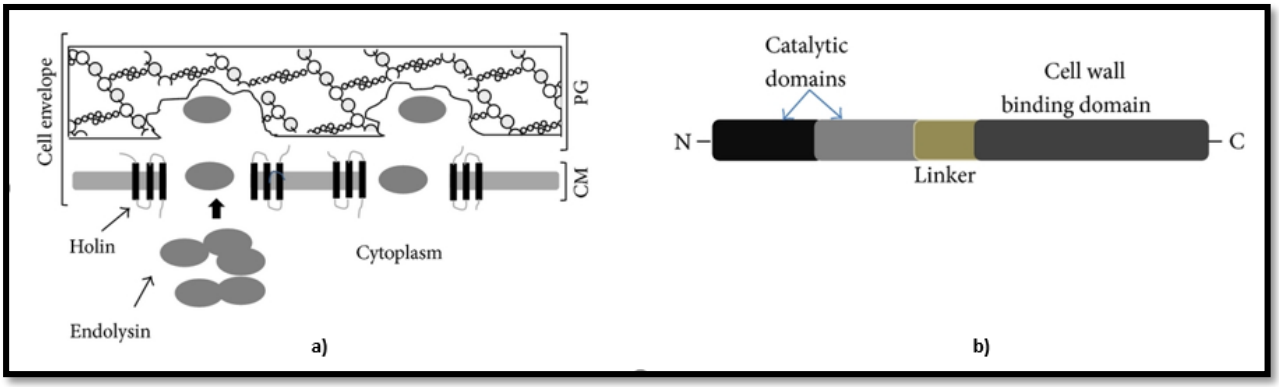


Figure 1.13: Schematic representation of phage encoded endolysins and their mode of action. a) The insertion of the holin into the cytoplasmic membrane is needed for the endolysins to access the peptidoglycan in the cell wall. b) Endolysin are generally characterized by one or two catalytic domains and cell wall binding domain that recognizes a specific substrate in the peptidoglycan of the bacterial cell wall (*Adapted from* Elbreki et al., 2014).

On a broader scope, endolysins belong to a group of bacterial cell wall hydrolases (BCWH's) that are deemed the most successful enzybiotics since they possess many clinically and therapeutically attractive features, including the ability to rapidly kill sensitive bacteria (Parisien et al., 2008). Endolysins have a narrow spectra of sensitive bacteria and due to the tremendous diversity of lytic phages in the biosphere, are readily available with great diversity. It is easy to detect bacteriolytic activity of phages to hosts, and that provides a solid basis to select phages for isolation of desired endolysins targeting specific pathogenic bacteria (Courchesne et al., 2009). Using endolysins in therapeutics has progressed over the years (Donovan et al., 2009; Nelson et al., 2012; Loessner, 2005). For example, Staphefekt^{TM}, a commercialised endolysin is now being used to treat skin infections by specifically targeting *S. aureus* and strains that are resistant to methicillin (MRSA). Thanks to its bacterial specificity it leaves beneficial bacteria unharmed, which is of great clinical importance considering longer term use. Another clinical example is an endolysin (LyS ABP-01) which specifically infects *Acinetobacter baumannii* and multi-drug resistant strains thereof (Thummeepak et al., 2016).

A. baumannii is one of the ESKAPE pathogens, a group of bacteria encompassing both Gram-positive and Gram-negative species, that includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae,* and *Enterobacter* sp. (Santajit et al., 2016). These bacteria are multi-drug resistant bacteria and the leading cause of nosocomial infections, which is one of the greatest challenges in clinical practice (Santajit et al., 2016). Lastly, there is a very low chance of bacteria acquiring resistance to endolysins, because to do that they would have make changes in the peptidoglycan which could be detrimental to the host itself. To date, no lysin resistant bacteria have been identified (Elbreki et al., 2014). Endolysins therefore offer an important advantage over classic antibiotics and are an attractive source of novel antimicrobial drugs due to their modes of action (Donovan et al., 2009).

1.6.2 Bacteriocins

Just like endolysins, certain classes of bacteriocins are highly specific enzymes and are possibly an alternative to conventional antibiotics. Bacteriocins are comprised of both, high and low molecular weight antimicrobial peptides or proteins, which generally exhibit bacteriacidal activity against other bacteria (Heu et al., 2001; Bastos et al., 2010). Bacteriocin production is a self-protection mechanism, employed by microorganisms to fight off closely related bacteria, conferring a survival advantage in their natural habitats (Bindiya et al, 2016). Bacteriocins generally target cells by binding to the receptors (Heu et al., 2001). They have a variety of bacteriacidal mechanisms such as pore formation, degradation of cellular DNA, disruption of specific cleavage of 16S rRNA and inhibition of peptidoglycan synthesis (Heu et al., 2001; Cotter et al., 2012). The bacteriocin family is comprised of many proteins that differ in size, microbial targets, modes of action and immunity mechanisms (Pattanayak, 2017). There are several classes of bacteriocins derived from Gram positive and Gram negative

bacteria that have greatly contributed to the pharmaceutical industry (Table 1.3).

Bacteriocins produced by Gram positive bacteria are divided into 3 classes, namely; Class I, Class II and Class III. Class I bacteriocins are comprised of post-translationally modified small peptides (<5kDa) called lantibiotics (Bindiya et al., 2016). This class is further divided into Type A and Type B, where Type A bacteriocins include positively charged, linear peptides (for example, nisin) (Flaherty et al., 2014) and Type B is comprised of neutral and negatively charged, globular peptides (mersacidin) (Meindl et al., 2010).

Class II bacteriocins are positively charged, heat stable peptides (< 10 kDa) that are not post-translationally modified (Gabrielsen et al., 2014). This class is subdivided into four subgroups; Class IIa, Class IIb, Class IIc and Class IId. Class IIa contains pediocin-like peptides which have a narrow spectrum of activity (Bindiya et al., 2016). Class IIb are comprised of bacteriocins that require the concertive action of two peptides to be fully active (Nissen-Meyer et al., 1992). Class IIc contains circular peptides such as carnocyclin A (Van Belkum et al., 2011). Class IId comprises single linear peptide bacteriocins such as epidermicin N101 (Sandiford & Upton, 2012).

Class III bacteriocins are heat sensitive peptides that are generally large (> 10 kDa). This class is further divided into 2 types; namely Type IIIa and Type IIIb. Type IIIa are comprised of bacteriolysins which are bacteriolytic enzymes which target the peptidoglycan in the cell wall of bacteria, for example, enterolysin (Nilsen et al., 2002). Type IIIb are non-lytic bacteriocins which rupture the cell membrane of the bacteria such as Helveticin (37kDa) (Joerger & Klaenhammer, 1986).

Table 1.3: Classes of bacteriocins in gram positive and negative bacteria (*Adapted from* Bindiya et al., 2016).

	Bacteriocins	Type/Class	Size	Example
Gram negative Bacteria	Colicins	Pore Formers	20-80	Colicins A, B
		Nucleases		Colicins E2, E3
	Colicin-like		20-80	S-pyocins Klebicins
	Phage-tail like		>80	Maltocin P28
	Microcins	Post translationally modified	<10	Microcin C7 Microcin B17
		Unmodified		Colicin V
		Class IIc - non-ribosomal siderophore-type post-translation modification		microcin E492
Gram positive Bacteria	Class I	Type A- Linear peptides, positively charged	<5	Nisin
		Type B- Rigid globular peptides, negatively or neutrally charged		Subtilosin A
	Class II	IIa - contain YGNGVxCxxxxCxV, Narrow spectrum of activity	<10	Pediocin, enterocin
		IIb – require concerted activity of 2 peptides		Lactacin F, Lactococcin G
		IIc – circular peptide bacteriocins		Carnocyclin A
		IId – linear, non-pediocin like, single peptide		Epidermicin NI01
	Class III	IIIa – bacteriolysin	>10	Enterolysin A
		IIIb – non-lytic bacteriocin		Helveticin A & J

Gram negative bacteriocins are divided into four classes, namely: microcins, colicins, colicin-like-bacteriocins (CLB's) and phage-tail-like bacteriocins also known as tailocins (Bindiya et al., 2016; Behrens et al., 2017). Microcins are low molecular weight antimicrobial peptides or natural products with modes of action similar to that of conventional antibiotics (Bastos et al., 2010), whereas colicins, first described in *E. coli*, are 25-90 kDa, heat and protease sensitive proteins. Colicins differ in their mode of action and can be divided into two types: nuclease colicins that kill bacteria by degrading nucleic acids in the cell; and pore forming colicins that kill other bacteria by forming pores in the cell membrane (Behrens et al., 2017). CLB's are

proteinaceous bacteriocins that have a similar structure and function to that of colicins, either acting as nucleases or pore formers. Examples of well-studied CLB's include S-pyocins produced by *Pseudomonas aeriginosa*, alveicins from *Hafnia alvei* and klebicins produced by *Klebsiella* sp (Bindiya et al., 2016).

Phage-tail-like bacteriocins (PTLBs) or tailocins are high molecular weight bacteriocins that are composed of multi-protein particles, morphologically similar to the tail structures of bacteriophages from the *Myoviridae* and *Siphoviridae* families (Patannayak, 2017). Examples of tailocins include; maltocin from *Strenotrophomonas maltophilia*, diffocin from *Clostridium difficile* and pyocins from *Pseudomonas aeruginosa* (Liu et al., 2013; Scholl, 2017). The best characterised tailocins are the R-type (related to contractile *Myoviridae* phage tails) and F-type (related to non-contractile *Siphoviridae* phage tails) pyocins (Behrens et al., 2017). Despite their large size, these bacteriocins and other phage lysins are highly potent and potential replacements for antibiotics because of their specificity and diverse action mechanisms (Behrens et al., 2017; Patannayak, 2017).

1.7 Bottlenecks in drug discovery and development

The development of many marine-derived bioactive compounds remains a challenging process. There are many bottlenecks that hinder the progression from natural compound discovery to their eventual commercialisation.

Assessing the various clinical applications of a secondary metabolite of interest, requires that the metabolite undergoes clinical trials such as phase 0, I, II, III and iv. Phase 0 involves toxicity tests to ensure that the drug is safe for humans to use. During phase I, the dosage and side effects of the drug is tested. In phase II, the efficacy of the drug is tested. Phase III evaluates

how the new drug works in comparison to existing medication for the same condition and in phase IV, the long term safety, effectiveness and any other benefits of the drug is tested (U.S food and drug administration, n.d). To successfully complete the various safety and efficacy trials, a large quantity of the compound is required. Thus, obtaining sufficient compound quantities from marine sources remains one of the biggest bottlenecks in the drug discovery pipeline (Macintyre et al., 2014). In some cases, where the compound of interest is thought to be produced by the macroorganism, the only solution is to harvest large amounts of the invertebrate to circumvent the supply limitations. For example, 13 tons of the bryozoan, *Bugula neritina* was needed to produce only 18 g of the cytotoxic agent, Bryostatin 1 (Piel, 2006). Harvesting such large quantities of the invertebrate negatively impacts the ecological balance in the natural environment, with detrimental ecological effects.

Since many of these natural products are produced by microorganisms, this provides the opportunity to produce them through microbial fermentation. For example, a precursor to Bryostatin 1 produced by the bacterial symbiont, *Candidatus Endobugula sertula*, is now being mass produced through microbial fermentation and the compound finished through chemical synthesis (Macintyre et al., 2014). The main limitation in trying to use the producing microorganism in fermentation, is that only an estimated 0.001-1% of microbial diversity has successfully been cultured in laboratories (Leal et al., 2013). The chances of being able to culture the producing organism are therefore slim. When it can be cultured, the organism may not be suited to current commercial fermentation processes necessitating additional steps such as heterologous expression of the gene(s) responsible for the compound production. However, there are many problems associated with heterologous expression. When a foreign gene or protein is expressed in a surrogate host it imposes a metabolic burden on that host thereby decreasing the available resources needed for the hosts' metabolic

activities. To maintain the natural function of the protein, the host chosen should have the same glycosylation, phosphorylation and acetylation patterns as the natural host (Glick, 1995). It is also important that the heterologous host recognise the same promoter regions as the native host so that the gene/s can be expressed (Gabor et al., 2004). If not, the pathway may have to be "re-factored' to provide promoters recognised by the heterologous host to enable expression of the pathway of interest. In addition to the metabolic load, the enzymatic activity or physical properties of some foreign proteins may also interfere with the cell functions of the host by converting an important and needed metabolic intermediate into a compound that is irrelevant, or may even be toxic to the cell (Glick, 1995). The generation of a construct encoding the secondary metabolite of interest is also a very challenging consideration because the biosynthetic pathway of the compound is often very large (Ongley et al., 2013; Pickens et al., 2011). Furthermore, regulating the expression of the pathway can be very difficult because in some cases the pathway relies on the products of other metabolic activities inside the cell and/or transcriptional factors that aid in the formation of the final product (Gabor *et al*., 2004). Thus, the absence of needed metabolic precursors might lead to the inability of the organism to produce the compound from the cloned pathway (Gabor *et al*., 2004; Ongley et al., 2013).

In the past bacterial isolations were based on their physical characteristics such as their morphology and pigment production and this has significantly contributed to the rediscovery of known compounds, now considered as the biggest bottleneck in the drug discovery pipeline. The reason for this is because bacteria that share similar morphological traits might have a different chemical profile and bacteria that differ in their morphology might still produce the same or known compound. To circumvent these issues, several dereplication

strategies such as High Performance Liquid Chromatography (HPLC), Liquid Chromatography-Mass Spectrometry (LCMS) and Nuclear Magnetic Resonance (NMR) have been employed to predetermine the possibility of identifying novel compounds from previously undescribed microbial communities (Harvey et al., 2015; Yang et al., 2013). The common denominator between the strategies is to exploit differences and similarities of molecules by observing whether they share physical characteristics such as UV-vis profiles, chromatographic retention times, mass to charge ratios and NMR chemical shifts to eliminate known compounds (Carnevale Neto et al., 2016). One of the biggest limitations using the afore mentioned dereplication strategies is that it examines the physiochemical differences and similarities of molecules and not the biological potential of the molecule. This results in the loss of compounds with significant biological functions. Thus to overcome this bottleneck different dereplication workflows need to be developed.

There are many dereplication workflows that have been developed to reach certain objectives. When searching for new bioactive molecules, the most commonly used workflow is the bioassay-guided fractionation procedure, in which biological or pharmacological assays are performed to target the isolation of the active molecule. This approach only regards biologically active fractions and disregards inactive compounds, saving time and resources (Guo et al., 2016). However, one of the drawbacks with this approach is that the purified biologically active molecule might be a known compound. To overcome this, dereplication analytical techniques can be integrated to overcome known compound rediscovery in bioactivity-guided fractionation procedures by rapidly setting aside undesired constituents (Hubert et al., 2017). Also, assays that target a specific property can be designed instead of using very general screens. In this way, the tedious isolation of known constituents can be

avoided so that the isolation, structural determination and pharmacological investigations can be carried out on novel bioactive compounds.

1.8 Progress in drug discovery

Recently, improvements in the drug discovery process have enabled the successful isolation and development of novel pharmaceutically active compounds despite the above-mentioned bottlenecks. This section discusses both culture-independent (Whole Genome Sequencing, genome mining and liquid chromatography-mass spectrometry) and culture-dependent strategies (the One Strain Many Compound method) that have been employed to overcome the challenges in natural product discovery.

1.8.1 Whole Genome Sequencing (WGS) and genome mining

The advent of genome sequencing of microorganisms has revealed a wealth of natural product biosynthetic genes that were previously unseen in culture dependent studies (Leal et al., 2012; Chaudhary et al., 2013). Genome sequencing holds great potential for unlocking novel chemistry and allows one to understand the genetic basis for the biosynthesis of novel drug molecules (Rutledge & Challis, 2015). In addition, it provides direct access to the genome and allows researchers to successfully “mine” for a variety of novel compounds at a nucleotide level, directing compound discovery away from previously described or known compounds (Pickens *et al*., 2011; Lane & Moore, 2012; Yamanaka et al., 2014). Genome mining of many *Streptomyces* species has revealed a number of cryptic yet novel secondary metabolite pathways that encode for a diverse range of previously undescribed compounds

(Table 1.4). These include bacteriocins, terpenoids, polyketides, non-ribosomal peptides, and other natural products (Chaudhary et al., 2013).

Table 1.4: A list of some of the natural product compounds identified in *Streptomyces* using genome mining (*Adapted from* Chaudhary et al., 2013).

Drugs	Strains	Applications
Aclacinomycin A	*S. galilaeus*	Antitumor
Actinorhodin	*S. coelicolor*	Antibacterial
Alnumycin	*Streptomyces* sp. CM020	Antitumor; gyrase inhibitor; topoisomerase inhibitor
Alpha-lipomycin	*S. aureofaciens*	Antibacterial
Amphotericin B	*S. nodosus*	Antifungal
Apramycin	*S. tenebrarius*	Antibacterial
Aranciamycin	*S. echinatus*	Antibacterial; Collagenase inhibitor
Ascomycin	*S. hygroscopicus* var. *ascomyceticus*	Immunosuppressive; antifungal
Asukamycin	*S. nodosus* subsp. *asukaensis*	Antitumor
Aureothin	*S. thioluteus*	Antitumor; antifungal; insecticidal
Avermectin	*S. avermitilis*	Anthelmintic
Benastatin	*Streptomyces* sp. A2991200	Antibacterial; apoptosis inducer; glutathione-S-transferase (GST) inhibitor
Bleomycin	*S.verticillus*	Antitumor
Borrelidin	*S. parvulus* Tü 4055	Angiogenesis inhibitor; antibacterial; antiviral; Antiproliferative
Chalcomycin	*S. bikiniensis*	Antibacterial
Chartreusin	*S. chartreusis*	Antibacterial; antitumor
Chlorothricin	*S. antibioticus*	Antibacterial
Chloramphenicol	*S. venezuelae*	Antibacterial
Chromomycin	*S. griseus*	Antibacterial; antitumor; antiviral
Coumermycin	*S. rishiriensis*	Antibacterial

To exploit the full natural product potential at a chemical level, there are several bioinformatics tools that can be used once an organism's genome has been sequenced. These include: antibiotics and Secondary Metabolite Analysis Shell (antiSMASH), Natural Product Domain Seeker (NaPDoS), NRPS predictor 2, the Database of BIoSynthesis clusters Curated and InTegrated (DoBISCUIT) and the PRediction Informatics for Secondary Metabolomes (PRISM). AntiSMASH is a database that bioinformatically identifies loci related to secondary metabolite classes such as polyketides, non-ribosomal peptides, bacteriocins, siderophores and others. It aligns the identified regions at the gene cluster level to their closest relatives from a database containing all other known gene clusters (Madema *et al*., 2011). NaPDos, analyses the domains architecture and phylogenetics of NRPS and PKS's and assesses the secondary metabolite biosynthetic gene diversity and novelty of strains (Ziemert et al., 2012;

Machado et al., 2015). NRPS predictor 2 is a database that predicts the amino acid substrate specificity of the adenylation (A) domains in NRPS, predicting possible monomer compositions of a compound and comparison between a potential novel and known pathway (Röttig et al., 2011). DoBISCUIT identifies domains in PKS/NRPS proteins and propose similar known biosynthetic clusters to their own (Ichikawa et al., 2013). PRISM predicts the chemical structure for genetically encoded NRPs and Type I and II PKs and predicts the bio-and cheminformatics dereplication of known NPs (Skinnider et al., 2015).

Using a combination of these computational tools, DNA sequence data can be exploited to allow new knowledge in many different areas relevant to medicinal chemistry. These include identifying and validating new drug targets and could also lead to the discovery of new chemical entities from natural products (Rutledge & Challis, 2015).

1.8.3 One Strain Many Compounds (OSMAC)

WGS studies have shown that microorganisms have the potential to generate a multitude of bioactive compounds (Imhoff et al., 2011). To realise the biosynthetic potential of marine microorganisms at a functional level, it is necessary to employ strategies to improve the production and concentration of the metabolites (Lane & Moore, 2012). Conventional laboratory culturing conditions are often used to selectively enrich microorganisms. However, it is often not conducive for secondary metabolite expression as cryptic pathways (unexpressed) go undetected during functional screening (Imhoff et al., 2011).

OSMAC is a simple and effective culture- based approach that aims at inducing secondary metabolite expression. It involves altering laboratory culturing parameters (such as media

composition, temperature, pH, pressure, aeration, culture vessel, etc.) to activate cryptic biosynthetic pathways and increase the production of secondary metabolites from one microbial source (Bode et al., 2002). Studies have shown that OSMAC approach is a powerful tool for bioprospecting microorganisms and for increasing the number of secondary metabolites from one species. For example, a study conducted by Bode and co-workers in 2002, proved that through using OSMAC they were able to isolate more than 100 compounds from only six different microorganisms and those compounds belonged to more than 25 different structural classes. This innovative "metabolic shunting" approach is a valuable tool to aid in the discovery of many novel compounds with pharmaceutical value.

1.9 Research objectives

The aim of this study was to screen a marine organism, *Thalassomonas viridans* XOM25^T for bioactivity and identify the biosynthetic gene responsible for the observed bioactivity in a using a bioassay-and-genome-guided isolation approach. This aim dictated the following objectives:

1. To functionally screen *T. viridans* for antimicrobial activity under different OSMAC conditions against a panel of indicator organisms.
2. To identify the biosynthetic gene in *T. viridans* responsible for the observed bioactivity under different OSMAC conditions through transcriptional profiling.
3. To perform a bioassay-guided fractionation using FPLC coupled with a proteomics and LCMS approach to identify the active compound.

Chapter 2 Materials and methods

2.1 General enzymes, chemicals and bacteria used in this study

All of the chemicals used in this study were supplied by Sigma Aldrich Chemical Company (Deissenhofen, Germany), Merck Chemicals and laboratory supplies (Darmstadt, Germany), and Kimix Chemical and Laboratory Supplies (South Africa). Culture media were supplied by Oxoid Ltd and Biolabs (Hampshire, England). Gene specific primers used in the polymerase chain reaction (PCR) were synthesized by Inqaba Biotech (Johannesburg, South Africa). DNA modifying enzymes (polymerases & restriction endonucleases) were purchased from Fermentas Life Sciences Ltd (Vilnius, Lithuania) and Thermo Scientific (USA).

For antibacterial screening purposes five indicator strains were used. For the detection of Gram positive antibacterial activity, strains *Bacillus cereus* ATCC10702 and *Staphylococcus epidermidis* ATCC14990 were used and for Gram negative activity screening the selected strains were *Pseudomonas putida* ATCC12633, *Vibrio neocistes* B-1037, *Vibrio percolans* B-781, *Vibrio rubicundus* B-782, *Vibrio chargasii* LMG 21353, *Pseudovibrio sp*. (PE 14-07, PE 14-03 and PE 5-40 from the IMBM collection) and *Vibrio sp*. (PE 14-63 from IMBM collection). *Escherichia coli* 1699 (Cubist, USA) was selected because it has been genetically engineered for resistance against 52 known antibiotics (Appendix A).

2.2 Genome mining for secondary metabolite pathways in *Thalassomonas viridans*XOM25^{T}

The genome of *T. viridans* was previously characterized (Olonade et al., 2015) and is available on NCBI (accession number: JYNJ00000000). In this study this genome sequence was submitted to antiSMASH 2.0 (http://antismash.secondarymetabolites.org [Accessed 2

February 2017]) or the detection and annotation of secondary metabolite pathways (Blin et al., 2013).

2.3 Antimicrobial activity screening of *T. viridans*

2.3.1 Culturing of bacterial strains

Thalassomonas viridans XOM25 was cultured on modified marine agar (MA) (Table 2.1) and incubated at either 15˚C or 28˚C until growth was observed and then transferred to medium and incubated at the same temperatures for 14 days while shaking at 150 rpm to enable production of secondary metabolites or antimicrobial proteins. Where indicated, the marine agar/broth was supplemented with different carbon sources. The yeast extract in the marine media was substituted with 0.1% of either sucrose, starch, cellobiose, maltose or glucose (Table 2.1).

The test organisms (*S. epidermidis*, *B. cereus*, *E. coli* 1699 and *P. putida*) used in this study were cultured in lysogeny broth (LB) medium (Table 2.2) and the *Vibrio sp*. were cultured in MB (Table 2.1). The test organisms cultured in LB were incubated at 30˚C and the *Vibrio sp* in MB were incubated at 15˚C until an optical cell density OD@600 nm of 0.3-0.4 was reached.

Table 2.1: Composition of Marine Broth (MB)

Chemical	Amount (g/l)
Peptone	5
Yeast extract	1
Ferric sulphate	0.1

Sodium chloride	19.45
Magnesium chloride	8.8
Sodium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Ammonium nitrate	0.0016
Disodium orthophosphate	0.008
Agar	15

Note: The marine broth was modified by replacing the yeast extract with 1 g/l of sucrose, maltose, glucose, starch and cellobiose.

Table 2.2: Composition of lysogeny broth (LB)

Chemical	Amount (g/l)
Yeast	5
Tryptone powder	10
Sodium chloride	10

2.3.2 Antibacterial activity screening using the well diffusion assay

Antimicrobial compounds were detected in cell-free culture broth using the well diffusion assay. Isolates were cultured in 50 ml of Marine Broth (MB) supplemented with one of six carbon sources (Section 2.3.1) for 14 days at 28˚C and 15˚C while shaking at 150 rpm in a 100 ml conical flask. Cells were separated from the broth by centrifugation at 10 000 *g* for 15 minutes at 4˚C. Ten millilitres of broth supernatant was concentrated until dry and the compound mixture was resuspended in 500 µl of 30% [v/v] DMSO. Indicator organisms, *E. coli* 1699, *B. cereus*, *S. epidermidis* and *P. putida* were cultured overnight at 37˚C in LB medium and adjusted to an OD_{600} between 0.3-0.4. *Vibrio* and *Pseudovibrio* test organisms were cultured for 48 hours in Marine Broth (MB) at 15˚C and adjusted to an OD_{600} between 0.3-0.4. Thereafter, 100 µl of culture was spread onto LB agar containing wells that were created using the wide end of a yellow pipette tip. The resuspended compound (100 µl) was loaded into the wells and allowed to stand at room temperature for 20-30 minutes, then incubated overnight at 37˚C. A clear zone around the well, in the bacterial lawn of the test strain was indicative of antimicrobial activity. The negative control contained 30% DMSO.

2.4 Nucleic acid extraction

2.4.1 Genomic DNA extraction

A single *T. viridans* colony was inoculated into 10 ml of the respective media and incubated at 30˚C on a shaking platform at 150 rpm, for 16 hours. The cell mass was harvested by centrifugation at 11 000 *g* for 5 minutes and the supernatant was discarded. The resulting pellet was resuspended in 500 µl of lysis buffer (25 mM Tris-HCl, pH 8; 50 mM glucose; 10 mM

EDTA; 25 mg/ml lysozyme powder) and incubated at 37˚C for 2 hours. A volume of 50 µl of 10% [w/v] SDS was added. The mixture was briefly vortexed, gently inverted and incubated at 65˚C for 20-30 minutes. This step was repeated until cell lysis was observed. An equal volume of chloroform-phenol (24: 1) was added and the solution was gently mixed by inversion followed by centrifugation at 11 000 *g* for 10 minutes. The upper aqueous phase was removed and transferred into a sterile microfuge tube. This step was repeated until the aqueous phase was clear. An equal volume of chloroform-isoamylalcohol (24:1) was added to the aqueous phase. The solution was gently inverted and centrifuged at 11 000 *g* for 2 minutes. Again, the aqueous phase was removed and transferred to a clean micro centrifuge tube. The DNA was precipitated by adding 1/10th of the volume of 3 M sodium acetate (NaAC), pH 5.2 followed by 0.6 volumes of 100% isopropanol. The mixture was gently inverted and allowed to precipitate at -20˚C overnight. The DNA was collected by centrifugation at 13 000 *g* for 30 minutes and the isopropanol was gently discarded. The DNA pellet was washed with 500 µl of ice cold 70% [v/v] ethanol, centrifuged at 12 000 *g* for 3 minutes and the ethanol was discarded. The DNA pellet was air-dried at room temperature for 20-30 minutes and suspended in 50 µl of 1× TE buffer, pH 8 (100 mM Tris-HCl, pH 8; 1 mM EDTA).

2.4.2 Total RNA extraction

A volume of 50 µl of the glycerol stock was inoculated into 50 ml of medium and cultured at 15˚C and 28˚C with shaking at 150 rpm. The cultures were cultured to the required growth stage (mid-logarithmic or stationary phase) and the cells were immediately harvested by centrifugation at 7 000 *g* at room temperature for 10 minutes. The supernatant was discarded and the cells were immediately resuspended in 0.75 ml of TRIzol reagent (Ambion, Life Technologies, USA). Total RNA was subsequently extracted from the cells as per the

manufacturer's instructions. The RNA was resuspended in 200 ul of 1× TE buffer.

2.5 Nucleic acid analysis

The purity and concentration of the DNA was determined by using the Nanodrop® ND-1000 (Nanodrop technologies, Inc., USA) and the Qubit™ Fluorometer (Life Technologies, USA).

2.5.1 DNA gel electrophoresis

A 0.8-3% [w/v] agarose gel was prepared in 1× TAE buffer (0.48% [w/v] Tris-base; 0.04 % [w/v] EDTA; 1.1% [v/v] glacial acetic acid). Ethidium Bromide (EtBr) was added to the solution at a final concentration of 0.5 µg/ml. The agarose gel solution was then poured into a gel tray and allowed to solidify. A concentration of 10× loading dye (20% [v/v] glycerol; 0.1 M EDTA, 1% [w/v] SDS; 0.25% [w/v] bromophenol blue) was added to the DNA samples. Electrophoresis was performed in 1× TAE buffer for 90 minutes at 90 volts. The size of the DNA was determined by comparing band sizes to a 100 bp molecular ladder (Biolabs) as well as phage lambda DNA completely digested with *Pst*I endonuclease (Thermo Scientific) as a molecular weight marker. The DNA was visualized using the Alpha Imager®HP 2000 (Alpha Innotech, USA).

2.5.2 RNA gel electrophoresis

RNA integrity was determined on a bleaching gel as described by (Aranda et al., 2012). A final concentration of 1% [v/v] bleach was added to the 1% [w/v] agarose and 1× TAE buffer mixture. The mixture was incubated at room temperature for 5 minutes with occasional swirling and then heated. Once the mixture was cooled, 5 µl of 0.5 µg/ml EtBr staining solution was added to the gel mixture. The solution was poured onto the gel tray and left to solidify. Electrophoresis was performed in 1× TAE buffer 90 volts/cm for 40 minutes. The size of the RNA was determined by

using lambda DNA completely digested with *Pst*I endonuclease as a molecular DNA ladder. The RNA was visualized under UV light (302nm) and images were obtained using the Alpha Imager®HP 2000 (Alpha Innotech, USA).

2.6 Polymerase chain reaction (PCR)

Primers used in this study, to amplify the 16S rRNA gene and the biosynthetic gene clusters, are listed in Table 2.3. The polymerase chain reaction (PCR) reactions were set up in 25 µl reactions. Each reaction contained 1× reaction buffer, 0.2 mM dNTP mix (dATP, dTTP, dCTP and dGTP), 1 µM of each of the forward and reverse primers, 0.25 U of a thermostable DNA polymerase (Dream Taq DNA polymerase) and 50-100 ng of the DNA/cDNA template. The reaction was adjusted to a final volume of 25 µl with nuclease free water. Amplifications were performed in an automated thermal cycler (T100 thermal cycler Bio-Rad). Table 2.3 summarises the PCR cycling parameters and the primer sequences.

Table 2.3: Primers and PCR cycling conditions

Primer	Target sequence	Sequence (5'-3')	Product size (bp)	Thermal cycling parameters	Reference
E9F U1510R	16S rRNA gene	GAGTTTGATCCTGGCTCAG GGTTACCTTGTTGTTACACTT	1500	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 55°C for 30 secs, 72°C for 1.30 min and final extension at 72°C for 5 min.	(Klindworth et al., 2013)
3aF 3aR	Cluster 3a: NRPS-T1 PKS Contig_22	TCCAATTTGA TTAAACGGGC TTCATCGTAGCCGGTCAAATC	479	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 56.3°C for 30 secs, 72°C for 29 sec and final extension at 72°C for 5 min.	This study
3bF 3bR	Cluster3b: NRPS-T1 PKS Contig_22	AAGCGTGTTT TGATAGATGG AACGGCTCGGCGATGGCGAT	479	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 56.3°C for 30 secs, 72°C for 29 sec and final extension at 72°C for 5 min.	This study
4F 4R	Cluster 4: other Contig_24	CTGCTGGC CGCCGGAGAATTC CAATAACTGGTAATGCTCCA	497	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 56.3°C for 30 secs, 72°C for 30 sec and final extension at 72°C for 5 min.	This study
5F 5R	Cluster 5: NRPS Contig_47	CCAGCGATTC ATCCTGCTCT GGTATTGCCGGATTATATGG	379	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 58.35°C for 30 secs, 72°C for 23 sec and final extension at 72°C for 5 min.	This study
6F 6R	Cluster 6: NRPS	CCTGATGGTG AAGGCCAGCT GTGAGCCCAGCACGATTGCC	349	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs,	This study

	Contig_49			64.5°C for 30 sec, 72°C for 21 sec and final extension at 72°C for 5 min.	
7F 7R	Cluster 7: NRPS Contig_74	TGCAGCCATT AACCCTGACA CAGCTCAAGGGTTCTTTCGC	529	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 60.4°C for 30 secs, 72°C for 32 sec and final extension at 72°C for 5 min.	This study
8F 8R	Cluster 8: NRPS Contig_98	CTGTATTCCA GGATCCTGAC CCGGCATTATTCCCGATGCC	489	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 60.4°C for 30 secs, 72°C for 30 sec and final extension at 72°C for 5 min.	This study
9F 9R	Cluster 9: NRPS-T1 PKS Contig_100	CCAATACTGCCAGGAAAAGT CGGCAATAGGCGAGTTGGTG	439	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 58.35°C for 30 secs, 72°C for 27 sec and final extension at 72°C for 5 min.	This study
10aF 10aR	Cluster 10a: NRPS-T1 PKS Contig_112	ATGGATACAAATATCACAGC CTAGCTGGCGCTATTTTCAA	209	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 54.25°C for 30 secs, 72°C for 13 sec and final extension at 72°C for 5 min.	This study
10bF 10bR	Cluster 10b: NRPS-T1 PKS Contig_112	TCCTCCGCCAATATTGCCCT CAGTACCGGCAGATGGCCTA	499	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 62.45°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 5 min.	This study
11aF 11aR	Cluster 11a: NRPS Contig_122	AGGCGGCCCA TAGGTTTCAG TAGTTACCGAGGTTGGCGAT	489	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 60.4°C for 30 secs, 72°C for 30 sec and final extension at 72°C for 5 min.	This study

11bF 11bR	Cluster 11b: NRPS Contig_122	ACTCTTCGTGGGCGTGATGG CGAAGCGTCTTCCACGGCCA	499	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 64.5°C for 30 secs, 72°C for 30 sec and final extension at 72°C for 5 min.	This study
12F 12R	Cluster 12: NRPS-T1 PKS Contig_134	CTGCGCAGCC ATGTCTCGCC GATCATCGACTGGACGGATTC	500	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 62.57°C for 30 secs, 72°C for 30 sec and final extension at 72°C for 5 min.	This study
14F 14R	Cluster 14: Lantipeptide Contig_152	CCAGGAAAACAATCTCGTGTT CGGGCATCTCCTATACCGACG	500	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 58.66°C for 30 secs, 72°C for 30 sec and final extension at 72°C for 5 min.	This study
15F 15R	Cluster 15: Bacteriocin Contig_159	CCAATGAACTACAACCCGAG ATTGTCATCGCGCTCTATCAT	500	Initial denaturation at 95°C, for 30 secs, 30 cycles of 95°C for 30 secs, 58.66°C for 30 secs, 72°C for 30 sec and final extension at 72°C for 5 min.	This study

2.6.1 Reverse transcription PCR

Prior to synthesising cDNA, the RNA samples were DNAse treated according to the DNAse I reaction protocol (BioLabs). RNA was precipitated from the DNAse mixture with 0.8 M lithium chloride (1/10th of the volume) and mixed by briefly vortexing or by flicking the tube several times with a finger. Twice the volume of 100% ethanol was added and the mixture was incubated at -20˚C for 90 minutes. The RNA pellet was collected by centrifugation at 13 000 *g* for 10 minutes at 4˚C and the supernatant was discarded. The RNA pellet was washed twice with 70% [v/v] ethanol and collected under the same centrifugation conditions. The supernatant was discarded and the RNA pellet was air-dried and resuspended in 50 µl of Nano pure water. The first strand cDNA synthesis kit (Roche) was used to synthesise complementary DNA (cDNA), according to the manufacturer's instructions from 1 µg of RNA, using random hexamers.

2.7 Identification of the bioactive protein

2.7.1 Sample Preparation

A volume of 1 L culture supernatant obtained from the 14-day old *T. viridans* cultures (MB supplemented with yeast extract, Section 2.3.1) was transferred to sterile glass beakers and concentrated at 40˚C in a drying oven until completely dried. The dried extract was resuspended in 30 ml of dH_2O. To remove particulate matter, the resuspended crude extract was transferred to sterile 50 ml Greiner tubes and centrifuged at 8000 *g* for 20 minutes. The supernatant was collected and filtered through a 0.45 µm filter to remove any remaining particles that could interfere with separation columns. Following filtration, the cell-free

extract (crude) was collected in a sterile 50 ml Greiner tube and stored at room temperature until further use.

2.7.2 Protein extraction

In this study, total protein was extracted from 6 cell-free extracts (prepared in section 2.7.1) produced by *T. viridans* under different culturing conditions (Section 2.3.1) by adding an equal volume of 100% acetone, followed by overnight incubation at -20˚C. After incubation, total protein was harvested by centrifugation at 12000 *g* for 15 minutes. The supernatant was discarded and the resulting protein pellet was resuspended in 100 mM Tris-HCl, pH 7.5. Crude extract proteins were quantified using the Bradford (Bio-Rad) Protein Assay 1.5 ml Protocol (Home.sandiego.edu, 1998). The crude extract protein refers to the total protein precipitated from the culture supernatant using acetone. The eluted proteins refer to the proteins eluted after semi-purification through FPLC.

2.7.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The integrity and purity of both extracted and eluted proteins (section 2.7.2) was assessed using SDS-PAGE. A SDS-PAGE typically consists of two parts, an upper stacking gel (Table 2.4) for protein loading and a lower resolving gel for protein separation (Table 2.5). A volume of 5 µl of 2× sample buffer (100 mM Tris-HCl, pH 8; 4% [w/v] SDS; 0.2% [w/v] bromophenol blue; 200 mM β-mercaptoethanol) was added to 10 µl of protein sample. The protein mixture was incubated in a heating block for 5-10 minutes at 95˚C followed by pulse centrifugation. Protein samples and 1 to 4 µl of protein molecular weight marker was transferred to the stacking gel

wells. Proteins were electrophoresed using 1× running buffer (25 mM Tris-HCl, pH 8; 200 mM glycine; 0.1% [w/v] SDS) in a Mini PROTEAN system (Bio-Rad Laboratories, Hercules, CA, USA) at 60mA for 30 minutes. Polyacrylamide gels were either silver stained or placed in Coomassie Brilliant Blue G250 for 10 minutes. To remove excess Coomassie stain (40% [v/v] methanol; 10% [v/v] acetic acid; 0.05% [w/v] Coomassie Brilliant Blue G250) gels were placed in a destaining solution (40% [v/v] methanol; 10% [v/v] acetic acid) until the bands became visible. For polyacrylamide gels that were silver stained, proteins were fixed in 30% ethanol; 0.5% acetic acid for 3× 30-minute intervals, and transferred to 20% ethanol for 10 minutes. Thereafter the gel was placed in distilled water for 10 minutes and later transferred to 0.2 g/L of freshly prepared sodium thiosulphate for 2 x 20 second washes. The gel was then placed in 1 g/L of silver nitrate for 30 minutes at room temperature with gentle swirling. Following incubation, the silver nitrate was discarded and the gel was rinsed with water for 5-10 seconds. To visualize the protein bands, a developer (20 g/L sodium bicarbonate$_3$; 973 µl/L formaldehyde) solution was added to the gel for 2 x 3 minutes or to the desired intensity. The staining reaction was terminated by the addition of Tris-base (50 g/L- 2.5% acetic acid) for 1 minute. Gel images were recorded using the Alpha- Imager® HP 2000 (Alpha, Innotech, USA).

Table 2.4: Preparation of stacking gel (4%) used in this study.

dH_20	3.075 ml
0.5M Tris-HCl, pH 6.8	1.250 ml
20% (w/v) SDS	0.025 ml
Acrylamide/ Bisacrylamide (30%/ 0.8% w/v)	0.670 ml
10% Ammonium Persulfate (APS)	0.025 ml
Tetramethylethylenediamine TEMED	0.01 ml

Table 2.5: Preparation of separating gel.

	10%	12%
dH_20	4.1	3.4
1.5M Tris-HCL, pH 8.8	2.5	2.5
20% (w/v) SDS	0.05	0.05
Acrylamide/ BisacrylamidE (30%/ 0.8% w/v)	3.3	4.3
10% Ammonium Persulfate (APS)	0.05	0.05
Tetramethylethylenediamine TEMED	0.01	0.01

2.7.4. Protein identification by liquid chromatography-mass spectrometry (LC-MS).

Total protein extracted in section 2.7.2 and the semi-pure fraction generated in section 2.7.7 were analysed using LC-MS.

LC-MS analysis of the total proteins extracts were conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-HPLC system. Peptides were dissolved in 0.1% (v/v) formic acid (FA; Sigma 56302), 2% (v/v) acetonitrile (ACN; Burdick & Jackson BJLC015CS) and loaded on a C18 trap column (300 µm × 5 mm × 5 µm). Chromatographic separation was performed with a PepAcclaim C18 column (75 µm × 25 cm × 2 µm). The solvent system employed was solvent A: LC water (Burdick and Jackson BJLC365); 0.1% (v/v) FA and solvent B: ACN, 0.1% (v/v) FA. The multi-step gradient for peptide separation was generated at 300 nL/min as follows: time change 6.2 min, gradient change: 3.5 – 11.4% Solvent B, time change 45.3 min, gradient change 11.4 – 24.6% Solvent B, time change 2 min, gradient change 24.6 – 38.7% Solvent B, time change 2.1 min, gradient

change 38.7 – 52.8% Solvent B. The mass spectrometer was operated in positive ion mode with a capillary temperature of 320°C. The applied electrospray voltage was 1.95 kV. Database interrogation was performed with Byonic Software (Protein Metrics, USA) using a *T. viridans* database sourced from UniProt (www.uniprot.org).

For the semi-pure *T. viridans* protein fraction in 100 mM Tris-HCl, pH 8 (section 2.7.7); 50 µg of protein was aliquoted into a new protein LoBind tube. Protein was reduced with TCEP which was added to a final concentration of 10 mM TCEP per sample, and incubated at 60˚C for one hour. Samples were cooled to room temperature and then alkylated with MMTS which was added to a final concentration of 10 mM MMTS per sample, and incubated at room temperature for 15 minutes. In preparation for the HILIC magnetic bead workflow, beads were aliquoted into a new tube and the shipping solution removed. Beads were then washed with 250 µl wash buffer (15% ACN, 100 mM Ammonium acetate, pH 4.5) for one minute. This was repeated once. The beads were resuspended in loading buffer (30% ACN, 200 mM Ammonium acetate, pH 4.5). The sample was transferred to a protein LoBind plate and 250 µg HILIC magnetic beads in 150 µl loading buffer were added and incubated on a shaker at 900 rpm for one hour for binding of protein to beads. After binding, the beads were washed twice with 500 µl of 95% (v/v) ACN for one minute. Thereafter, the beads were resuspended in 200 µl of 0.025 µg/µl trypsin made up in 50 mM TEAB and incubated at 37˚C on the shaker for four hours. After digestion, the supernatant containing peptides was removed and dried down. Thereafter, samples were resuspended in LC loading buffer: 0.1% (v/v) FA, 2.5% (v/v) ACN.

Proteins, containing at least two unique peptides and that were above the first false positive protein identified for each sample, were reported. The raw data was searched against a *T. viridans* (which is the exact strain sequenced by IMBM) database (Thalassomonas_viridans_uniprot-proteome_Unrev_14072017.fasta) of unreviewed

proteins downloaded from UniprotKB.

2.7.5 Size fractionation

Prepared crude cell-free extract (Section 2.7.1) was first transferred to a 50 kDa Amicon cut-off filter (Amicon®Ultra, Merck, USA) and centrifuged at 5000 *g* for 5 minutes until 1 ml was left in the upper reservoir. To ensure that all the smaller proteins had passed through the filters, 5 ml of dH_2O was added followed by another centrifugation step at 5000 *g* until the initial volume of 1 ml was left in the upper reservoir of the Amicon filter. These steps resulted in a fraction with proteins > 50 kDa and another with proteins and compounds < 50 kDa. The < 50 kDa fraction was added to a 3 kDa cut-off Amicon filter and the same series of centrifugation and washing steps as for the 50 kDa filter was repeated. This resulted in 2 fractions containing material between 3-50 kDa and < 3 kDa. After centrifugation, the different fractions (< 3 kDa, > 3 kDa- < 50 kDa and > 50 kDa) were collected and transferred to sterile 15 ml Greiner tubes. Each collected fraction was assayed using the well-diffusion bioactivity assay against a range of pathogenic indicator organisms (Section 2.3.2; Figure 2.1). dH_2O was used as a negative control for anti- microbial activity.

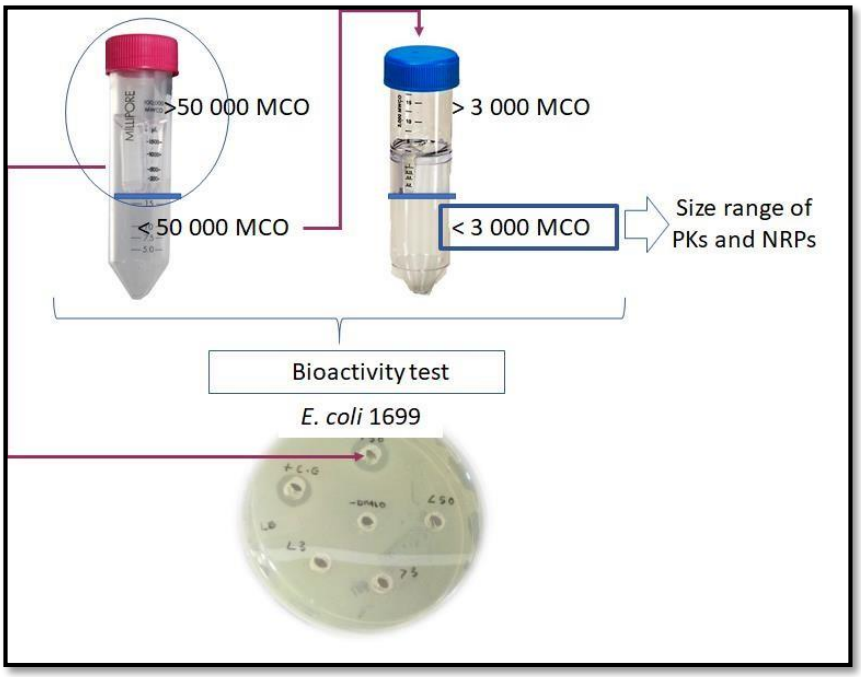


Figure 2.1: Flow diagram for the size fractionation process. MCO refers to molecular weight cut-off.

2.7.6 Ion exchange chromatography

To further purify the fraction in which the active compound was retained (> 50 kDa), ion exchange chromatography was used to separate the proteins based on charge. A volume of 1ml of the partially purified concentrated extract was injected onto the column (mono Q HiPrep™ 16/10 QFF; Amersham Biosciences AB, Sweden). The column was attached to an AKTA™ FPLC UPC 900 system (Amersham Biosciences AB, Sweden). The column was equilibrated with 50% Buffer B (100 mM Tris-HCl, pH8 + 1 M NaCl) then washed with 20 column volumes of Buffer A (100 mM Tris-HCl, pH8) at 5 ml/min to remove unbound protein (positively charged proteins). Bound fractions (negatively charged proteins) were eluted with 100% Buffer B at a flow rate of 2 ml/min. A volume of 40 ml of both bound and unbound protein fractions were collected in 50 ml Greiner tubes and then transferred to a 50kDa cut-off Amicon filter (Amicon@Ultra, Merck, USA), and concentrated to a final volume of 1 ml. Each protein fraction (100 µl) was subjected to a well diffusion bioactivity assay (Section 2.3.2) to

determine the fraction in which the active compound was retained. Buffer A was used as a negative control for antimicrobial activity tests. Purity of the collected fractions were assessed by SDS-PAGE (Section 2.7.3).

2.7.7 Gel permeation chromatography

The fraction(s) that retained bioactivity after ion exchange chromatography was further purified using gel permeation chromatography. A volume of 1 ml of the partially pure extract was subjected to separation using the AKTA™ FPLC UPC 900 system (Amersham Biosciences AB, Sweden) with a gel permeation Superdex™ 200 10/300 GL column (GE Healthcare Bio-sciences AB, Sweden) equilibrated with 10 column volumes of Buffer B (100 mM Tris-HCl, pH8; 50 mM NaCl) and protein eluted with 30 column volumes. Two millilitre fractions were collected and each was concentrated using a 50 kDa cut-off Amicon filter (Amicon®Ultra, Merck, USA) to a final volume of 1 ml. The concentrated fractions (100 µl) were then subjected to a bioactivity assay (Section 2.3.2) to identify the fraction in which active compound was retained. Buffer A and B were used as negative controls for antimicrobial activity. The purity of the active fraction was assessed on a 12% SDS-PAGE gel (Section 2.7.3).

2.7.8 Zymography

The bacterium, *P. putida* was cultured in 10 ml of LB media and incubated at 37˚C overnight for 16 h. Following incubation, cells were harvested by centrifugation at 12000 *g* for 15 minutes before the supernatant was discarded. A 10% resolving acrylamide SDS-PAGE gel, was spiked with 2 mg dry weight *P. putida* cells, which is the substrate. A standard stacking gel, lacking substrate cells was placed on top of the resolving gels. Sample buffer was added

to 250 ng of the > 50 kDa active crude protein fraction (prepared in Section 2.7.5), produced under marine broth supplemented with yeast extract. Electrophoresis was conducted using the method described in section 2.7.3. After electrophoresis, the gel was placed and incubated in a renaturation buffer (100 mM Tris-HCl, pH 8; 2.5% Triton X-100), overnight at 37˚C. Thereafter, the gel was rinsed with Milli Q water and transferred into an incubation buffer (100 mM Tris-HCl, pH8; 1% Triton X-100) for 24 hours at 37˚C. The gel was rinsed with Milli Q water and stained with Coomassie Brilliant Blue G- 250 for 2 hours at room temperature. After staining, the gel was placed in a destaining solution for 1 hour at room temperature. A white zone against a blue background was indicative of hydrolytic activity.

2.7.9 In gel trypsinisation

The semi-pure fraction obtained in section 2.7.7 was subjected to SDS-PAGE (section 2.7.3) for further purification.

The active gel band (50.5 kDa) was excised and destained with 200 µl of 100mM ammonium bicarbonate in 50% (v/v) acetonitrile (ACN) until clear. Gel pieces were dehydrated and desiccated with 100 µl ACN followed by reduction with 2 mM Tris (2-carboxyethyl) phosphine (TCEP) for 15 min at room temperature (with agitation). Thereafter the gel pieces were alkylated with 20 mM iodoacetamide for 30 min in the dark. Following alkylation, gel pieces were washed with 25 mM ammonium bicarbonate for 15 min, treated with 0.4 µg of trypsin (Promega, USA) and incubated on ice for 1 hr. After incubation, excess trypsin solution was discarded and 20 µl of 50 mM ammonium bicarbonate was added to the gel pieces. The samples were then allowed to incubate at 37°C for 16 hr. After complete tryptic digestion, peptides were extracted from the gel by adding 20 µl of extraction solution (0.1% (v/v) TFA in

30% (v/v) acetonitrile). The solution was placed on a shaking platform for 45 min at room temperature. The supernatant was transferred to a clean Eppendorf tube and the extract was dried down. The dried pellet was resuspended in 0.1% (v/v) FA, 2.5% (v/v) ACN. The resuspended peptides were analysed using LC-MS (Section 2.7.4) to identify the active protein.

Chapter 3: Dereplication as a tool for the discovery of novel secondary metabolite pathways from a marine invertebrate associated bacterium.

3.1 Introduction

The discovery and development of novel bioactive compounds is challenging as one of the biggest problems in the drug discovery pipeline is the rediscovery of previously identified compounds (Yang et al., 2013; Harvey et al., 2015). The marine environment has been shown to harbor exceptionally novel molecules, distinct from those identified in terrestrial environments (Pickens et al., 2011). Whole genome sequencing (WGS) coupled with computational mining of microbial genomes has become an important part in the discovery of novel natural products as drug leads (Lane & Moore, 2012). Thousands of bacterial genome sequences are publicly available these days containing an even larger number and diversity of secondary metabolite gene clusters that have not been linked to their encoded natural products. With the development of high-throughput sequencing methods and the wealth of genomic data available, a variety of genome mining methods and tools have been concurrently developed to guide the discovery of new drug candidates, aiming at avoiding wasted effort and resources on the rediscovery of known compounds (Yamanaka et al., 2014).

To realise the biosynthetic potential of marine microorganisms at a functional level, it is necessary to employ strategies and improve the production of the metabolites (Lane & Moore, 2012). The genomes of most readily cultured bacteria contain cryptic biosynthetic pathways, representing a large reservoir for the discovery of novel antimicrobial or bioactive compounds (Milshteyn et al., 2014) since traditional culturing methods restrict the expression of some

biosynthetic pathways (Leal et al., 2012; Chaudhary et al., 2013; Okada & Seyedsayamdost, 2017).

Finding optimal conditions required to activate biosynthetic pathways has been difficult and therefore no direct one-size-fits-all one strain many compounds (OSMAC) approach (Section 1.8.3) has been developed to induce the expression of these silent gene clusters (Bode et al., 2002). One approach (Section 1.8.3) to induce the expression of secondary metabolites is by altering the constituents in culture media, such as the carbon and nitrogen source, inorganic phosphate, inorganic salts, trace metals, precursors, inhibitors, inducers and others (Iwai & Omura, 1981; Bode et al., 2002; Sánchez et al., 2010; Milshteyn et al., 2014). Among these nutrient sources, the downstream effect of the carbon sources has been the subject of continuous studies for both industry and academic research groups. For example, Amagata and colleagues (2007) showed that *Gymnascella dankaliensis* produces unusual steroids, dankasterone A (which is active against *E. faecalis* and the vancomycin resistant (VRE) *E. faecalis*) (Kumla et al., 2017) and B when grown in a modified malt extract medium containing soluble starch instead of glucose (Amagata et al., 2007). Also, a study conducted by Matobole et al (2017) showed that *Bacillus zhangzhouensis* isolated from the sponge, *Isodictya compressa* only produced activity against *S. epidermidis* when cultured in succinic acid as the carbon source. However, when *B. zhangzhouensis* was cultured in mannitol containing media, a wide range of bioactivity was exhibited against *S. epidermidis*, *P. putida*, *B. cereus*, *E. coli* 1699 and *Mycobacterium smegmatis* (Matobole et al., 2017).

Marine microorganisms possess a wealth of novel natural product chemistry. Many compounds previously isolated from macroorganisms such as sponges and tunicates, are actually metabolic products of associated

microbes (Fisch et al., 2009). As part of a large screening program at the Institute for Microbial

Biotechnology and Metagenomics (IMBM), *Thalassomonas viridans*, isolated from oyster larvae (*Ostrea edulis*) off the coast of Spain, Valencia (Pujalte et al., 1999) was identified to show some antimicrobial activity. *T. viridans* belongs to the order *Alteromonadales* (Park et al., 2011) in the class *Gammaproteobacteria* and has been characterised as a Gram negative, rod shaped, chemoorganotrophic, halophilic, mesophilic and strictly aerobic bacterium (Macian et al., 2016; Yi et al., 2004; Thompson et al., 2006; Hosoya et al., 2009). Genome sequencing data generated by Olonade *et al*. (2015) revealed that *T. viridans* is a novel bacterium with an average nucleotide identity of 81% to its closest relative *T. actiniarum,* also indicating that it likely produces novel chemistries and bioactive compounds (Olonade et al., 2015). To our knowledge, this species has not previously been exploited for its biological activity. Hence, we were particularly interested in the antimicrobial potential of the marine bacterium *T. viridans*.

In this chapter, an invertebrate-associated bacterium, *T. viridans* was screened for antimicrobial activity using the OSMAC strategy, previously described in section 1.8.1. In an attempt to identify the secondary metabolite pathway responsible for the inhibitory activity against the indicator test microorganisms (*P. putida*, *S. epidermidis*, *B. cereus*, *E. coli* 1699), transcriptional profiling and secondary metabolite pathway prediction as well as a bioassay-guided fractionation was employed using WGS data as a guide.

Results and Discussion

3.2Assessing the antimicrobial potential of *Thalassomonas viridans*, using an OSMAC approach.

To exploit the antimicrobial potential of *T. viridans* and induce production of a variety of potential bioactive compounds, the carbon sources (sugars) of the medium were altered to determine whether the expression of bioactive compounds could be induced. In this study, *T. viridans* was cultured in marine broth (MB) supplemented with one of six different carbon sources (yeast extract, sucrose, maltose, cellobiose, starch and glucose; Table 2.1). Each crude extract of *T. viridans* generated in the different culture media was concentrated and tested for bioactivity. In this study all bioactivity experiments were conducted using the well-diffusion assay (Section 2.3.2), where a zone of clearance around the well represents antibacterial activity (Figure 3.1).

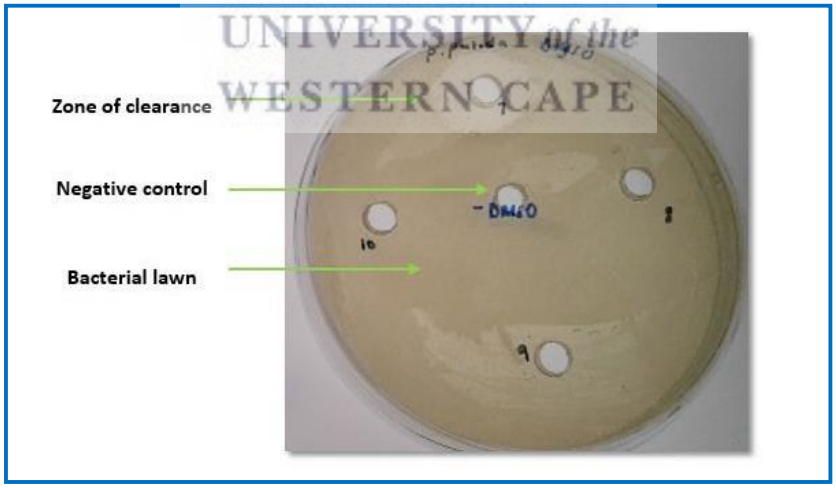


Figure 3.1: Detection of antibacterial activity using the well diffusion assay. The zone of clearance surrounding the well represents antibacterial activity.

From the antimicrobial profile in Table 3.1, it is evident that a change in carbon source, and additional nitrogen source in the case of yeast extract, influences the induction or expression of different biosynthetic pathways in *T. viridans* generating different antimicrobial profiles.

The functional screening data revealed that the crude extract from *T. viridans* was active against a range of indicator organisms in five of the six carbon sources. The antimicrobial activity was highest in yeast extract-containing medium and no bioactivity was observed when sucrose was served as a carbon source in the culture medium.

Table 3.1: Antibacterial profile of *T. viridans* influenced by different carbohydrate sources in Marine Broth (MB).

Culturing condition	*E. coli* 1699	*S. epidermidis*	*B. cereus*	*P. putida*
MB+ yeast extract	++	+++	++	+++
MB+ maltose	++	+++	-	++
MB+ cellobiose	+	++	+	++
MB+ sucrose	-	-	-	-
MB+ glucose	+	++	-	++
MB+ starch	+	++	-	++

The (+) sign represents antibacterial activity and the number of (+) signs represents the intensity of antibacterial activity; where + represents a zone clearance diameter of 1mm, (++) represents 4mm and (+++) represents a zone diameter of 7mm. The (-) sign is indicative of no antibacterial activity after 14 days of growth.

The lack of bioactivity in sucrose-supplemented MB could be due to the fact that *T. viridans* did not grow well in this medium as observed by experimental growth curves (Figure 3.2). However, when the genome of *T. viridans* was submitted to the Rapid Annotation using Subsystem Technology (RAST), it was discovered that *T. viridans* contains the necessary genes to metabolise sucrose such as sucrose phosphorylases, sucrose permeases and fructokinases and hence should be able to metabolise this substrate (Reid & Abratt, 2005). The inability of

S. viridans to produce substantial biomass in sucrose-containing media could possibly have resulted in a low production of the bioactive compound/ (s), and no detection thereof in the antimicrobial assay. In contrast to low yields of *T. viridans* obtained in sucrose containing media, high cell yields and antimicrobial activity against all test organisms was obtained in MB with yeast extract as the sole carbon source (Table 3.1; Figure 3.2). This result could be explained by the fact that yeast extract has been proven to be a good substrate for many microorganisms as it not only serves as a carbon source, but also contains amino acids, peptides and water-soluble vitamins (Costa et al., 2002). In addition, yeast extract also serves as an organic nitrogen source (Kumar et al., 2012), which has been shown to provide good growth yields and influence the accumulation of metabolic products (Costa et al., 2002).

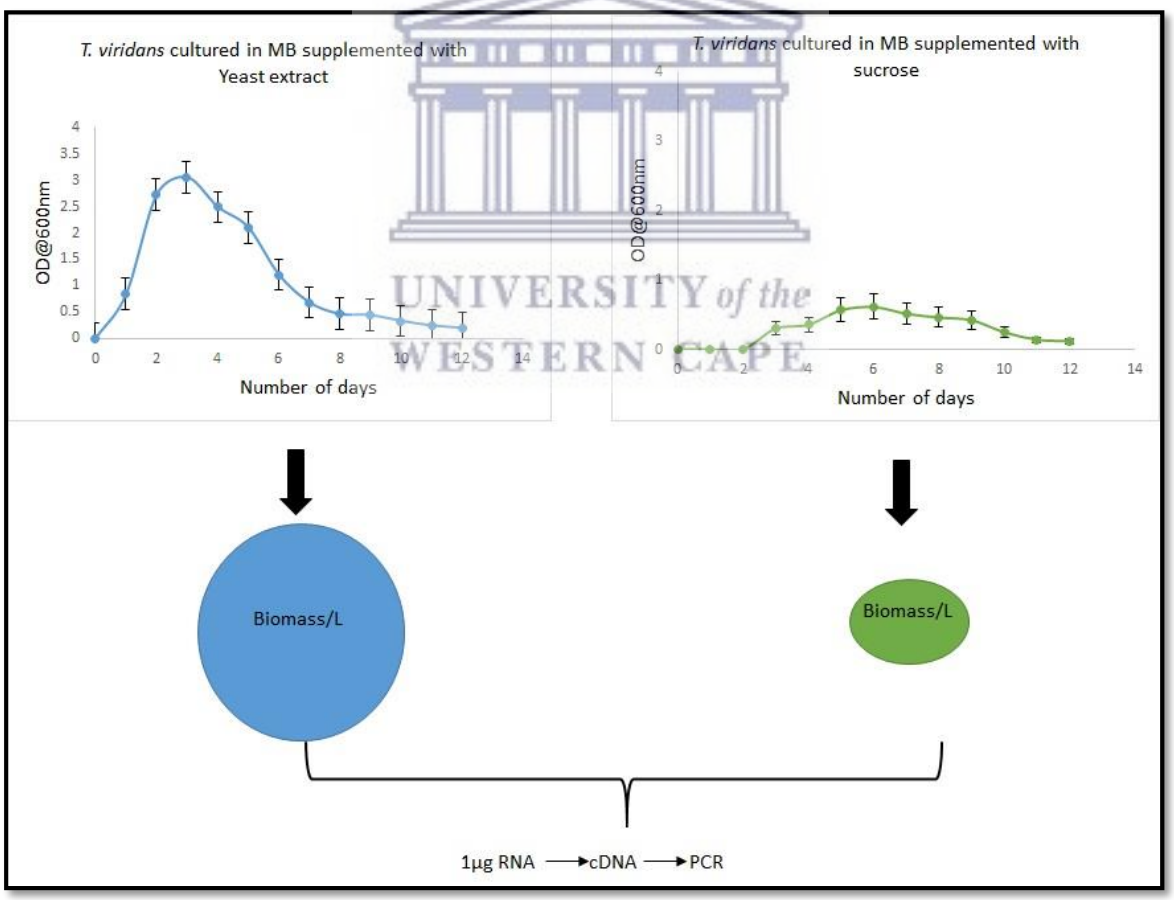


Figure 3.2: Illustration of *T. viridans* growth rate in MB supplemented with yeast extract and sucrose.

Another remarkable finding was that the *T. viridans* cell-free extract was active against the multi-drug resistant microorganism, *E. coli* 1699 (Table 3.1). This organism has been engineered to be resistant to over fifty known antibiotics (Appendix A). Including a multi-drug resistant strain such as *E. coli* 1699 is important to detect bioactive compounds with potentially novel modes of action and therefore there is a good chance that *T. viridans* produces a novel compound.

3.2.1 Genome mining of *T. viridans*

To link the biosynthetic pathway to the observed activity and to assess whether the bioactivity (Table 3.1) was representative of a novel drug candidate, the genome of *T. viridans* was "mined" for secondary metabolite pathways using antiSMASH 2.0 (Madema *et al.,* 2011). AntiSMASH analysis demonstrated that the genome of *T. viridans* contains a total of 19 putative pathways belonging to the following classes: 11 NRPS pathways, 3 T1-NRPS-PKS pathways, 2 bacteriocins, 2 lantipeptides and one "other" or unknown secondary metabolite cluster. Many of these pathways represent truncated/split pathways due to incomplete genome assembly. This is evident in Table 3.2 where 17 of the gene clusters start at position 1 of the contig. All of the secondary metabolite pathways that were identified have very low (< 40% similarity at the nucleotide level) to no similarity to any known or characterised biosynthetic clusters, indicating that these are novel, at least at the sequence level (Table 3.2), and thus could potentially encode the synthesis of novel compounds. In addition, a nucleotide NCBI BLAST analysis revealed that many of the secondary metabolite gene clusters identified in *T. viridans* were not highly similar to biosynthetic gene clusters on other bacterial genomes (Table 3.2) and are thus not widely distributed. Overall, genome interrogation data revealed that ±20% of the *T. viridans* genome is dedicated to secondary metabolite production which is greater than the 9.9% that *Salinospora tropica* (marine bacterium that has the highest secondary metabolite capacity) dedicates to its natural product assembly (Udwary et al., 2007). *T. viridans* also has a higher secondary metabolite capacity than all other actinomycetes, many of which are well-known to be exceptionally prolific in secondary metabolite production, accounting for more than half of all the microbial antibiotics discovered to date (Bérdy, 2005; Udwary et al., 2007).

Table 3.2: Secondary metabolite pathways identified in the genome of *T. viridans* using the antiSMASH2.0 software.

Contig no. and size	BGC no. and type	Cluster position	% identity to known BGC's	% identity to pathways on other bacterial genomes (Nucleotide NCBI BLAST)
Contig_19 (20765bp)	1. NRPS	1..20765	Nosptopeptolide BGC (37% genes show similarity)	85% similar to *Pseudomonas fluorescens* strain UK4, complete genome Accession no: CP008896.1
Contig_21 (13433bp)	2. NRPS	1..13433	Nosptopeptolide BGC (28% genes show similarity	80% similar *to Photorhabdus* sp. PB45.5 NRPS Pb45A, NRPS Pb45B, and NRPS Pb45C genes, complete cds Accession no: KR871230.1
Contig_22 (143973bp)	3. Type-I-NRPS-PKS hybrid	1..98063	N/A	73% similar to *Oceanimonas* sp. GK1, complete genome Accession no: CP003171.1
Contig_24 (78115bp)	4. Other	1..23185	N/A	78 % similar to *Escherichia coli* strain 2015C-4136CT1 chromosome, complete genome Accession no: CP027550.1
Contig_47 (101381bp)	5. NRPS	1..46266	N/A	88% similar to *Colwellia* sp. PAMC 21821, complete genome Accession no: CP014943.1
Contig_49 (32827bp)	6. NRPS	1..32827	N/A	74% similar to *Serratia rubidaea* strain 1122, complete genome Accession no: CP014474.1
Contig_74 (43625bp)	7. NRPS	1..43625	N/A	80% similar to *Pseudomonas* sp. DRA525 genome Accession no: CP018743.1
Contig_98 (47082bp)	8. NRPS	1..26076	N/A	75% similar to *Ferrimonas balearica* DSM 9799, complete genome

				Accession no: CP002209.1
Contig_100 (70028bp)	9. NRPS	1..27561	N/A	75% similar to *Thalassospira* sp. CSC3H3 chromosome, complete genome Accession no: CP024199.1
Contig_112 (88508bp)	10. Type-I-NRPS-PKS hybrid	1..34959	N/A	80% similar to *Colwellia* sp. PAMC 20917, complete genome Accession no: CP014944.1
Contig_122 (30224bp)	11. NRPS	1..30224	N/A	94 % similar to *Yersinia frederiksenii* strain FDAARGOS_418 chromosome, complete genome Accession no: CP023964.1
Contig_134 (35337bp)	12. Type-I-NRPS-PKS hybrid	1..35337	N/A	75% similar to *Xenorhabdus bovienii* str. CS03 chromosome, complete genome Accession no: FO818637.1
Contig_151 (40681bp)	13. Bacteriocin	1..8262	N/A	No significant results found
Contig_152 (62958bp)	14. Lantipeptide	33492..57824	N/A	73% similar to *Pseudoalteromonas arctica* A 37-1-2 chromosome I, complete sequence Accession no: CP011025.1
Contig_159 (33123bp)	15. Bacteriocin	25359..33123	N/A	76% similar to *Yersinia kristensenii* strain Y231, complete genome Accession no: CP009997.1
Contig_184 (8666bp)	16. NRPS	1..4379	N/A	74% similar to *Colwellia* sp. PAMC 20917, complete genome Accession no: CP014944.1
Contig_191 (8277bp)	17. Lantipeptide	1..8277	N/A	No significant similarity found

Contig_192 (2753bp)	18. NRPS	1..2753	N/A	98% similar to *Saccharopolyspora erythraea* NRRL2338 complete genome Accession no: AM420293.1
Contig_238 (2993bp)	19. NRPS	1..2993	N/A	100% similar to *Paenibacillus mucilaginosus* KNP414, complete genome Accession no: CP002869.1

In addition to the secondary metabolite pathways, the *T. viridans* genome was mined for the presence of bacteriophages and submitted to PHAge Search Tool Enhanced Released (PHASTER). PHASTER is a bioinformatic tool that rapidly identifies prophage sequences within bacterial genomes and plasmids by searching against a custom phage database that combines protein sequences from the NCBI database. PHASTER analysis revealed that the *T. viridans* genome also harbours two putative prophage regions, one intact prophage (with a prophage scoring region of >90) and one incomplete prophage (with a prophage scoring region of <70) (Figure 3.3). Bacteriophages (viruses that infect bacteria) and their derived proteins such as tailocins (phage-like bacteriocins) and endolysins are known to be highly potent antimicrobial proteins (Sulakvelidze, 2011; Behrens et al., 2017). These classes of proteins have been considered potential replacements to overcome the drug resistant problem due to their high host specificity and different mechanisms of action (Pattanayak, 2017).

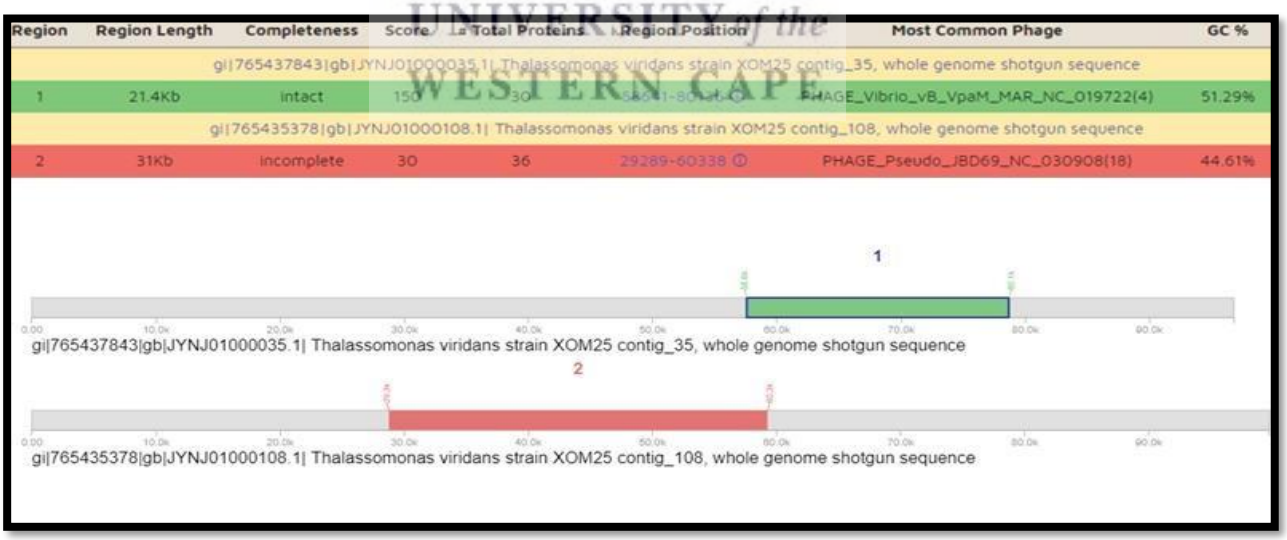


Figure 3.3: Prophage regions identified in *T. viridans*. Two prophage regions have been identified, of which one region is considered intact (green) and the other incomplete (red) by PHASTER (https://tinyurl.com/yawbk8qc).

3.2.2 Identification of bioactive pathways through targeted transcriptional profiling

RNA expression profiles or transcriptional profiling is one means of attempting to match the biosynthetic pathways responsible for the bioactivities observed. To discover whether bioactive compound/ (s) responsible for the observed bioactivity was synthesized from one or more of the secondary metabolite pathways present in the genome of *T. viridan*s, transcriptional profiling was selected to screen for the activation of biosynthetic pathways under different culturing conditions. It stands to reason that if such a large portion (±20 %) of the genome was designated for secondary metabolite synthesis, these pathways offer a competitive niche advantage, and allows the organisms to adapt to changing environmental conditions. Extraction of RNA which could be converted to cDNA and from which gene transcription could be detected from *T. viridans* in certain carbon sources (maltose, glucose, starch, cellobiose) was unsuccessful due to the production of polysaccharides that interfered with the integrity of the RNA (Dos Reis Falcão et al., 2008). Consequently, the expression profiles of *T. viridans* cultured in MB supplemented with yeast extract (wherein bioactivity was observed against all of the test organisms) and sucrose (where *T. viridans* failed to produce bioactivity) were the only two usable transcriptional profiles generated.

Based on the RNA expression profile of *T. viridans*, seven of the screened pathways (Appendix B, Figure B.1A and Figure B.1 A; Table 3.3) were seen to be activated when cultured with yeast extract, any one of which, or none, could be responsible for producing the active compound/s. When cultured in sucrose, all of the PCR targeted biosynthetic gene clusters were activated, with the exception of cluster 10A (Appendix B, Figure B.2; Table 3.3). This was not the expected outcome. If a secondary metabolite was responsible for the observed bioactivity,

then the expected result would be to detect transcription. Since no bioactivity was detected when *T. viridans* was cultured in sucrose (Table 3.1), it would suggest that none of the clusters targeted encode the bioactivities detected, except possibly cluster 10A. Further support for this conclusion is that all the pathways that were activated in the yeast extract profile were also active under sucrose growth conditions.

Table 3.3: Transcriptional profile of *T. viridans* cultured in marine broth with yeast extract and sucrose as the sole carbon sources.

			Activated pathways in *T. viridans*	
Contig	**Gene Cluster**	**Type**	**MB + yeast extract**	**MB + sucrose**
19	**1**	**NRPS**		
21	**2**	**NRPS**		
22	**3A**	**T1-PKS-NRPS**	**X**	✓
22	**3B**	**T1-PKS-NRPS**	**X**	✓
24	**4**	**Other**	✓	✓
47	**5**	**NRPS**	✓	✓
49	**6**	**NRPS**	**X**	✓
74	**7**	**NRPS**	✓	✓
97	**8**	**NRPS**	✓	✓
100	**9**	**NRPS**	✓	✓
112	**10A**	**T1-PKS-NRPS**	**X**	**X**
112	**10B**	**T1-PKS-NRPS**	**X**	✓
122	**11A**	**NRPS**	**X**	✓
122	**11B**	**NRPS**	**X**	✓
134	**12**	**T1-PKS-NRPS**	✓	✓
151	**13**	**Bacteriocin**		
152	**14**	**Lantipeptide**	✓	✓
159	**15**	**Bacteriocin**		✓

184	**16**	**NRPS**
191	**17**	**Lantipeptide**
192	**18**	**NRPS**
238	**19**	**NRPS**

Gene clusters highlighted in blue were not PCR screened as those were deemed incomplete biosynthetic gene clusters. Clusters 3, 10 and 11 were named A and B because each of those biosynthetic pathways contained genes that were divergently transcribed.

✓ Represents the transcription of secondary metabolite gene clusters predicted by antiSMASH.
X Represents no transcription of secondary metabolite gene clusters predicted by antiSMASH.

The activation of pathways obtained in the sucrose profile could suggest that the enzyme complexes did not form antibacterial compounds (hence no bioactivity detected) and possibly formed other compounds used for other functions. It is therefore most likely the case that the bioactive compounds are encoded by pathways which were deemed incomplete and not assessed through RNA analysis or encoded by other gene clusters that were not identified through antiSMASH 2.0. For example, a monorhamnolipid synthesized by a *Pseudomonas* sp was not detected by antiSMASH, yet, exhibited high antibacterial activity against *Burkholderia cepacia* (Tedesco et al., 2016). Overall, we were unable to unequivocally establish whether or not the activated antibacterial pathways were responsible for synthesizing the compounds responsible for the bioactivities observed.

Moreover, a single gene was used as a proxy for the entire biosynthetic gene cluster, and there is no guarantee that every other gene in the pathway is under the same transcriptional regulation. Also, the necessary post-translational modifications may be under different regulation to the target gene.

While the applied methodology improved our understanding of *T. viridans* secondary metabolite pathway expression, we were unable to deduce precisely which pathway/(s) resulted in the synthesis of the active compound/(s) using a targeted transcriptomic approach.

Essentially, our genomic-based approach limited us to the antiSMASH-identified biosynthetic gene clusters. It did however guide us to focus on a different approach to identify the genetic determinants responsible for the bioactivity. Considering the limitations with targeted transcription profiling, we applied a bioassay-guided-fractionation approach to identify the bioactive compound/s, described in the following chapter.

Chapter 4: Isolation of bioactive compounds: bioassay-guided fractionation using fast performance liquid chromatography (FPLC) coupled with LCMS

4.1 : Introduction

Bioassay-guided fractionation is a procedure whereby an extract is fractionated and refractionated until a pure biologically active compound is isolated. In this procedure, each fraction is evaluated in a bioassay system and only active fractions are further fractionated (Malviya & Malviya, 2017). This method has been shown to be quite effective in isolating biologically active compounds due to the ability to directly link the analysed extract to the target compound and has thus been commonly employed in drug discovery research. In this chapter, a bioassay guided fractionation assay was conducted using fast performance liquid chromatography (FPLC) coupled to liquid chromatography mass spectrometry (LCMS/MS) to aid in the identification of the bioactive compound.

4.2 Size fractionation assay

A size fractionation assay was conducted on the crude bioactive extract (generated from *T. viridans* cultured in MB containing the variety of carbon sources used in this study) using Amicon molecular weight cut-off filters (Section 2.7.5) to identify the approximate size of the active compound. The bioactivity assay results revealed that fractions < 3 kDa and 3 kDa- 50 kDa were inactive against all of the indicator organisms while the > 50 kDa fraction retained the antimicrobial activity (Figure 4.1; Table 4.1). These findings indicated that the compound producing the bioactivity was most probably a protein or a polysaccharide, and not a secondary metabolite since these are typically low molecular weight compounds less than 3

kDa in size (Zahner, 1979) with the 5.033 kDa, non-ribosomal peptide, Polytheonamide A being an exception (Hamada et al., 2005)**.** These results further highlight the limitations of taking a genome mining approach to targeting secondary metabolite gene clusters, none of which appeared to be responsible for producing the biologically active compound in our study.

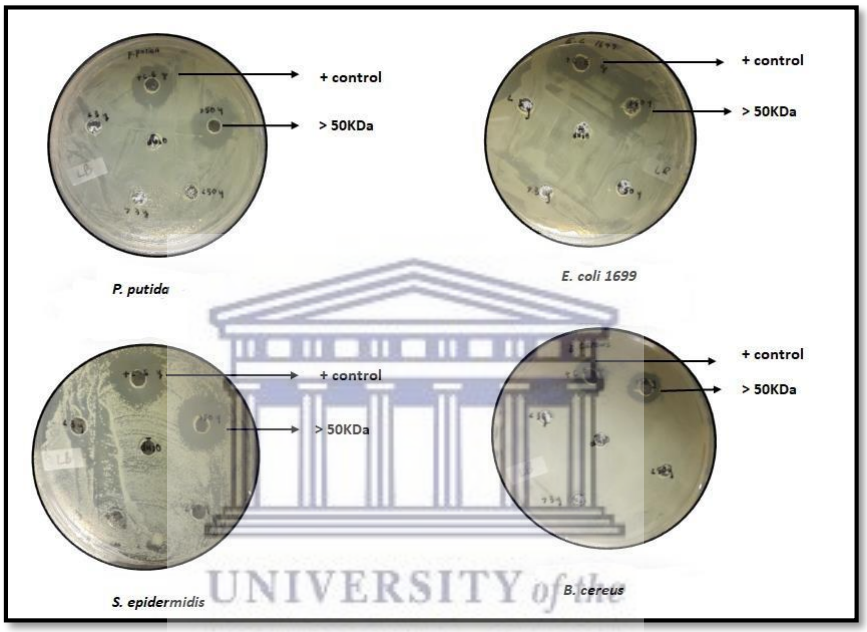


Figure 4.1.: Representation of size fractionated extracts from *T. viridans* cultured in yeast extract-supplemented MB, assessed using the well diffusion assay. Crude extracts produced *by T. viridans* when cultured in the selected carbon source were subjected to size fractionation and tested against the indicator organisms; *P. putida*, *E. coli* 1699, *S. epidermidis* and *B. cereus*. All of the bioactivity was retained in the > 50kDa fraction for all of the carbon sources. Bioactivity is indicated by the clearance zone around the well. The positive control consisted of the crude extract prior to size fractionation, and the negative control contained distilled water in which the dried crude extract was resuspended.

Table 4.1: Size fractionation of crude extracts produced by *T. viridans* supplemented with different carbon sources.

Culture media	Size fraction in kDa	*P. putida*	*B. cereus*	*S. epidermidis*	*E.coli* 1699
Yeast	> 50	+++	++	+++	++
	< 50	-	-	-	-
	> 3	-	-	-	-
	< 3	-	-	-	-
Positive control	Crude extract	+++	++	+++	++
Maltose	> 50	++	+	++	++
	< 50	-	-	-	-
	> 3	-	-	-	-
	< 3	-	-	-	-
Positive control	Crude extract	++	-	+++	++
Cellobiose	> 50	++	-	++	+
	< 50	-	-	-	-
	> 3	-	-	-	-
	< 3	-	-	-	-
Positive control	Crude extract	++	+	++	+
Sucrose	> 50	-	-	-	-
	< 50	-	-	-	-
	> 3	-	-	-	-
	< 3				
Positive control	Crude extract	-			-
Glucose	> 50	++	-	+	+
	< 50	-	-	-	-
	> 3	-	-	-	-
	< 3	-	-	-	-
Positive control	Crude extract	++	+	++	+
Starch	> 50	++	-	+	+
	< 50	-	-	-	-
	> 3	-	-	-	-
	< 3	-	-	-	-
Positive control	Crude extract	++	+	++	+

The (+) sign represents antibacterial activity and the number of (+) signs represents the intensity of antibacterial activity; where + represents a zone clearance diameter of 1mm, (++) represents 4mm and (+++) represents a zone diameter of 7mm. The (-) sign is indicative of no antibacterial activity.

Considering the size fractionation and carbon-supplementation results (Table 4.1), subsequent experiments were conducted on the > 50 kDa fraction of the crude extract, generated by *T. viridans* cultured in yeast extract-supplemented MB. The yeast extract profile seemed more consistent, in that the > 50 kDa activity profile closely resembled the bioactivity profile of the positive control (crude extract before size fractionation) (Table 4.1). Essentially, the antimicrobial activity was retained in the > 50 kDa fraction. In addition, the intensity of the antibacterial zones for the > 50 kDa fraction had a larger zone diameter than the > 50 kDa fractions generated by *T. viridans* when cultured in the other carbon sources (Table 4.1).

In parallel to the antimicrobial tests performed on the > 50 kDa yeast extract fraction, a zymography assay was conducted to assess the active fraction at a protein level and also conclude whether the antimicrobial activity was bacteriostatic (growth inhibition) or bacteriolytic (killing cells by lysis). Zymography is an electrophoretic technique used for the detection of hydrolytic enzymes, on the basis of substrate degradation (Vandooren et al., 2013). In this study the substrate was one of the indicator organisms, *P. putida*. From the results obtained in Figure 4.2, we deduced that the antimicrobial activity is a hydrolytic enzyme (approximately 52 kDa) causing bacteriolytic (cell lysis) activity, indicated by the white zone observed on the zymogram (Figure 4.2).

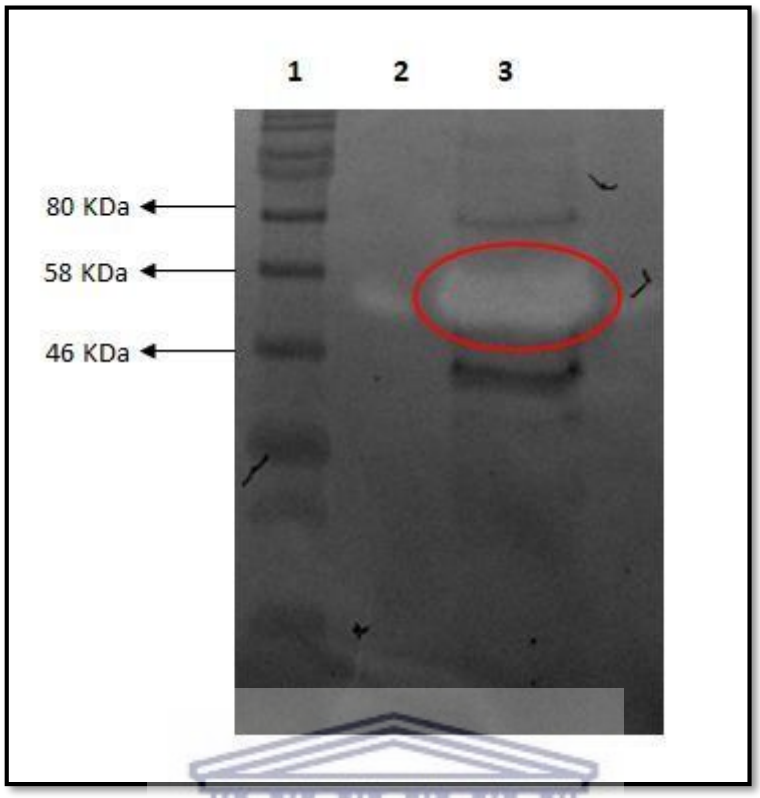


Figure 4.2: Zymogram of > 50 kDa fraction, generated by *T. viridans* cultured in yeast extract-supplemented MB. Lane 1: Molecular weight marker, lane 2: > 50 kDa (MB+ yeast extract) fraction approximately 52 kDa in size. The white zone encircled in red depicts antimicrobial activity.

4.3 Fast protein/performance liquid chromatography (FPLC)

Fast protein liquid chromatography (FPLC, formerly named as “fast performance liquid chromatography) is a form of medium to high performance liquid chromatography originally developed to purify or separate biopolymers, including proteins at a high resolution (Walls & Loughran, 2011). FPLC allows the use of different chromatography modes such as gel filtration, ion exchange, chromatafocusing, hydrophobic interaction and reverse phase. However, anion exchange and gel filtration chromatography are the most commonly used modes (Kosanović et al., 2017).

4.3.1 Ion exchange and gel permeation chromatography

The >50kDa fraction was subjected to ion exchange chromatography (IEC) for further separation and purification (Section 2.7.6). IEC is a process that involves the separation of ionisable molecules based on their total charge (Coskun, 2016). Both the positive and negatively charged molecules were collected and assessed for antimicrobial activity. Bioactivity screening results showed that both fractions had antimicrobial activity, with more pronounced bioactivity retained in the positively charged fraction indicated by a larger clearance zone (Figure 4.3).

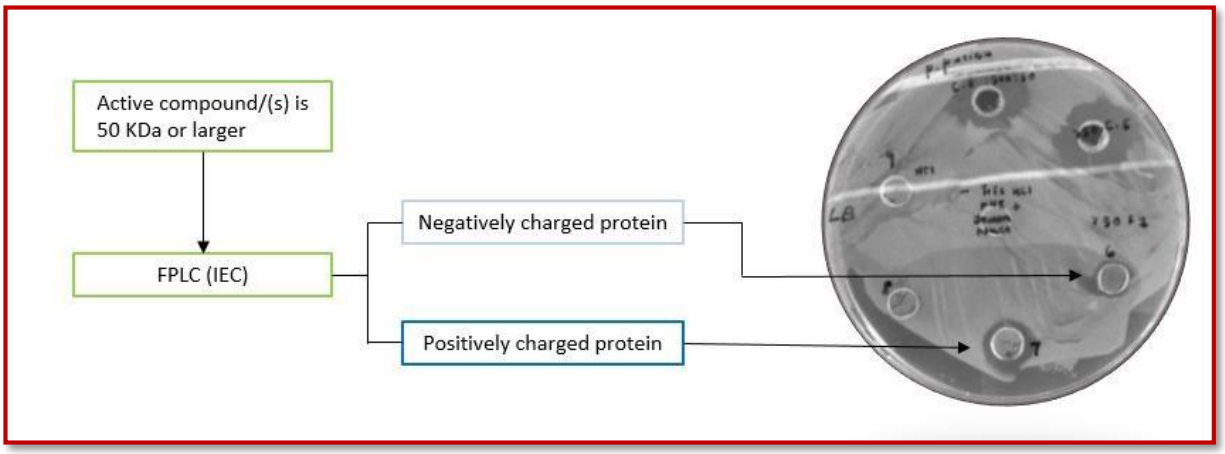


Figure 4.3: Anion exchange chromatography and bioactivity test of the >50kDa yeast-extract fraction. Both positive controls (crude extract and the >50kDa fraction retained bioactivity). The buffer in which the proteins were eluted (100mM Tris-HCl, pH8) was tested as the negative control. The positively charged protein fraction retained more bioactivity than the negatively charged protein. fraction indicated by the difference in zone diameter.

The bioactive positively-charged protein fraction was subjected to gel permeation chromatography (GPC) for further separation and purification (Section 2.7.7). GPC separates dissolved macromolecules by size, based on their elution from columns filled with a porous gel matrix (Walls & Loughran, 2011). In this process, larger molecules are the first to elute from the column as they are excluded from the pores, resulting in high molecular weight peaks on the chromatogram. Smaller molecules have high access to the pores and are the last to elute, represented by low molecular weight peaks. Figure 4.4 shows the GPC chromatogram output of the positively charged bioactive fraction. The four fractions that were generated were collected and assessed for antimicrobial activity. The bioactivity test results showed that peak 2 was the only fraction that retained the active antimicrobial protein (Figure 4.4). This peak represents proteins > 50 kDa.

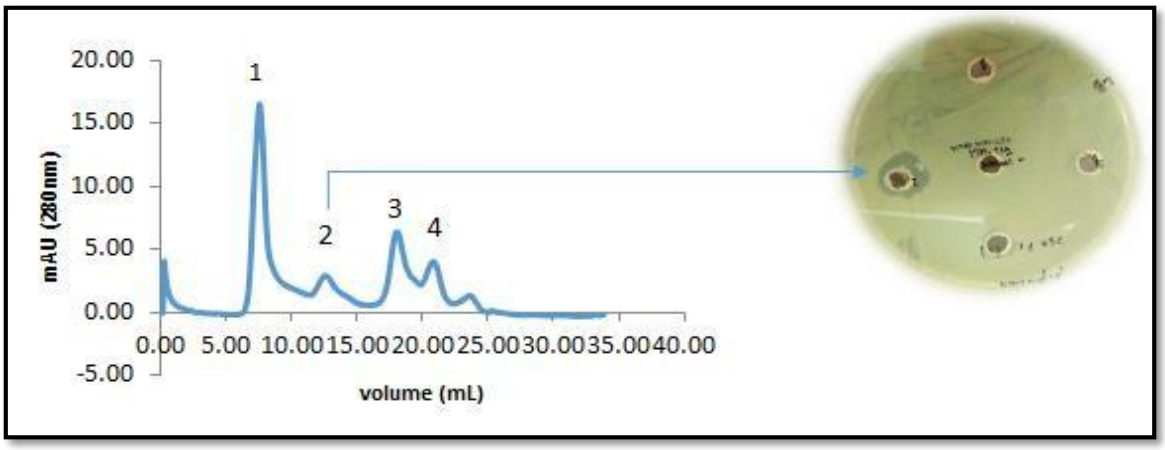


Figure 4.4: Illustration of the chromatogram output of the positively charged bioactive fraction on GPC. The peaks show different molecular weights with peaks on the left having higher molecular weights and peaks on the right, lower molecular weights. Broad molecular weight distribution peaks indicate that there are many different molecules with different molecular weights. A sharp peak is indicative of one narrow molecular range.

4.4 Protein identification by liquid chromatography-mass spectrometry (LC-MS/MS).

To assess the purity, the bioactive fraction 2 generated from GPC (Figure 4.4) was subjected to SDS PAGE in a 12% acrylamide gel (Section 2.7.3) to detect the number of proteins present in the fraction. Results obtained revealed the presence of approximately four protein bands indicating that bioactive fraction 2 was “semi-pure” (Figure 4.5). Subsequently, liquid chromatography mass spectrometry (LCMS/MS) was used to identify the proteins in the “semi-pure” fraction. LCMS/MS is an analytical technique used for protein identification, whereby a purified protein or complex mixture of proteins are digested into peptide fragments. During LCMS/MS precursor mass data and fragment ion spectra data are obtained and are matched to known sequence databases to identify the peptide and consequently the protein (Hird et al., 2014). In addition to the “semi-pure” bioactive fraction, total protein isolated from the crude extract

produced by *T. viridans* cultured in selected carbon sources (Figure 4.6) were also subjected to LCMS/MS to aid in the identification of the bioactive protein.

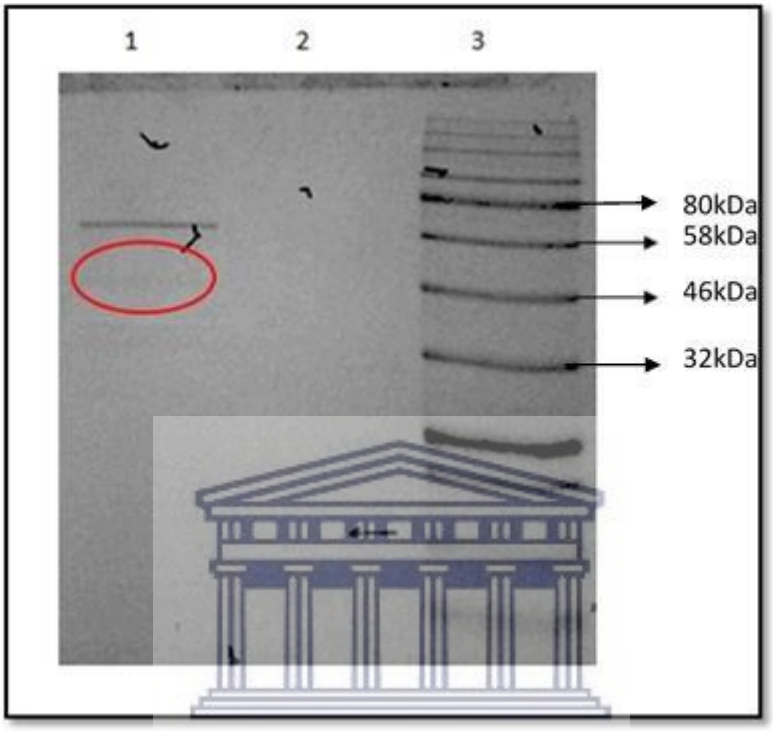


Figure 4.5: A 12% Coomassie stained SDS-PAGE illustrating the number of protein bands present in GPC bioactive fraction 2. Lane 1: semi-pure protein fraction generated from gel permeation chromatography (GPC) and lane 3: molecular weight marker. The faint band encircled in red at approximately 52 kDa is the target band (that corresponded to the zone of clearance in Figure 4.2) that was excised and subjected to LCMS/MS for identification.

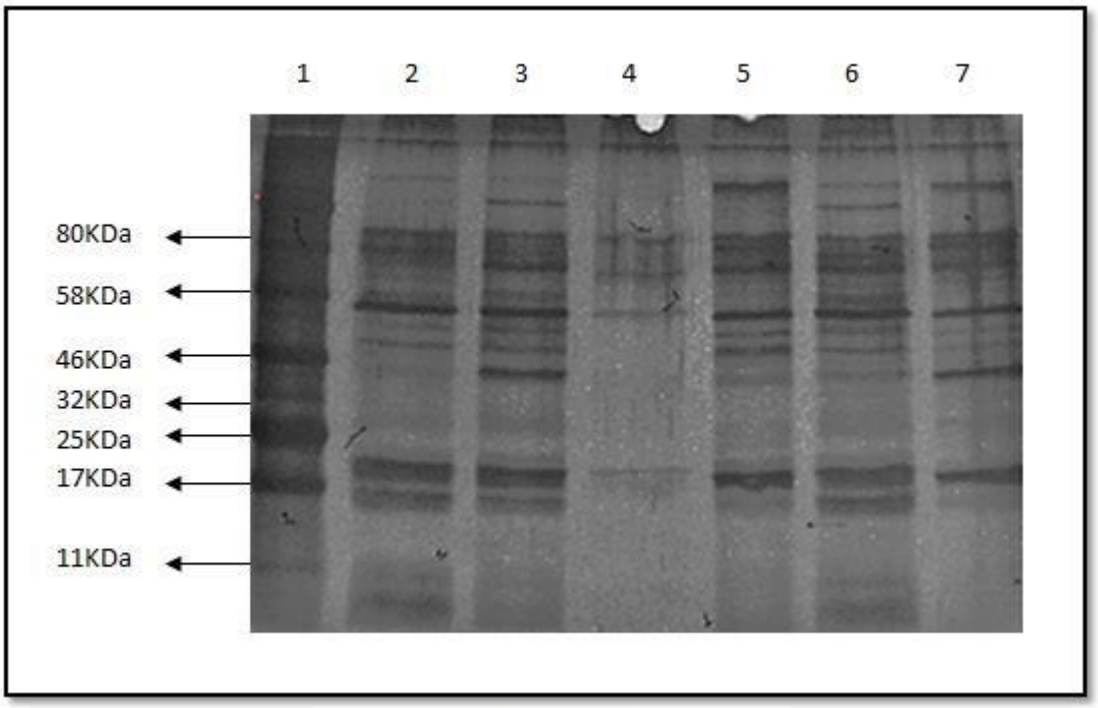


Figure 4.6: A 12% silver stained SDS-PAGE gel representing total protein extracted from *T. viridans* cultures (marine broth supplemented with different carbon sources). Lane 1: Molecular weight marker, lane 2: MB + yeast, lane 3: MB + maltose, lane 4: MB + sucrose, lane 5: MB + starch, lane 6: MB + glucose, lane 7: MB + cellobiose.

LCMS/MS data generated from the "semi-pure" bioactive fraction (generated from GPC in figure 4.4) revealed the presence of five proteins (Table 4.2). Considering these findings, the proteins identified in the "semi-pure" bioactive fraction were compared to the LCMS/MS protein profiles generated from the other carbon sources. The protein highlighted in Table 4.2 was the only protein in the "semi-pure" bioactive fraction that was absent in the sucrose profile (where no bioactivity was observed) and present in all the other carbon source protein profiles (where bioactivity was observed). In addition, the size of the identified protein corresponded to our size fractionation assay and to the size of the clearance zone depicted in the zymogram at approximately 52 kDa (Figure 4.2). To further validate the identification of the bioactive protein, the faint band circled in Figure 4.5 at approximately 52 kDa was excised

and subjected to LCMS/MS. The result generated in Table 4.2 (bottom row) provided secondary confirmation which identified the same protein.

Table 4.2: LCMS/MS profile of proteins produced from *T. viridans* when cultured in MB supplemented with different carbon sources.

Cellobiose	Accession number	Protein description	# of unique peptides	# of mod peptides	# AA's in protein
	A0A0D8DCA8	Uncharacterized protein	8	1	729
	A0A0D8DGN4	Aminopeptidase B	7	2	428
	A0A0D8DJI6	Uncharacterized protein	6	0	911
	A0A0D8CQW3	Dihydrodipicolinate synthetase	4	1	300
	A0A0D8DFY0	Uncharacterized protein	2	1	462
	A0A0D8CUE1	TonB-dependent receptor	7	0	932
	A0A0D8DBM8	Membrane protein	11	0	1070
	A0A0D8D9K3	Glutathione reductase	5	1	455
Yeast extract					
	A0A0D8DKN2	Uncharacterized protein	3	0	460
	A0A0D8DGN4	Aminopeptidase B	12	3	428
	A0A0D8DI24	Flagellin	7	2	481
	A0A0D8CUE1	TonB-dependent receptor	8	1	932
	A0A0D8DFL8	Uncharacterized protein	9	2	963
	A0A0D8DFY0	Uncharacterized protein	6	1	462
	A0A0D8DFX0	Tail protein	3	0	387
	A0A0D8D9K3	Glutathione reductase	4	0	455
Maltose					
	A0A0D8DKN2	Uncharacterized protein	3	0	460
	A0A0D8DGN4	Aminopeptidase B	14	3	428
	A0A0D8DI24	Flagellin	11	3	481
	A0A0D8DBM8	Membrane protein	7	0	1070
	A0A0D8CUE1	TonB-dependent receptor	7	0	932
	A0A0D8DFY0	Uncharacterized protein	6	1	462
	A0A0D8CQW3	Dihydrodipicolinate synthetase	4	1	300
	A0A0D8D2Q4	TonB-dependent receptor	8	1	865

Sucrose					
	A0A0D8DHG6	Dihydrolipoamide dehydrogenase	4	1	476
	A0A0D8DEW3	Methyltransferase	4	0	249
	A0A0D8CQW3	Dihydrodipicolinate synthetase	2	1	300
	A0A0D8D9K3	Glutathione reductase	2	0	455
	A0A0D8DIC3	Probable cyt	2	1	502
	A0A0D8D987	6,7-dimethyl-8-ribityllumazine synthase	2	0	154
	A0A0D8DFW6	Superoxide dismutase	2	0	193
Starch					
	A0A0D8DKN2	Uncharacterized protein	4	0	460
	A0A0D8CUE1	TonB-dependent receptor	17	2	932
	A0A0D8DBM8	Membrane protein	18	1	1070
	A0A0D8DCA8	Uncharacterized protein	13	2	729
	A0A0D8DG46	Uncharacterized protein	14	1	900
	A0A0D8DGN4	Aminopeptidase B	10	2	428
	A0A0D8DFY0	Uncharacterized protein	4	1	462
	A0A0D8D6I8	Uncharacterized protein	11	2	892
Glucose					
	A0A0D8DKN2	Uncharacterized protein	3	0	460
	A0A0D8DGN4	Aminopeptidase B	14	4	428
	A0A0D8DFY0	Uncharacterized protein	7	2	462
	A0A0D8DI24	Flagellin	11	4	481
	A0A0D8DEA7	Tail protein	8	2	167
	A0A0D8CUE1	TonB-dependent receptor	11	0	932
	A0A0D8D1G1	Uncharacterized protein	3	1	264
	A0A0D8DI75	Flagellin	9	4	471
Semi pure protein fraction					

	A0A0D8CQW3	Dihydrodipicolinate synthetase	4	2	300
	A0A0D8DFY0	Uncharacterized protein	4	1	462
	A0A0D8DHW1	Uncharacterized protein	3	1	600
	A0A0D8DDZ1	Uncharacterized protein	2	0	155
	A0A0D8DH33	Uncharacterized protein	2	0	223
Target band excised					
	A0A0D8DFY0	Uncharacterized protein	5	1	462

The different coloured blocks in the table represent some of the proteins that were identified to be produced in the respected carbon sources. The "semi-pure" protein fraction and target band excised sections are highlighted in the same colour as the yeast extract results since those fractions were generated from *T. viridans* cultured with yeast extract. The protein proposed to be responsible for the bioactivity is highlighted in grey.

4.5 Bioinformatic analysis of the identified antimicrobial protein

The novelty of the antimicrobial protein identified in this study was assessed through bioinformatic analyses using the basic local alignment search tool (BLAST). A BLASTp search was conducted using this protein sequence as query against the NCBInr, Uniprot and IMG/VR 700 000 metagenomic viral contig databases. The BLAST results revealed that the antimicrobial protein had no similarity to any proteins in any of these databases, highlighting its novelty. To identify the genomic origin of the protein, additional genomic analyses were conducted using CLC genomic workbench 11.0 where the target sequence was aligned to the genome sequence of *T. viridans*. The analysis suggested that the bioactive protein is located on a predicted bacteriophage integrated into the genome of *T. viridans* (Figure 4.7). From here onwards the identified unknown bioactive protein highlighted in Figure 4.7 will be referred to as TVP1.

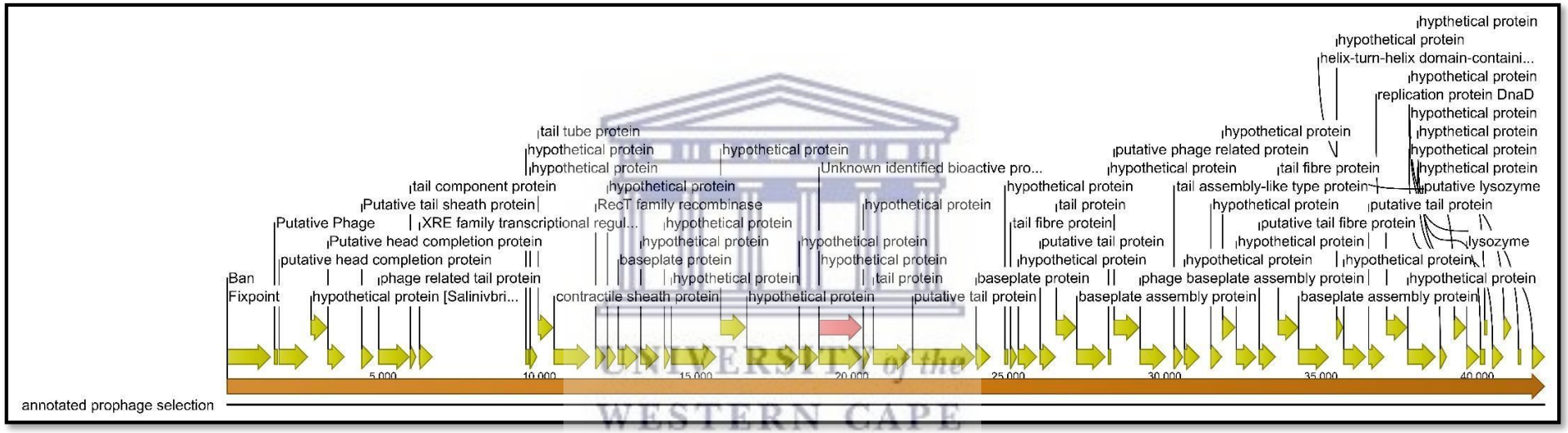


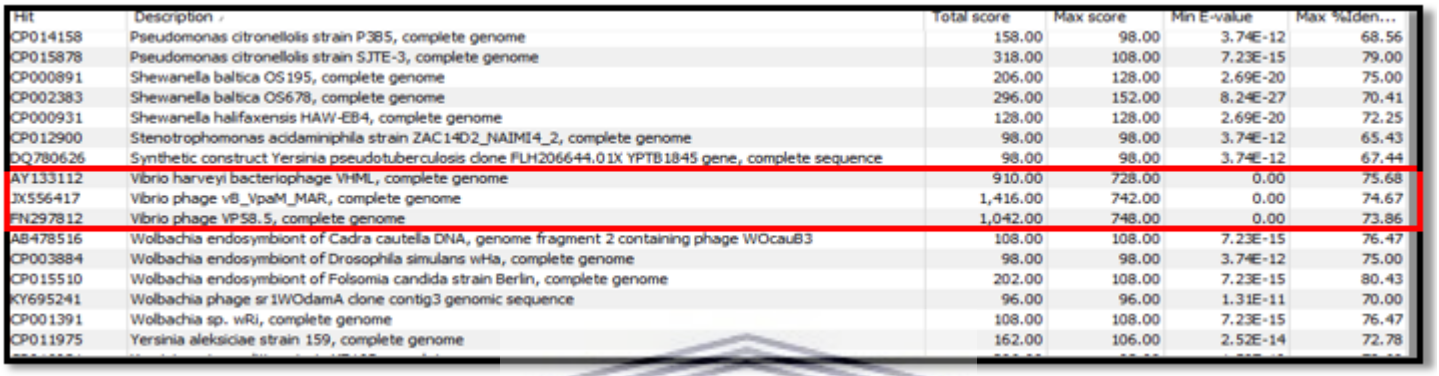
Figure 4.7: Linear representation of the annotated putative prophage integrated in *T. viridans* with the highlighted target protein (TVP1) located on the tail region of the prophage.

Interestingly, analysis of the prophage sequence predicted that TVP1 was located in the tail region. Bacteriophage tail regions are comprised of proteins that are responsible for the physical structure of the phage tail (sheath protein, tail protein), determining phage tail length (tape measure protein), recognising and attaching to a specific host (Fokine & Rossmann, 2014) as well as genes coding for lytic proteins (holins, spannins and endolysins) whose function is to degrade the bacterial host cell wall (Section 1.7). Based on the genomic loci and observed bioactivity, we assume that TVP1 might be used by the phage as a mechanism of infection. Another possibility is that TVP1 might be responsible for reducing the integrity of the hosts cell wall by attacking one of the four major bonds in the peptidoglycan, thus functioning as either an endo-β-N-acetylglucosiminidase or N-acetylmurimidase (lysozyme) (both of which act on the sugar moiety of the bacterial cell wall); an endopeptidase (which acts on the peptide moiety); or an N-acetylmuramoyl-L- alanine amidase (amidase), which hydrolyses the amide bond connecting the glycan strand and peptide moieties (Young, 1992; Fischetti, 2005; Courchesne et al., 2009) thereby effecting it's antimicrobial activity. Another plausibility is that TVP1 is acting as a holin or a type of pore forming bacteriocin (forming "holes" in the cytoplasmic membrane of the bacterial host) or as a spannin disrupting the outer membrane of the cell (Young, 2002; Rajaure et al., 2015).

Bioinformatic analysis of the identified phage in *T. viridans* revealed that the proposed prophage was similar to three other *Vibrio* phages namely: *Vibrio harveyi* phage VHML, *Vibrio* phage vB_Vpam_MAR and *Vibrio* phage VP58.5 (Table 4.3). While it is well established that phages are very specific to their hosts, recent literature indicates that some phages are polyvalent or have a broader host range (Ross et al., 2016). Given the high percentage identity to other *Vibrio* phages, we pursued antimicrobial testing of the >50 kDa fraction against other *Vibrio* and *Pseudovibrio* species. Functional screening results presented in Table 4.4 showed

that the extract (>50kDa size fraction) from which TVP1 was derived, was not only active against the organisms presented in Table 4.1 but also exhibited activity against *Vibrio sp*, *Pseudovibrio sp* as well as *T. viridans* (the producer of TVP1) (Table 4.4).

Table 4.3: NCBI nucleotide BLAST of the bacteriophage identified on contig 35 in the bacterial genome of *T. viridans*.



Hit	Description	Total score	Max score	Min E-value	Max %iden...
CP014158	Pseudomonas citronellolis strain P3B5, complete genome	158.00	98.00	3.74E-12	68.56
CP015878	Pseudomonas citronellolis strain SJTE-3, complete genome	318.00	108.00	7.23E-15	79.00
CP000891	Shewanella baltica OS195, complete genome	206.00	128.00	2.69E-20	75.00
CP002383	Shewanella baltica OS678, complete genome	296.00	152.00	8.24E-27	70.41
CP000931	Shewanella halifaxensis HAW-EB4, complete genome	128.00	128.00	2.69E-20	72.25
CP012900	Stenotrophomonas acidaminiphila strain ZAC14D2_NAIMI4_2, complete genome	98.00	98.00	3.74E-12	65.43
DQ780626	Synthetic construct Yersinia pseudotuberculosis clone FLH206644.01X YPTB1845 gene, complete sequence	98.00	98.00	3.74E-12	67.44
AY133112	Vibrio harveyi bacteriophage VHML, complete genome	910.00	728.00	0.00	75.68
JX556417	Vibrio phage vB_VpaM_MAR, complete genome	1,416.00	742.00	0.00	74.67
FN297812	Vibrio phage VP58.5, complete genome	1,042.00	748.00	0.00	73.86
AB478516	Wolbachia endosymbiont of Cadra cautella DNA, genome fragment 2 containing phage WOcauB3	108.00	108.00	7.23E-15	76.47
CP003884	Wolbachia endosymbiont of Drosophila simulans wHa, complete genome	98.00	98.00	3.74E-12	75.00
CP015510	Wolbachia endosymbiont of Folsomia candida strain Berlin, complete genome	202.00	108.00	7.23E-15	80.43
KY695241	Wolbachia phage sr1WOdamA clone contig3 genomic sequence	96.00	96.00	1.31E-11	70.00
CP001391	Wolbachia sp. wRi, complete genome	108.00	108.00	7.23E-15	76.47
CP011975	Yersinia aleksiciae strain 159, complete genome	162.00	106.00	2.52E-14	72.78

Table 4.4: Antimicrobial screening results of the >50kDa fraction (generated by *T. viridans* in yeast supplemented MB) against *Pseudovibrio, Vibrio* bacterial species as well *T. viridans* (the native host from which the extract is produced).

Indicator organisms	Level of bioactivity detected (>50kDa)
PE 14-07 (*Pseudovibrio* sp.)	+ + + +
PE 14-03 (*Pseudovibrio* sp.)	+
PE 14-63 (*Pseudovibrio* sp.)	+ + +
PE 5-40 (*Pseudovibrio* sp.)	+ +
B781 (*Vibrio percolans*)	+ + + +
B782 (*Vibrio rubicundus*)	+ + +
B1037 (*Vibrio neocistes*)	+ +
Vibrio chagasii	+ + +
T. viridans XOM25	+ +

The (+) sign represents antibacterial activity and the number of (+) signs represents the intensity of antibacterial activity; where (+) represents a zone clearance diameter of 1mm, (++) represents 4mm, (+++) represents a zone diameter of 7mm and (++++) represents a zone diameter of 10mm.

These results suggest that TVP1 (assuming it is the only bioactive compound in the >50 kDa fraction) could be responsible for lysis from within since TVP1 is active against its producing host. To lyse Gram negative bacteria, along with endolysins, a holin protein or spannin is required to kill the host cell because of the outer membrane cell layer that they possess. However, when the amino acid sequence of TVP1 was analysed with a variety of software programs such as TMMOD (a hidden Markov Model that predicts transmembrane topology and identifies transmembrane proteins) (Kahsay et al., 2005), TOPCONS (a web server for consensus prediction of membrane protein topology and signal peptides) (Bernsel et al., 2009), DAS (a dense alignment surface method that predicts transmembrane domains in protein sequences) (Cserzö et al., 2002) and Signal P 3.0 (a web server that predicts signal peptides from amino acid sequences) (Bendtsen et al., 2004), this ruled out our endolysin, holin or spannin hypothesis. This was chiefly due to the lack of a signal peptide, transmembrane, glycosyl hydrolase, protease or amidase domains which are well described features of endolysins, holins and/or spannins (Oliveira et al., 2013). At this point the lysis mechanism of the antimicrobial protein, TVP1 is completely unknown.

In a parallel study, our colleagues (D Isaacs, unpublished results) detected the presence of tailocins in the > 50 kDa fraction through transmission electron microscopy (TEM) (Figure 4.8). This same fraction showed broad-range antibacterial activity against indicator organisms used in this study (Table 4.1; Table 4.4).

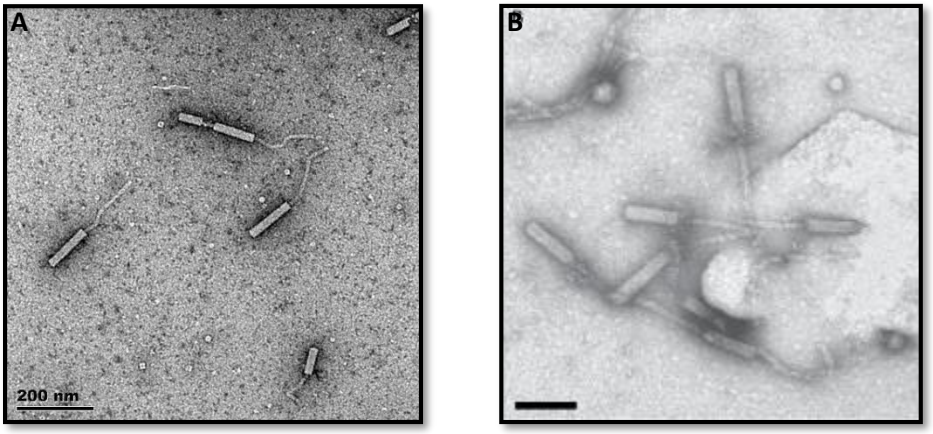


Figure 4.8: TEM Images of tailocins. A) Tailocins found in the > 50kDa fraction of *T. viridans* (D Isaacs, 2017). B) Tailocin elements found in bacteriocin preparations of *P. luteoviolacea* (adapted from Freckelton *et al*., 2017).

Tailocins as defined in section 1.6.2 are bacteriocins that morphologically resembles the tail structures of defective prophages (Ghequire & De Mot, 2015). They function primarily by puncturing the bacterial cell membranes, resulting in membrane depolarization and eventually cell death (Michel-Briand & Baysse, 2002; Scholl & Martin, 2008; Ling et al., 2010). This finding raises the question as to whether TVP1 was solely responsible for the observed antimicrobial activity, or if the tailocins (of which this protein may be a part) were responsible. However, when TVP1 was purified from the > 50 kDa fraction through bioassay- guided fractionation (Section 4.1.2) it still exhibited antibacterial activity against *P. putida*, thus suggesting its bioactivity against this test organism in the absence of the larger tailocin structures.

To slow down the development of antimicrobial resistance, antimicrobial compounds with novel modes of action, that are less susceptible to microbial resistance are required to reduce

the spread of infective agents (Brüssow, 2017). Phage proteins have been shown to meet these requirements and are now being investigated as alternatives to antibiotics due to their high specificity and diverse action mechanisms that makes them less susceptible to antimicrobial resistance (Behrens et al., 2017; Pattanayak, 2017). In light of TVP1 being highly novel and exhibiting antibacterial activity, it could contribute to the development of novel therapeutic drugs required by the public health sectors to combat antimicrobial resistance (Levy, 1998; Saha et al., 2014). More interestingly, although the mechanism of action is unknown, the fact that TVP1 was found to be a phage derivative is an added advantage because phage proteins generally have a reduced risk of resistance evolution (Young, 2002; Rajaure et al., 2015).

Should TVP1 be found to exhibit antibacterial activity against an even broader range of bacteria then this protein may not be considered suitable as oral administration since a broad antimicrobial spectrum would not only target the pathogenic bacteria but also kill the normal microbiota inhabiting the body, which is likely to increase the antibiotic resistant rate (Leekha et al., 2011; Rea et al., 2011). However, TVP1 could be developed into a potential therapeutic drug to treat bacterial infections when the infecting bacterium has not been identified (broad spectrum antibiotic). It could potentially also be used topically on skin or mucous membranes to treat ailments. In addition, there are many other applications that would appreciate broad-range antimicrobials. For example, it can be used in the food industry (to control food pathogens during food processing), as a green disinfectant (to decontaminate environmental pathogens), in veterinary science (to prevent zoonotic disease and avoid pathogen transmission through food) and in agriculture (to eliminate phytopathogenic bacteria). In contrast, TVP1 exhibit a narrow-spectra of antibacterial activity, it can be used as a potential drug candidate to treat infections caused by *Pseudomonas* sp without causing damage to the

host's microbiota. However, further experimentation investigating the true antimicrobial range of TVP1 will better inform the range of biotechnological applications it is suitable for.

Apart from the antimicrobial activity displayed by TVP1, the role it plays in the prophage and perhaps more broadly, in its host bacterium, was also of interest. *T. viridans*, from which TVP1 is derived, was isolated from oyster larvae of *Ostrea edulis* (Pujalte et al., 1999). Studies have shown that marine invertebrates and their associated bacteria share a mutualistic relationship where the invertebrate hosts provide a favourable habitat and sufficient nutrients while the symbionts in turn eliminate pathogens and/or reduce the settlement of pathogens (Sneed et al., 2014). These symbionts present in complex biofilms have also been shown to promote the settlement and metamorphosis of marine invertebrate larvae, a crucial process for the persistence of benthic marine populations (Freckelton et al., 2017). For example, bacterial biofilms have been shown to induce the settlement of larvae of sea sponges (Whalan et al., 2008; Abdul Wahab et al., 2014; Whalan & Webster, 2014), bryozoans (Dobretsov & Qian, 2006), cnidarians (Webster et al., 2004), molluscs (Bao et al., 2007; Gribben et al., 2009), annelids (Hadfield et al., 2014), echinoderms (Dworjanyn & Pirozzi, 2008; Huggett et al., 2006) and crustaceans (Khandeparker et al., 2006). Although recent literature has highlighted a range of microorganisms that induce ecological metamorphosis and settlement of larvae, very few studies have isolated the inducing agent. In one successful study by Sneed et al (2014), the settlement of larvae of some corals were shown to be partially or completely induced by a single small, non-polar bacterial metabolite, tetrabromopyrole (Sneed et al., 2014). Exopolysaccharides of the bacterial glycocalyx (a glycoprotein) was also shown to be responsible for the induction of larval settlement of the barnacle, *Balanus amphitrite* and the tunicate, *Ciona intestinalis* (Szewzyk et al., 1990). More interestingly, phage tail derivatives, such as tailocins, have also been shown to induce larval settlement

followed by metamorphosis (Freckelton et al., 2017). With the microscopic identification of tailocins being produced by *T. viridans*, together with the marine invertebrate origin of *T. viridans*, it raises the question whether these structures are also involved in inducing larval settlement in oysters. Moreover, what role, if any, does TVP1 contribute towards such a function? Clearly, further studies are required to identify whether this protein acts as an environmental cue for the settlement of oyster larvae and its true ecological role, and opens up a new exciting line of investigation.

4.6 Conclusion

In conclusion, we have linked the observed antimicrobial activity to a potential gene using a bioassay-guided fractionation approach coupled with LCMS and genome sequencing. Here we report the identification of a novel phage tail protein (TVP1) found in the genome of *T. viridans* that exhibits bioactivity against *P. putida* and possibly other indicator organisms including *B. cereus, S. epidermidis, E. coli* 1699*, Vibrio* sp*, Pseudovibrio* sp and *T. viridans* itself. Future work would entail the following: 1) Determining whether the tailocin (assuming it incorporates TVP1) identified in the >50kDa fraction is responsible for any of the antimicrobial activity detected in this study, 2) investigating the production of tailocins under sucrose (where no bioactivity was detected) to determine whether the tailocin-TVP1 activities are linked and 3) characterising TVP1 towards understanding its structure and mechanism of action to elucidate the biological function and also inform future biotechnological applications. Furthermore, this study has shown that *T. viridans* harbours the potential to produce a number of secondary metabolites, potential bioactive proteins and phage proteins, painting *T. viridans* as a promising source of novel chemistry.

Chapter 5: General discussion and final conclusion

Antibiotics remain a crucial tool in the treatment of pathogenic bacterial infections. However, more than 70% of known pathogenic bacteria are already resistant to some antibiotics (Bérdy, 2012). Therefore, the continued emergence of therapy resistant bacteria has led to a global urgency for the identification of novel antibiotics and antimicrobial treatment options. Natural products produced by microorganisms are well established as a source of most commercially available antibiotics (Bérdy, 2012; Villa & Gerwick, 2010), however the rediscovery of previously described antimicrobial compounds from microbes inhabiting terrestrial environments, has inspired a growing number of research groups to explore the oceans for new bioactive compounds (Debbab et al., 2010). Marine bacteria are of great interest as novel and rich sources of biologically active compounds as they constitute a promising source of unique and structurally diverse metabolites with considerable pharmaceutical and therapeutic potential (Debbab et al., 2010; Villa & Gerwick, 2010).

Here we used the OSMAC approach to assess the potential for the production of antimicrobial activity by *T. viridans*, and the most optimal conditions for such. Antimicrobial activity displayed by *T. viridans* was particularly interesting in that, in addition to its activity against wild-type strains, activity against the multi drug-resistant bacterium (*E. coli* 1699) was exhibited, indicating that the observed bioactivity was most likely caused by a novel biomolecule/(s). Since secondary metabolites produced by marine microorganisms exhibit a wide range of pharmaceutical activities (Egan et al., 2008; Williams et al., 2008) and in light of 20% of *T. viridans* genome being dedicated to potential secondary metabolites, we assumed that the observed activity was a product of one or more of the secondary metabolite gene clusters. To identify the biosynthetic pathway responsible for the observed activity, we used

a transcriptional profiling approach, targeting the secondary metabolite gene clusters of *T. viridans* cultured under the OSMAC conditions. As discussed in Chapter 3, this approach was not successful in our study as we could not precisely deduce the potential gene cluster responsible for the observed activity.

Size fractionation analysis presented in Chapter 4, indicated that the bioactive compound was greater than 50 kDa suggesting that the target antibacterial compound was not likely to be a secondary metabolite which are generally classified as being < 3 kDa in size (Zahner, 1979). Using a genome-and-bioassay-guided fractionation approach, performed in conjunction with LCMS/MS, showed to be more effective in isolating and identifying the antimicrobial compound. With these methods, we were able to identify the antimicrobial agent as a novel protein, TVP1. TVP1 is proposed to be encoded by a phage tail gene, integrated in the genome of *T. viridans*. Despite the bioactive compound being a phage derived protein and not a secondary metabolite, TVP1 is still a good candidate for therapy development. Moreover, phage derived antimicrobials are attractive alternatives to antibiotics due to their specificity and reduced risk of resistant evolution (Garcia et al., 2013; Behrens et al., 2017). In comparison, the use of whole phages used as a therapy is more prone to resistance (same as antibiotics). Phage-derived proteins on the other hand have a lower chance of triggering resistance evolution due to their modes of action, which is to degrade the cell wall of the target bacterium before the host has the opportunity to develop resistance (Donovan et al., 2009; Kiros, 2016). Consequently, phage derived products (and not the entire phage) are attractive options as antimicrobials since the functional protein cannot be assimilated into the host genome (Lin et al., 2017). With proper testing and further validation, TVP1 could be a formidable antimicrobial candidate. Among phage proteins known for their antimicrobial

activity, TVP1 appears to be novel, in that it cannot be classed with any known phage proteins based on sequence comparisons. TVP1's novelty warrants future experiments to improve yields through microbial fermentation or heterologous expression for further development and clinical trials. Of further interest would be to determine the structure and chemistry of TVP1 to identify the mechanism of action. Therefore, some fundamental understanding still needs to be established before clinical trials can be considered to assess TVP1's pharmaceutical suitability as an antimicrobial candidate. Furthermore, characterising TVP1 will also give insight into understanding its role in the natural environment and help to better understand the function of lysogeny and the relationship between the putative phage and the bacterial host and their role together in the natural environment. For example, it is not clear whether the phage-derived proteins confer a survival advantage for the host to occupy a particular niche. Considering the study conducted by Freckelton et al (2017), phage-derived proteins were shown to be the inducing agents of larval settlement in the natural environment and since the role of TVP1 is still uncharacterised, it remains a speculation that it may offer a similar function to oysters.

In addition to identifying a novel prophage and novel phage-derived proteins in the genome of *T. viridans*, WGS coupled with computational analysis of the microbial genome revealed a wealth of potentially novel chemistry in the secondary metabolite pathways of *T. viridans*. Therefore, although none of the secondary metabolite gene clusters were responsible for the production of the active compound under our laboratory conditions, we cannot exclude these pathways as potential antibacterial synthesis pathways since we did not test against other pathogenic bacteria such as *Klebsiella* sp. and *Acinetobacter* sp., which may show sensitivity to some of the compounds produced by this microorganism. Since secondary metabolites

from other marine microorganisms have exhibited anti-cancer, anti-inflammatory and anti-convulsant agents (as examples), it remains possible that the *T. viridans* metabolites may have even wider therapeutic application, and it would be prudent to screen *T. viridans* metabolites as broadly as possible. Hence, we advise continued experimentation to maximally exploit the secondary metabolite production of these pathways. These experiments should include culturing *T. viridans* under a variety of conditions such as altering the media composition (carbon and nitrogen source), incubation temperature including heat and cold shocks, salt concentration, amino acid supplementations and addition of antimicrobial agents. These factors have been shown to affect or induce the expression of secondary metabolite pathways and result in the formation of an array of bioactive compounds (Bode et al., 2002), possibly including other antimicrobial proteins, as it triggers the induction of different bioactive gene clusters.

In conclusion, through using WGS accompanied by computational genome mining, this study has revealed a wealth of novel chemistry in *T. viridans* that should be exploited for other therapeutic and biotechnological applications. Furthermore, this study has highlighted the significance of marine invertebrate associated microorganisms as novel sources of antimicrobial compounds and novel chemistries. Lastly, this study has also highlighted the importance of taking a parallel and integrated approach rather than a single approach. Our initial thoughts entailed taking a genome and transcriptomic approach for quicker identification of the target gene cluster/(s) instead, the standard bioassay and genome guided approach is the method that worked in the end and led to a quicker route for identification of the target bioactive gene cluster. To our knowledge, this is the first study to assess the antimicrobial potential of *T. viridans* and link bioactivity to a putative tailocin gene cluster.

Appendices

Appendix A

Table A1: List of antibiotics to which the multi-drug resistant *E. coli* 1699 exhibits resistance

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

Appendix B

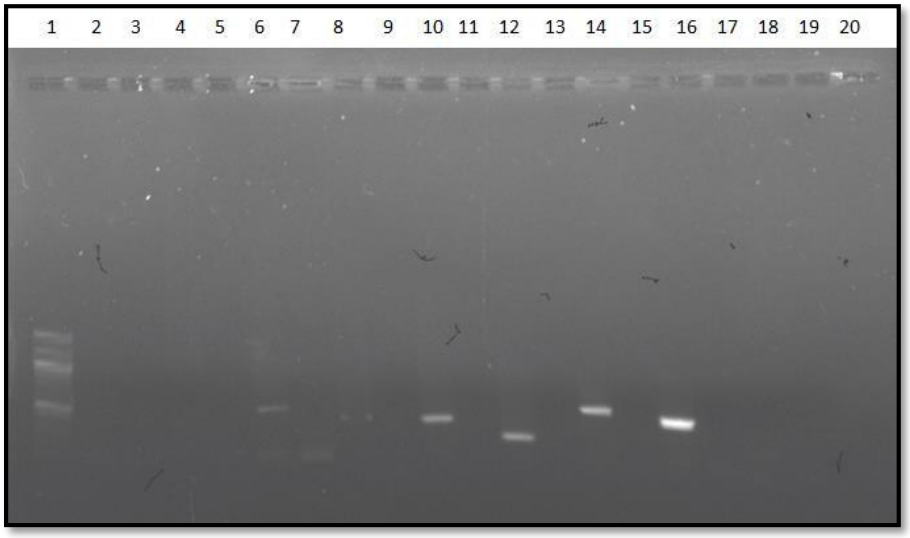


Figure B.1A: Transcriptional profile of *T. viridans* when cultured in MB supplemented with yeast extract as the sole carbon source. Lane 1: 100bp marker, lane 2: BGC 3a, lane 3: BGC 3a (-AMV), lane 4: BGC 3b, lane 5: BGC 3b (-AMV), lane 6: BGC 4, lane 7: BGC 4 (-AMV), lane 8: BGC 6, lane 9: BGC 6 (AMV), lane 10: BGC 8, lane 11 BGC 8 (-AMV), lane 12: BGC 5, lane 13: BGC 5 (-AMV), lane 14: BGC 7, lane 15: BGC 7 (-AMV), lane 16: BGC 9, lane 17: BGC 9 (-AMV), lane 18: BGC 10a, lane 19: BGC 10a (-AMV) and lane 20: BGC 10b.

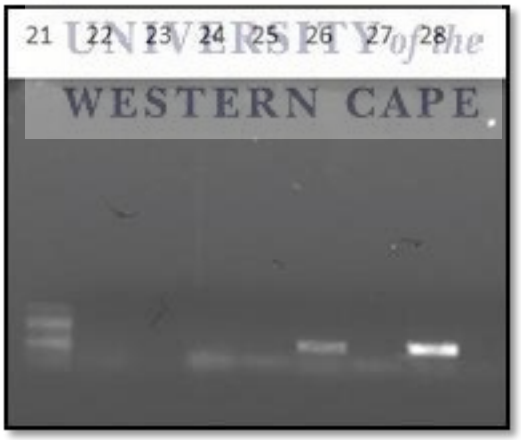


Figure B.1B: Transcriptional profile of *T. viridans* when cultured in MB supplemented with yeast extract as the sole carbon source. Lane 21: 100bp marker, lane 22: BGC 10b (-AMV), lane 23: BGC 11a, lane 24: BGC 11a (-AMV), lane 25: BGC 12 (-AMV), lane 26: BGC 12, lane 27: BGC 14 (-AMV) and lane 28: BGC 14.

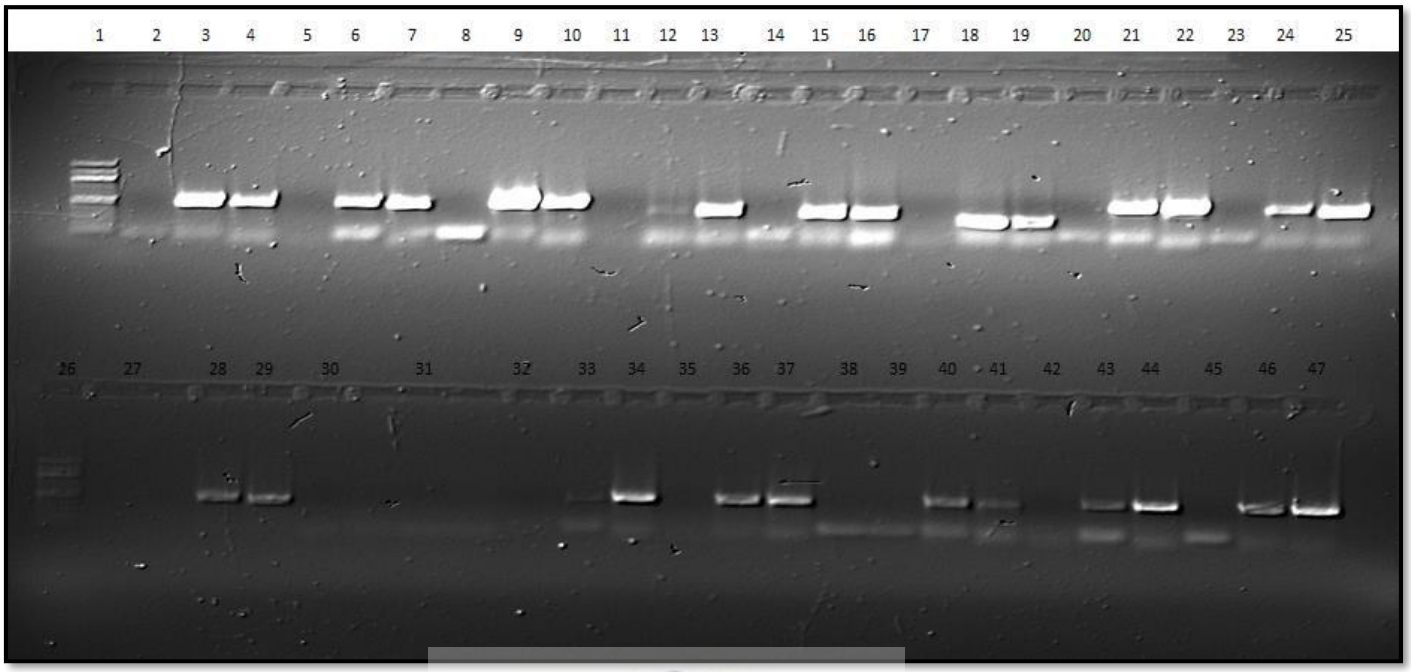


Figure B.2: Transcriptional profile of *T. viridans* in MB supplemented with sucrose as the sole carbon source. Lanes 1 and 26: 100bp marker, lanes 2 (BGC 3A), 5 (BGC 3B), 8 (BGC 4), 11 (BGC 5), 14 (BGC 6), 17 (BGC 7), 20 (BGC 8), 23 (BGC 9), 27 (BGC 10B), 30 (BGC 10A), 32 (BGC 11A), 35 (BGC 11B), 38 (BGC 12),39 (BGC 12), 42 (BGC 14) and 45 (BGC 15): -AMV controls, lanes 4 (BGC 3A), 7 (BGC 3B), 10(BGC 4), 13 (BGC 5), 16 (BGC 6), 19 (BGC 7), 22 (BGC 8), 25 (BGC 9), 29 (BGC 10B), 34 (BGC 10A), 37 (BGC 11) , 41 (BGC 12), 44 (BGC 14) and 47 (BGC 15): positive controls. Lanes 3 (BGC 3A), 6 (BGC 3B), 9 (BGC 4), 12 (BGC 5), 15 (BGC 6), 18 (BGC 7), 21 (BGC 8), 24 (BGC 9), 28 (BGC 10B), 33 (BGC 11A), 36 (BGC 11B), 40 (BGC 12), 43 (BGC 14) and lane 15 (BGC 46): activated pathways

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