UV mutagenesis to unlock cryptic secondary metabolism in streptomycetes, and the identification of novel antibiotics against Gram-negative pathogens

Asma Akter

Submitted in accordance with the requirements for the degree of

Doctor of Philosophy

The University of Leeds

School of Molecular and Cellular Biology

Faculty of Biological Sciences

January 2018

The candidate confirms that the work submitted is her own and that the appropriate credit has been given where reference has been made to the work of others.

Part of the research within Chapter 5 was carried out with the help of, Dr Richard Barton (Principal Clinical Scientist, Mycology Reference Centre, LGI, Leeds) and Sannia Farooque (School of Chemistry, University of Leeds). Credit has been given to the work carried out by Dr Richard Barton and Sannia within the chapter.

Acknowledgements

First and above all, I thank and praise the Almighty Allah for providing me this opportunity and granting me with blessings throughout my work to complete the research successfully.

I am extremely indebted to my supervisor Dr. Alex O'Neill, and wish to thank him beyond words for his invaluable support, advice and patience throughout my study period. I would also like to take this opportunity to thank him heartily for his generous support during my financial difficulties.

I would like to thank Dr. Chris Randall for his support and valuable advice from the beginning of my PhD study. Special thanks to Dr. Ryan Seipke for being kind to me in every aspect, and for providing me with his expert advice and suggestions. I would like to thank everybody in the O'Neill, Seipke & McDowall labs, and special thank goes to Elham Elkrewi, Fatma Salama, and Luiza Galarion for being such a nice heart to me.

I would like to thank Dr Richard Barton (Principal Clinical Scientist, Mycology Reference Centre, LGI, Leeds) for his kind assistance in the identification of the fungal isolate. I would also like to thank Sannia Farooque, and Dr. Chris Rayner from the School of Chemistry for their assistance in the purification of the antibacterial compound from my fungal isolate. I would like to extend my thanks to Martin Huscroft (School of Chemistry) for his training and assistance in LC-MS, and HPLC purification.

I would also extend my appreciation to Leeds International Research Scholarship (LIRS) to support my PhD study at Leeds.

No words can explain my thankfulness to my Husband S. M. Ahad Ali for this continuous support and patience throughout my PhD research. And a heartfelt apology to my two lovely daughters Anaum and Aalimah for not being by their side when they needed me most.

Abstract

Gram-negative multi-drug resistant (MDR) bacteria are resistant to most of our existing antibiotic arsenal, and consequently represent a major threat to clinical practice. Target-based synthetic drug design has been unsuccessful in delivering new antibacterial agents to combat these pathogens. The presence of ample cryptic/silent biosynthetic gene clusters (BGCs) that direct the production of antibiotics in different species of *Streptomyces* suggest that new antibiotics could be discovered simply by devising effective strategies to switch on these cryptic BGCs. Whilst approaches to switch on cryptic antibiotic production already exist, these are not generally a form that could be translated into an antibiotic discovery platform for large-scale mining of new antibiotics from *Streptomyces*. The present study describes UV induced mutagenesis as a new platform approach to activate cryptic BGCs to discover new antibiotics.

Initial studies established that UV irradiation could increase the frequency of mutation in phylogenetically distant *Streptomyces* species by 8-50,000-fold. Subsequently, screening for antibacterial activity against MDR *Escherichia coli* strain MG1699 established that UV mutagenesis enables the activation of cryptic antimicrobial activity in diverse streptomycetes. Comparative metabolomics was used to demonstrate that these UV-induced metabolites were compounds not previously linked to the species that produced them, revealing that this approach does allow access to new antibiotics from well-characterized species of *Streptomyces*.

MS/MS fragmentation and chemical de-replication of ten mass/metabolites with antibacterial activity indicated that seven of them may not have been discovered previously. Phenotypic characterization of UV-induced mutants (UVMs) from a given *Streptomyces* species also revealed diversity in their spectrum of antimicrobial activity against indicator microorganisms, implying that multiple new antibiotics can be recovered from a single strain using the UV mutagenesis approach.

Alongside these studies, this thesis also investigated the basis for the antibacterial activity observed for a fungal contaminant identified during the project. This organism, which was found to be a strain of *Penicillium dipodomyicola*, exhibited broad-spectrum, selective, antibacterial activity (including against MDR strains). Preliminary characterization of the agent responsible suggests that it could be a novel analogue of dimeric xanthone.

Table of Contents

List of Figures

List of Tables

List of abbreviations

ACN	Acetonitirile
antiSMASH	antibiotic and Secondary Metabolite Analysis Shell
BLAST	Basic local alignment search tool
BGC	Biosynthetic gene cluster
°C	Celsius
CFU	colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulphoxide
EIC	Extracted ion chromatogram
GNPS	Global natural products social molecular networking
HPLC	High performance liquid chromatography
HRMS	High-resolution accurate mass spectrometry
HTS	High-throughput screening
LB	Luria-Bertrani
LC-MS	Liquid chromatography–mass spectrometry
MDR	Multi drug resistance
MeCN	Acetonitrile
MeOH	Methanol
MF	Mutation frequency
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW/mw	Molecular weight
m/z	Mass to charge ratio
NMR	Nuclear magnetic resonance
NRPS	Non-ribosomal peptide synthetase
PCR	Polymerase chain reaction
pH	Potential hydrogen

PKSs	Polyketide synthases
rRNA	ribosomal ribonucleic acid
SkMs	UV-induced mutants from *S. kanamyceticus*
SaMs	UV-induced mutants from *S. antibioticus*
SgMs	UV-induced mutants from *S. griseoluteus*
SfMs	UV-induced mutants from *S. ficellus*
TLC	Thin layer chromatography
UV	Ultra-violet light
UVMs	UV-induced mutants
v/v	volume per volume
WHO	World Health Organization

Chapter 1 Introduction

1.1 Background to the study

Antibiotics have been one of the most successful forms of chemotherapy over the past ~80 years, enabling the treatment and prevention of infections caused by pathogenic bacteria (Wright, 2010; Davies and Davies, 2010). They have contributed to a rise in the life expectancy by decreasing the number of deaths from infectious diseases, and by reducing the risk of infections in post surgeries, organ transplantations and cancer chemotherapy (Adedeji, 2016). However, bacterial infection remains a significant healthcare challenge worldwide (WHO, 2017). This is particularly the case given the emergence and spread of antimicrobial resistance (AMR) among pathogens, and the scarcity of new antibiotics in the discovery pipeline (Ventola, 2015). It was recognized after the introduction of penicillin that bacteria can evolve under selection to become resistant to antibiotics (Payne *et al.,* 2007). Surprisingly, in less than a century, bacterial resistance to all the clinically used antibiotics has emerged. Several recent articles (CDC, 2013; O'Neill, 2016; WHO, 2017) have highlighted the need for immediate action to this globally rising health threat with improved surveillance and infection control. Though 22 new antibiotics have been introduced in the period between 2000-2014, these are limited to the treatment of Gram-positive infections; thus, infections caused by resistant Gram-negative bacteria currently pose a greater threat than Gram-positive pathogens.

Gram-negative bacteria possessing genes for extended spectrum β-lactamases (ESBLs) have become a major therapeutic challenge for treating both hospitalized- and community-based patients (Paterson and Bonomo, 2005). ESBLs are a group of diverse and complex enzymes which are capable of hydrolysing third generation cephalosporin and aztreonam, and eventually cause resistance against them (Bradford, 2001). These enzymes are typically encoded in plasmid DNA, and spread among bacteria by horizontal transfer (Rawat, 2010). ESBLs producing bacterial infections have been reported globally caused mainly by *Escherichia coli* and *Klebsiella* spp. (Rawat, 2010). Surveillance study by Mitra *et al*. (2011) in neonatal units of England and Wales had revealed the prevalence of ESBLs (26%) among new-born baby, most of them were *E. coli* and *Klebsiella*.

Carbapenem- and colistin-resistant *Escherichia coli* and *Klebsiella* spp. (carbapenem-resistant enterobacteriaceae/CRE), are another growing public health concern because they are resistant to carbapenem antibiotics (Capone *et al.,* 2013; Stein, 2016), as carbapenem and colistin were considered drugs of last resort for treating infections caused by these pathogens (Yu *et al.,* 2016; Zeng *et al.,* 2016; Granata and Petrosillo, 2017). The CRE has been detected in 44 states of the USA and around the world (CDC, 2013). According to the WHO (WHO, 2017), the critical priority pathogens of urgent demand for new antibiotics are the Gram–negative species *Acinetobacter baumannii* (carbapenem-resistant), *Pseudomonas aeruginosa* (carbapenem-resistant), and *Enterobacteriaceae* (carbapenem- or extended spectrum β-lactamase resistant). Therefore, development of new antibiotics against Gram-negative bacteria is now imperative. The following section will give an overview of antibiotic discovery to highlight the failure of current approaches and to convey the rationale for re-exploring natural products (NPs) to discover novel antibiotics.

1.2 An Overview of Antibiotic Discovery - Successes and Failures

Antibiotic discovery (Figure 1.1) began with the concept of the 'magic bullet' proposed in 1906 by Paul Ehrlich, which he defined as a chemical that selectively kills only disease-causing organisms but not the cells of the host (Aminov, 2010). After developing hundreds of derivatives of Atoxyl (sodium arsanilate), compound 606 (arsphenamine) was discovered and was successfully used to treat syphilis (Heynick, 2009). In the early 1930s, the synthetic compound sulfonamidochrysoidine (also named as Prontosil) was found to be effective against *Streptococcus pyogenes* infection in mice (Kimmig, 1969), and led to the development of a new class of antibacterial agents- the sulphonamides (Silver, 2011). Arguably, the most important breakthrough in the field was the discovery of penicillin by Alexander Fleming in 1928 (Fleming, 1929). Though Fleming first reported the activity of this compound, he neither successfully purified it nor tested it against bacterial infections in animals or humans; it was Florey's team at Oxford who achieved the latter and thereby established the therapeutic potential of penicillin for the first time (Lobanovska and Pilla, 2017). Penicillin was introduced into the market in the early 1940s and this was the real beginning of effective antibacterial chemotherapy.

In the 1940s, Selman Waksman developed a systematic approach to screen soil actinomycetes, a milestone in the discovery of natural antibiotics. Soil-derived actinomycetes were screened for antimicrobial activity against a susceptible test organism by detecting zones of growth inhibition on an agar overlay plate (Lewis, 2013). This approach discovered streptomycin, the first member of the

aminoglycoside class and heralded the start of the “Golden Era” of antibiotic discovery (Chopra, 2013; Brown and Wright, 2016). This era lasted over twenty years and saw the discovery of most of the antibiotic scaffolds used today to treat bacterial infection (Lewis, 2013). Later, semi-synthetic approaches became another platform for developing new antibiotics. The semi-synthetic approach involves the chemical modification of existing antibiotic chemical scaffolds to increase their activity, lower their toxicity and overcome bacterial resistance mechanisms (Chopra *et al.,* 2002; Lewis, 2012). Many of these semi-synthetic antibiotics were very successful and continue to be used; for example, the macrolide derivative, azithromycin (Lewis, 2013; Wright *et al.,* 2014).

Another approach to antibiotic discovery was proposed by Cohen in 1977 based on the concept of screening for inhibitors of bacterial essential enzymes in a cell-free assay (Cohen, 1977). This led to the development of the high-throughput screening (HTS) approach from the late 1980s (Payne *et al.,* 2007). In the 1990s, the antibiotic discovery field widely adopted target-based approaches, introducing HTS of synthetic compound libraries to identify potential molecules that bound to these targets (Mishra *et al*., 2008). Determination of the whole genome sequence of *Haemophilus influenzae* initiated the post-genomic era of antibiotic discovery (Figure 1.1) when comparative genomics of pathogens provided a way of identifying potential new antibacterial drug targets (Donkor, 2013). The targets chosen were often enzymes that are essential in pathogenic bacteria but absent in humans.

During this period, many of the major pharmaceutical companies abandoned natural product research in favour of screening large libraries of synthetic molecules for antibacterial activity (Shen, 2015). The complex extracts from microbial broths were incompatible with the modern detection techniques for HTS, and it was considered too time-consuming and costly to continue routinely screening NPs. For example, Glaxo, SmithKline Beecham (now part of GSK), Merck & Co. Pfizer, Inc. and Wyeth all pursued HTS technology to discover novel antibacterial drug candidates (Macarron *et al.,* 2011). The SmithKline Beecham antibiotic development program using HTS was active from 1995 to 2000, and has been described in detail (Payne *et al.,* 2007). Although this program identified potential targets and ran 67 high-throughput screens (~530,000 compounds), none of their 'hit' compounds progressed to the development candidate stage (Payne *et al.,* 2007). The team concluded that a lack of biologically relevant chemical diversity in their screening set was a major contributor to the poor outcome (Hansford, Blaskovich, and Cooper 2016). Most of the compounds active against the purified targets lacked antibacterial activity, as they were unable to accumulate inside bacterial cells. Thus, over 20 years after its advent, no antibiotic developed by HTS has reached the market (Payne, 2007; Silver, 2011; Lewis, 2013).

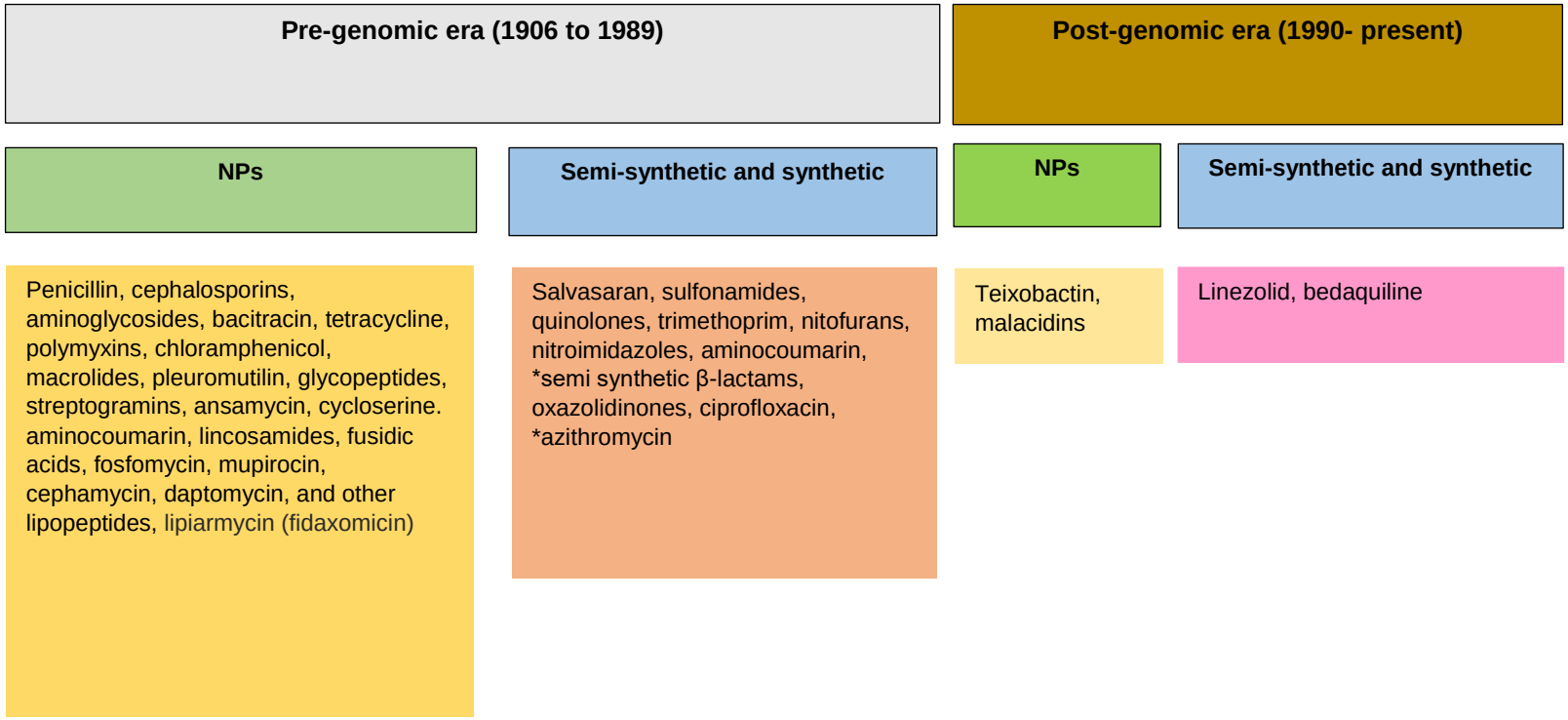


Figure 1.1 Antibiotics from natural products Vs synthetic compounds in the pre- and post-genomic eras of antibiotic discovery (major breakthroughs are shown here). NPs: Natural products. * denotes semi-synthetic compound (Adapted from Lewis, 2013; Fischbach and Walsh, 2009).

Only five new classes (linezolid, daptomycin, retapamulin, fidaxomicin, and bedaquiline) were introduced over a period of 16 years (2000-2015), (Butler *et al.,* 2013; Brade *et al.,* 2016). Three of them have a natural origin and/or scaffold, and were in fact discovered in the pre-genomic era. The breakthroughs in recent years were the discovery of teixobactin from uncultivable soil bacteria (Ling, 2015) and culture-independent metagenome based discovery of malacidins (Hover *et al*., 2018). Therefore, it is obvious that NPs have been a continuous source for new antibiotics, either in their own right or as precursors for semi-synthetic antibiotics (Figure 1.1). However, the overall reduction in new 'antibiotic lead' in post-HTS is mainly due to the down scaling or abandoning of natural product antibiotic discovery program by Big pharma (Li and Vederas, 2009). The shortage of new Gram-negative specific antibiotic candidate in the clinical pipeline and the emergence of antimicrobial resistance (AMR) among pathogenic bacteria emphasize on the necessity of re-exploration of NPs for the discovery of novel antibiotics.

Microbial NPs are an established source of diverse bioactive natural products (Huang and Lin, 2017). Both bacteria and fungi have been contributed into the arsenal of natural product antibiotics. Whereas antibiotics like streptomycin, daptomycin, and chloramphenicol are produced by bacteria, penicillins and cephalosporins (member of the beta-lactam class of antibiotics), the remarkable NPs discovered from fungi (Elander, 2003). Other antibiotics from fungi include griseofulvin (Richardson and Warnock, 2012), fusidic acid (Biedenbach *et al*., 2010) and pleuromutilin (Novak and Shlaes, 2010). In the next section, the scope of microbial natural product antibiotic discovery in post-genomic era is described.

1.3 Microbial NPs for the discovery of novel antibiotics

1.3.1 Unexploited natural sources

It is likely that most easily accessible natural products with antibiotic properties have already been identified. Nevertheless, unexplored natural sources such as uncultivable microorganisms (Singh and Macdonald, 2010; Taylor, 2013), microbes from arctic regions (Liu *et al.,* 2013), and other extreme environments (Roberts, 2013) could be sources of new chemistry. It is currently estimated that less than 1% of the total microbes in the environment are cultivable by traditional techniques, and that 1 gram of fresh soil contains 10^{11} uncultivable organisms (Stewart, 2012). These calculations indicate the likelihood that useful secondary metabolite producers exist that have yet to be cultivated (Baltz, 2006; Joshi *et al.,* 2014).

Biotechnology and pharmaceutical companies like TerraGen Discovery, Xoma, Diversa and Millennium and Rhône-Poulenc Rorer have all pursued metagenomic approaches to discover new antibiotics from these uncultured microorganisms (Lewis, 2013). NovoBiotics has developed a special programme for isolating metabolites from uncultivable organisms by mimicking their natural environment in the laboratory (Kaeberlein *et al.,* 2002). Texicobactin, a new class of antibiotic from an undescribed soil bacterium (*Eleftheria terrae*) was discovered using a new tool, the iChip, for bacterial cultivation in the laboratory (Ling, 2015). The iChip is a high-throughput technological platform to cultivate unculturable microbes *in situ* (Nichols *et al*., 2010). In brief, this multi channel device was employed to isolate and grow uncultrable soil bacteria by inoculating diluted soil sample (20 µl aliquot) in each of the tiny wells (total 384 wells) and then incubated in soil after covered with two semi-

permeable membranes (Ling *et al*., 2015). A substantial number of microbes were grown, and among these ~10,000 cultures were screened for activity against Gram-positive *S. aureus*. The extract from *Eleftheria terrae*, produced a large zone of inhibition against *S. aureus*. Later the compound responsible for this activity was identified and chracterized as teixobactin (Ling *et al*., 2015). Malacidins, a calcium-dependent peptide antibiotic was discovered from environmental DNA by metagenomic approach that showed activity against methicillin resistant *S. aureus* (MRSA) in animal model of skin infection without any detectable resistance (Hover *et al*., 2018).

The marine environment is a rich source of novel chemical classes (Zhao, 2011; Ziemert *et al.,* 2014), marine microbes could be a source of new antibiotics (Mayer *et al.,* 2010; Taylor, 2013; Skropetaa *et al.,* 2014). Abyssomicin C was discovered from a marine bacteria *Verrucosispora* spp. (Riedlinger *et al.,* 2004) that inhibits *para*-aminobenzoic acid biosynthesis in the folic acid pathway in staphylococci (Manivasagan *et al.,* 2014). Marine actinobacteria especially the genus *Salinispora* has been demonstrated as a source of structurally diverse compounds with bioactivity (Fenical and Jensen, 2006). Secondary metabolite investigation by Mincer *et al*. (2002) from over 100 *Salinispora* spp. showed more than 80% of strains showed anti-tumor aactivity and 35% showed antibacterial activity including drug-resistant bacteria. Later same group discovered arenimycin from marine *S. arenicola*, which is an antibacterial against rifampicin- and methicilin resistant *S. aureus* (Asolkar *et al.,* 2010). New antibacterial activity against drug resistant human pathogen from *Salinispora* spp. are being reported periodically (Singh *et al*., 2014; Bose *et al*., 2015; Cardoso-Martínez *et al*., 2015).

Comparative genomics of marine-derived sponge associated *Streptomyces* with closely-related *S. albus* J1074 showed hidden potential of secondary metabolites (Ian *et al*., 2014). Another promising compound, patellemides from a cyanobacteria *Prochloron didemni* (in symbiosis with *Lissocolinum patella*) was discovered with potential anti-cancer activity against multi-drug resistant cancer cell lines (Schmidt *et al*., 2005)

Both terrestrial and marine endophytic fungi have demonstrated a source of new antimicrobial compound (Deshmukh *et al*., 2015). For instance, from 1991 to 2014 total 390 new natural products were discovered from marine *Penicillium spp.*, among these 5% of NPs possess antimicrobial activity (Ma *et al.,* 2016). Many of these NPs are active against drug resistant Gram-positive pathogens, for example, methicillin-resistant *S. aureus* (MRSA) *(*Koyama *et al*., 2005; Yang *et al.,* 2011; Li *et al*., 2013; Khamthong *et al*., 2014). Other NPs exhibited activity against Gram-negative bacteria, for instance, a novel phenolic compound*,* 4-(2,4,7-trioxa-bicyclo [4.1.0] heptan-3-yl) phenol exhibited activity against *E. coli, P. aeruginosa, and K. pneumoniae* by causing cytoplasmic agglutination and membrane damage (Subban *et al.,* 2013).

1.3.2 Cryptic biosynthetic gene clusters (BGCs)

The genes required to direct the synthesis of an antibiotic are usually clustered together as several co-ordinately regulated transcriptional units in the chromosome, or occasionally on a plasmid, in the producing organism. In general, these clusters of genes are termed as biosynthetic gene clusters (BGCs).

Whole genome sequence of microorganisms, and *in silico* analysis have revealed the presence of several BGCs with biosynthetic capabilities of different secondary metabolites, in a particular species (Tracanna *et al*., 2017). For instance, *Streptomyces coelicolor* A3(2) is a model organism from the genus *Streptomyces*, and was known to produce four secondary metabolites before the availability of its whole genome sequence data (Challis, 2008). But the genome sequence revealed an additional ~20 BGCs in this species (Bentley *et al*., 2002). Thus, it was apparent that this species has far more biosynthetic capabilities beyond our knowledge. Those newly detected BGCs were not linked to any known metabolites, and subsequent studies revealed that those BGCs are either remain unexpressed or poorly expressed under typical laboratory conditions (Challis, 2008). BGCs that remain unexpressed or poorly expressed under typical laboratory conditions have been called ‘silent’ or ‘cryptic’ and represent a potential reservoir of novel antibiotics (Zerikly and Challis, 2009).

Several databases and *in silico* computational tools have been developed to analyse BGCs encoding secondary metabolites (Weber and Kim, 2016). ClusterFinder is a standalone software that can identify putative BGCs by utilizing a non-rule based probabilistic approach (Cimermancic *et al*., 2014). It is also integrated into Antibiotics and secondary metabolite analysis shell (antiSMASH) (Blin *et al.,* 2017); an automated pipeline for mining of secondary metabolite encoded gene clusters from bacteria and fungi to predict genes from the input nucleotide sequence, and identifies gene clusters by specific domain analysis and annotation, in some cases predicting the core chemical structure of cryptic secondary metabolites. All the gene cluster mining tools follow the standard from Genomic Standards Consortium (GSC) for the prediction of BGCs to ensure the consistent and systemic data incorporation and retrieval into the BGCs database. The Minimum Information about a Biosynthetic Gene cluster standard (MIBiG) is the standard approved by GSC that defines the minimal set of information to describe a BGC.

It is worth noting that the number of cryptic pathways apparently coding for the biosynthesis of putative natural products far outnumbers the number of currently known metabolites for a given organism (Nett, Ikeda, and Moore, 2009). Analyses of more than 500 microbial genome sequences currently in the publicly accessible databases have revealed numerous examples of gene clusters encoding enzymes similar to enzymes involved in the biosynthesis of important natural products (Tracanna *et al.,* 2017). Examples of such enzymes include non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs) and terpene synthases (TSs), as well as enzymes belonging to less thoroughly investigated families (Lou, 2014; Tracanna *et al.,* 2017).

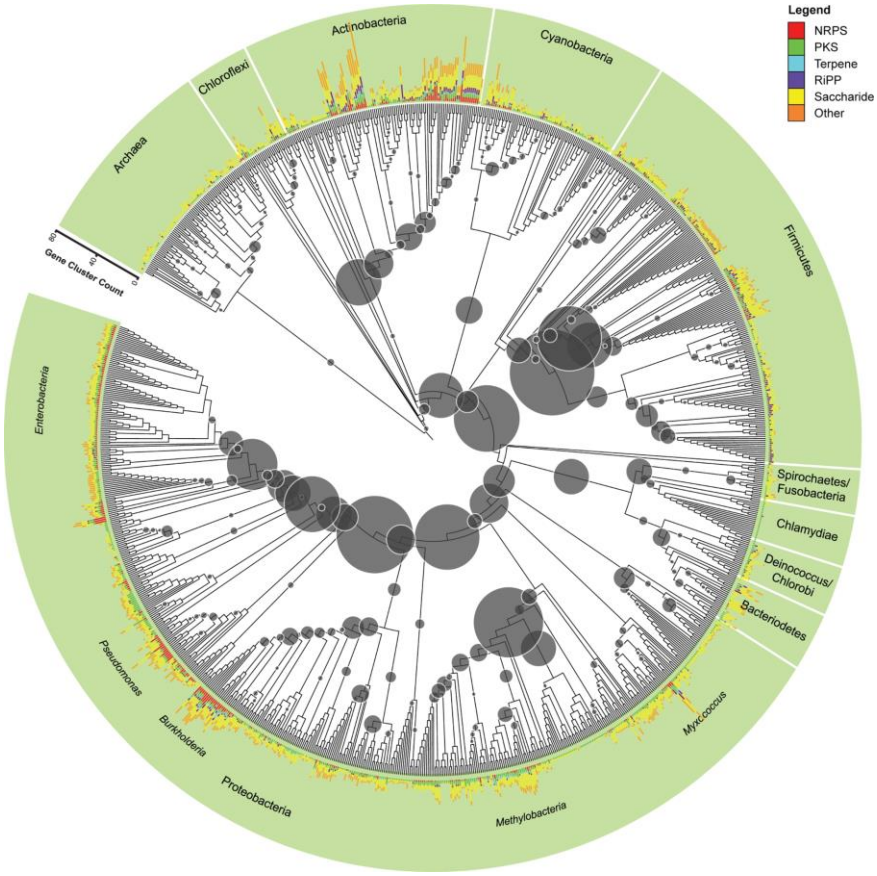


Figure 1.2 Taxonomic diversity of biosynthetic gene clusters (BGCs) across bacteria and archaea. Bar plots indicating gene cluster counts as detected by antiSMASH+ClusterFinder are plotted onto prokaryotic taxonomy. Circles on the tree indicate the amount of within-taxon variation (Adapted from Tracanna *et al.*, 2017).

Figure 1.2 shows the predicted number of BGCs across the prokaryotic taxa where *Actinobacteria*, *Mycobacteria, Burkholderia* and *Cyanobacteria* possess the greatest numbers of BGCs that might produce new antibiotics (Cimermancic *et al.,* 2014). Genome mining and activation of silent gene cluster in *Burkholderia gladioli* led to the discovery of 'gladiolin', which showed promising activity against *Mycobacterium tuberculosis* (Song *et al*., 2017). The discovery of these numerous pathways represents a treasure trove, and innovative approaches should to be employed to access these cryptic BGCs to uncover novel antibiotics.

Despite success of fungal NPs, until recent large-scale investigation of secondary metabolites, they were less studied and characterised at the genomic level. The antiSMASH genome analysis and comparison of 34 sequenced fungal strains (11 *Aspergillus* strains) revealed 44 unique genes encoding polyketide synthases (PKSs), non-ribosomal peptide synthases (NRPSs), and dimethylallyl tryptophan transferases (DMATs), with no orthologues across the analysed *Aspergillus* spp., which anticipates the chances of discovering new chemical entity from these species (de Vries *et al.,* 2017). Intriguingly, horizontal gene transfer from other bacteria into fungi in nature has also been reported. Studies of 60 fungal genomes showed that 713 bacterial genes had been transferred to fungal genomes (Marcet-Houben and Gabaldón, 2010). Early studies hypothesized that bacterial β-lactam antibiotic genes were transferred to fungal species such as *A. nidulans*, *Penicillium chrysogenum*, and *Acremonium chrysogenum* either from Gram-positive (e.g. *Streptomyces* spp.) or Gram-negative (e.g. *Lysobacter* spp.) bacteria (Kück *et al*., 2014). Although *Aspergillus* and *Penicillium* species have 30–40 Mb genomes containing 30–80 PKS and NRPS genes, most of their BGC-encoded products have yet to be discovered (Alberti *et al.,* 2017).

1.4 Approaches to activate cryptic BGCs in streptomycetes

Actinomycetes, and specifically the genus *Streptomyces,* have considerably enriched the arsenal of natural product antibiotics from the early 1940s onwards (Procópio, 2012; Nett *et al.,* 2009). It is predicted that this genus of over 500 described species (Manivasagan *et al.,* 2014) can produce as many as 150,000 natural products (Clardy *et al.,* 2006; Baltz, 2006). However, Baltz (2006) has estimated that $>10^7$ strains of soil actinomycetes would need to be screened to discover the next novel antibiotic with clinical utility. When Waksman discovered streptomycin and tetracycline from ~10^3 random soil actinomycetes (Baltz, 2007), the task was easy; however, screening $>10^7$ random soil strains for the next therapeutic antibiotic candidate would be an incredible challenge. As mentioned in the previous section, actinomycetes possess the highest number of BCGs found in bacteria, although most of these BGCs are not expressed in the laboratory condition. For instance, whole genome sequences of three *Streptomyces* species (*S. coelicolor*, *S. avermitilis* and *S. griseus)* were analysed by antiSMASH revealing the presence of an average of 34 BGCs in each strain (Nett *et al*., 2009). These BGCs have the capability to produce secondary metabolites such as polyketides and non-ribosomally synthesized peptides, bacteriocins, terpenoids, aminoglycosides and other natural products (Nett *et al*., 2009). Previously, only four to five metabolites were known from these species. Thus, genomic analysis indicates that the biosynthetic capability of these bacteria far exceeds compare to the known secondary metabolite reported. Researchers have been trying to trigger the expression of cryptic BGCs in *Streptomyces* to discover new antibiotics; some of these contemporary approaches of activating silent BGCs (Table 1.1) are discussed below.

1.4.1 Genome-driven approaches

Genomic approach involves direct cloning or synthetic refactoring of cryptic BGCs, manipulation of global/pathway specific regulators and expression of the clusters in native organisms or in heterologous hosts. Typically, a natural BGC is larger than 20 kb (sometimes up to 100 kb; Nah *et al.,* 2017). Larionov and co-workers developed a transformation-associated recombination (TAR) cloning method in *S. cerevisiae* to isolate large chromosomal fragments or entire gene clusters from mammalian and bacterial genomes (Cocchia *et al.,* 2000; Noskov *et al.,* 2002; Noskov *et al*., 2003; Feng, Kim and Brady, 2010). In 2009, Shao *et al.* (2009) developed the DNA assembler approach to assembling multiple gene expression cassettes either on a plasmid or a chromosome in *Saccharomyces cerevisiae*. Both TAR cloning and the DNA assembler approach have shown the ability to capture BGCs from *Streptomyces* spp. (Yamanaka *et al.,* 2014; Shao, Luo and Zhao, 2011; Luo *et al.,* 2013). Once cloned, manipulation of silent BGCs can be performed by optimising promoters, manipulating cluster specific regulatory genes, ribosome-binding sites, and even codon usage (Medema *et al.,* 2011). For example, overexpression of *sanG* (a pathway specific regulator) significantly increased the production of nikkomycin in *Streptomyces anoschromogenes* (Zhong *et al.,* 2013), and the overexpression of LAL family regulatory genes (LuxR-like gene) activated stambomycins A-D from silent BGC in *S. ambofaciens* (Laureti *et al.,* 2011). The manipulation of pleiotropic regulators has also been shown to activate silent gene transcription; for example, disruption of *adp*A encoding a global regulator (AdpA) resulted in the activation of cryptic oviedomycin gene cluster in *S. ansochromogenes* (Xu *et al.,* 2017). Another genomic approach for cryptic gene cluster activation is the application of CRISPR–Cas9 strategy to generate mutants in *Streptomyces* (Huang *et al.,* 2015). This strategy has achieved activation of multiple BGCs of different classes in five *Streptomyces* species, leading to the

production of unique metabolites such as a novel pentangular type II polyketide in *Streptomyces viridochromogenes* (Zhang *et al.,* 2017). However, genome driven approaches either require genetically tractable *Streptomyces* strains and/or the *de novo* development of genetic protocols optimised for each native producer strain. Although there is no universal heterologous host but *S. coelicolor* M1146 and *S. coelicolor* M1152 (Gomez and Bibb, 2011), *S. albus* J1074 (Zaburannyi *et al.,* 2014), and *S. avermitilis* (Komatsu *et al*., 2010) have been developed to express specific BGC heterologously.

1.4.2 Environmental cues and co-culture approaches

In the one strain-many compounds (OSMAC) approach, host strains are grown under different fermentation conditions to express new compounds by manipulating growth parameters (media composition, temperatures, pH, aeration, etc.). This can be done in two main ways; (i) manipulation of growth conditions without prior knowledge about the genomic information of a strain, and (ii) first prediction of physiochemical characteristics of putative product of a silent BGC, and then addition of nutrients provided with in the media to obtain desired metabolites from fermentation. Using the first approach, several novel compounds were identified from *S. flaveolus* through screening six different media, and expression of corresponding BGC was confirmed by transcriptomics (Qu *et al*., 2011). Ecopia BioSciences discovered a novel antifungal agent from *Streptomyces arzunensis* (McAlpine *et al.,* 2005) and a novel antibacterial agent from *Amycolatopsis orientalis* (Banskota *et al.,* 2006) utilizing the second approach.

Chemicals ‘elicitors’ such as histone deacetylases (HDAC) inhibitor sodium butyrate has shown to influence the production of actinorhodin in *S. coelicolor* in a culture-dependent manner (Moore *et al*., 2012; Zhu *et al*., 2014). Sodium butyrate can apparently disrupt chromatin structure and trigger global changes in gene expression in *Streptomyces.* The cell-wall component *N*-acetylglucosamine (GlcNAc) has also been used to activate the expression of antibiotic encoding gene clusters in *S. coelicolor* and other four *Streptomyces* spp. (Rigali *et al*., 2008). GlcNAc can serves as a signalling molecule in streptomycetes, and the accumulation of GlcNAc under nutrient limitation promotes morphological development and antibiotic production.

Microbial interaction can also be an inductive cue for the biosynthesis of novel natural products such as antibiotics (Schroeckh *et al.,* 2009). This ‘co-culture’ approach has the potential to activate novel antibiotic gene clusters in *Streptomyces;* for instance*,* a novel antibiotic was obtained from a *Streptomyces* strain after co-culture with a mycolic acid-containing *Tsukamurella pulmonis strain* from soil samples (Marmann *et al.,* 2014). In a different study, the antibiotics istamycin A and B production from the marine bacterium *Streptomyces tenjimariensis* was induced by co-culturing with Gram-positive and negative marine bacteria (Bader *et al.,* 2010).

1.4.3 Mutagenic approaches

Mutations in the *rpsL* gene, which encodes ribosomal protein S12 (Ochi *et al.,* 2004), and mutations in *rpoB* that encodes a subunit of RNA polymerase (RNAP) (Chiang *et al.,* 2011; Craney *et al.,* 2013) have the ability to activate silent BGCs for secondary metabolite production. A mechanistic study indicated that the effect observed was due to a mutation at Lys-88 to either Glu or Arg in the ribosomal protein S12, a substitution which enhances protein synthesis in stationary growth phase (Hosaka *et al.* 2009). In the case of RNAP, the bacterial alarmone ppGpp was found to bind the enzyme (Artsimovitch *et al.,* 2004), thereby initiating the production of antibiotics (Bibb, 2005; Ochi, 2007). Ribosomal engineering has been proposed by Ochi as a method to induce the expression of *Streptomyces* cryptic gene clusters (Ochi and Hosaka, 2013). It is worth to note that these ribosomal mutations have been incorporated into the heterologous host *S. coelicolor* M1154 for increasing the production of secondary metabolites with the benefit of global regulation of BGCs (Gomez and Bibb, 2011).

A recent targeted activation of a specific silent gene cluster (the *pga* cluster) in *Streptomyces* sp. PGA64 was achieved by genome-scale random mutagenesis (by UV irradiation), using a reporter-guided selection system to identify mutants (Guo *et al.,* 2015). These mutants produced two new anthraquinone aminoglycosides, gaudimycin D and E. However, this approach relied on the availability of genomic information and the need to clone the promoter sequence of the targeted BGC.

Table 1.1 Approaches for the activation of silent BGCs, and their disadvantages. (Adapted from Rutledge & Challis, 2015)

Strategies	Principle	Disadvantages
Environmental cues & Co-culture	Variation in growth conditions, for example, by adding competing species, chemical elicitors or other additives) to induce changes in BGC expression	Limited by the range of conditions under which the organism will grow and difficulties to predict which variables will influence gene expression.
Ribosomal engineering	Induction of mutations in RNA polymerase and ribosomal proteins to cause upregulation of BGC expression	Only a small number of ribosome modulating antibiotics trigger changes in a restricted group of bacteria, potentially limiting broad applicability.
Manipulating global regulators	Alteration of expression levels of pleiotropic transcriptional regulators to modulate BGC expression	Only a few pleiotropic transcriptional regulators are known in most microorganisms, so scope is limited.
Epigenetic perturbation	Use of mutagenesis or small-molecule inhibitors to disrupt chromatin structure and thus trigger global changes in gene expression	Sites at which histone methylation or acetylation may be altered are limited by accessibility.

Table 1.1 Approaches for the activation of silent BGCs, and their disadvantages (continued)

Strategies	Principle	Disadvantages
Manipulating pathway-specific regulators	Identification of genes encoding putative pathway-specific transcription factors within a BGC of interest, followed by overexpression (for an activator) or deletion (for a repressor) of these genes as appropriate	Requires that the BGC of interest be controlled by an identifiable transcription factor, that its genetic manipulation is readily achievable, and that perturbation does not cause other effects that complicate identification of the target metabolite
Reporter-guided mutant selection	Genome-scale random mutagenesis to introduce genetic diversity, while coupling a target BGC to a promoter–reporter system that visualizes mutants in which target BGC transcription is activated	Requires cloning of promoter sequences from a target BGC into a reporter cassette and generality has not yet been demonstrated beyond a single example.
Refactoring	Replacement of the natural promoters within a BGC with constitutive or readily inducible promoters	Natural promoters must be readily identified and the BGC must be amenable to genetic manipulation.
Heterologous gene expression	Expression of an entire BGC in a heterologous host	Traditional methods for heterologous expression are limited to small gene clusters (<40 kb), but many BGCs are considerably larger than this; recent advances are addressing this limitation.

1.5 Random mutagenesis to access cryptic secondary metabolites in *Streptomyces*?

Activation of silent BGCs by different approaches is being studied extensively as a route to identifying novel NPs (Ren *et al.,* 2017). Nevertheless, the current approaches have several drawbacks (Table 1.1). As mentioned above, genomic approaches rely on the genomic data and genetic amenability of strains, and/or utilization of universal heterologous hosts (for expression of specific gene cluster). They also require the knowledge of cluster specific or global regulatory systems. Other approaches like OSMAC plays with environmental conditions by trial and error and ribosome engineering uses only certain ribosome modifying antibiotics. These approaches have activated only few silent BGCs with antibiotic activity. For example, a new antibiotic keyicin (Adnani *et al*., 2017) was selectively active against Gram-positive bacteria. It was activated by co-culturing of a *Rhodococcus* sp. and a *Micromonospora* sp. It is apparent that current approaches cannot meet the urgent demand of discovering new Gram-negative antibiotics for clinical practice. A new approach to achieve large-scale and comprehensive activation of cryptic BGCs by inducing changes in their gene expression is imperative for the discovery of novel antibiotics. Although different approaches have been utilized to activate silent BGCs from streptomycetes, except for OSMAC, none of them have shown the ability to activate multiple BGCs. Furthermore, except for a few examples, none of these approaches discovered clinically relevant antibiotics.

In streptomycetes, nutrient depletion or environmental changes start the stationary growth phase that leads to the activation of secondary metabolite production and morphological differentiation via complex series of changes in global gene expression (McCormick and Flärdh, 2012; Nieselt *et al.,* 2010). Moreover, BGCs often contain pathway-specific regulatory genes, as well as genes involved in antibiotic export and self-resistance. Thus, the regulation of antibiotic production involves multiple factors and regulatory cascades (Figure 1.3) that create a complex network. This network of regulation is controlled at two main levels. At the lower level, the pathway-specific regulator can modulate the antibiotic biosynthetic genes of the BGC, for example, ActII-ORF4 for the production of actinorhodin in *S. coelicolor* (Arias *et al*., 1999); and at the upper level production of more than one antibiotics is often co-ordinately controlled by pleiotropic or global regulatory genes. DasR, a DNA binding protein of *S. coelicolor*, is an example of such a pleiotropic regulatory protein that links antibiotic production to the nutritional status of the cell (Rigali *et al*., 2008). Antibiotics can also be regulated by small diffusible extracellular signalling molecules, for instance, γ-butyrolactone A-factor from *Streptomyces griseus* was shown to regulate the production of several secondary metabolites and the onset of morphological differentiation (Ohnishi *et al*., 2005).

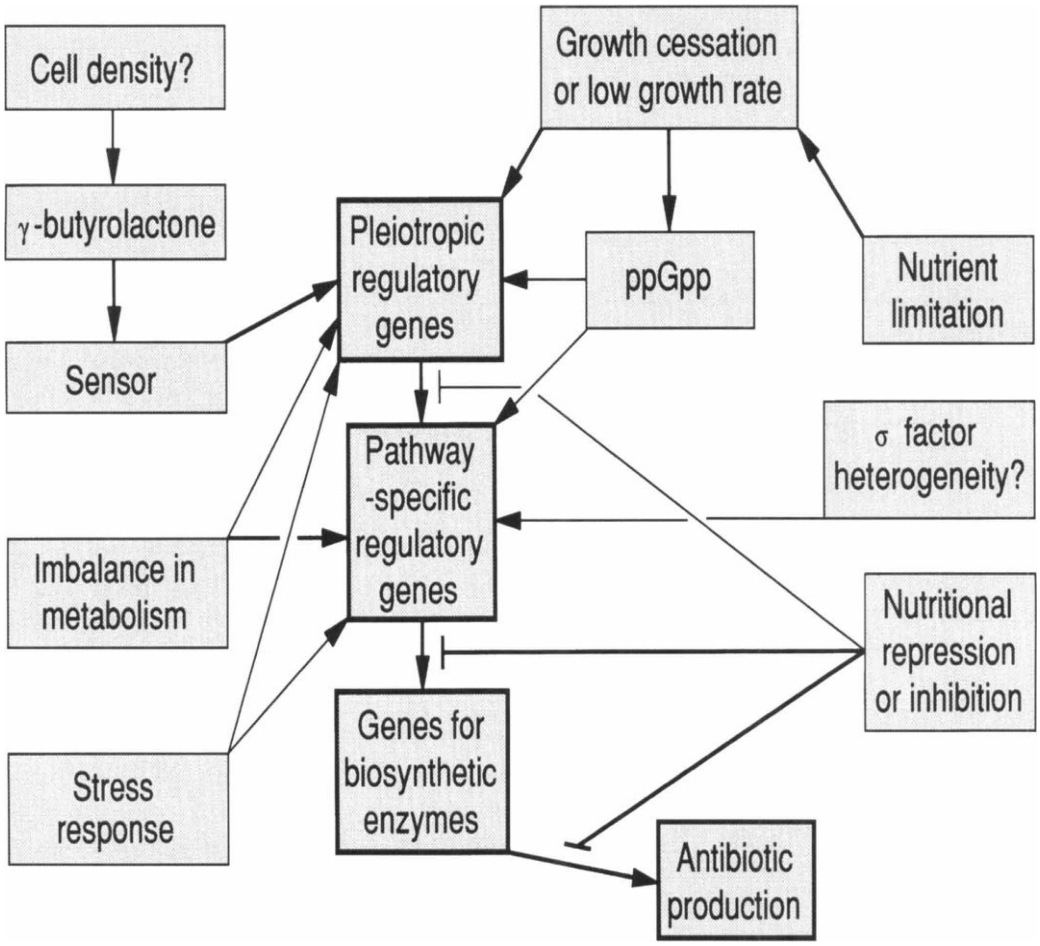


Figure 1.3 Factors and regulatory cascade involved in the regulation of antibiotic production on streptomycetes. Regulatory cascade: Pleiotropic and pathway specific regulation of genes for biosynthetic enzymes. In general, the interaction of different intra and extra cellular factors at different levels within the cellular regulatory network or even with the secondary metabolite biosynthesis forming a complex regulatory system for the production of antibiotics in *Streptomyces* (Adapted from Bibb, 1996).

Random mutagenesis is a widely applied approach for improving the yield of antibiotics in *Streptomyces* spp. (Siddique *et al.,* 2014; Gao *et al*., 2010; Korbekandi *et al.,* 2010). Typically, random mutation can be induced by either physical mutagens (UV light, X-ray and gamma ray) or by chemical mutagens (Kodym and Afza, 2003). These mutagens induce changes in the DNA by substitution, frame-shift mutation or deletions that remain unrepaired and produce a mutant derivative of the parent strain with high yielding capability of a metabolite (Kieser *et al.,* 2000). These changes lead to an increase of the number of antibiotic biosynthesis enzymes, deactivation of negative regulators, and/or increase of positive regulatory effect (Paradkar, 2013; Parekh *et al.,* 2000; Stephanopoulos *et al*., 2000; Jia *et al.,* 2010).

A recent genomic study on antibiotic biosynthesis investigated by Xu *et al*. (2017) revealed the complex regulation of tripyrrole antibiotic undecylprodigiosin (RED) production in *S. coelicolor*. In this study, large-scale transposition mutagenesis resulted in the identification of hundreds of genes that influence the production of RED apart from pathway specific transcription regulators; 365 genes were identified that could alter the level of RED production. After the gene complementation, they found that more than two-third genes were responsible for the altered RED production. Interestingly, these genes participated in different biosynthetic pathways (such as amino acid biosynthesis, DNA metabolism, and protein modification), and from those responsible for signalling, stress and transcriptional regulation. Therefore, it is evident that regulation of antibiotic production is not confined to some global regulatory genes and pathway specific genes in streptomycetes. Herein lies the potential of random mutagenesis to trigger expression from silent BGCs by inducing genome-wide global changes.

1.5.1 UV-induced mutagenesis and its use to mutagenize streptomycetes

UV induces all type of base pair substitutions by error-prone repair or replication of lesions that bypass the error-free excision repair system (Rastogi *et al.* 2010). The general mechanism of UV induced DNA damage and DNA repair have been described at length (Leclerc, Borden and Lawrence 1991; Lee *et al*., 2000; Chandrasekhar and Van, 2000; Li *et al*., 2010; Ikehata and Ono, 2011; Lin and Wang, 2011; Greinert *et al*., 2012; Shibai *et al.,* 2017). Briefly, DNA damage is predominantly induced by formation of a pyrimidine dimer between adjacent pyrimidines (thymines TT / cytosines CC) on a single DNA strand (Ikehata and Ono, 2011). These can be repaired either by PR (photoreactivation) or NER (nucleotide excision repair) directly. If this does not happen at the first instance, an SOS response is induced and these photoproducts are bypassed with modest efficiency by DNA polymerase and give rise to mutations. This process has been termed translesion replication (TR) or bypass synthesis, and mutations are caused by the tendency of DNA polymerase to insert an incorrect nucleotide opposite the lesion during TR (Li *et al.*, 2010; Ikehata and Ono, 2011).

UV radiation has been successfully used for strain improvement purposes in streptomycetes, especially for increasing the yield of desired antibiotics (Lee and Rho, 1999; Cheng *et al.,* 2001; Korbekandi *et al.,* 2010). Most of the early studies on UV induced mutation were conducted with *S. coelicolor* (Clarke and Hopwood 1976), *S. clavuligerus* (Saunders and Holt, 1982), *S. catteleya* (Coyne *et al*., 1984), and *S. fradiae* (Baltz and Stonesifer, 1985; Stonesifer and Baltz, 1985) using auxotrophic markers and mutation frequency against streptomycin, rifampicin and

spectinomycin as resistance markers. UV had proven to be a potential mutagen for *S. coelicolor*, and *S. clavuligerus,* but a weak mutagen for *S. fradiae* (Baltz, 1986).

S. coelicolor A677, a polyauxotrophic strain showed high induction of Arg* and Cys* revertant after UV treatment (Clarke and Hopwood, 1976). Interestingly, Saunders and Holt (1982) demonstrated that caffeine has a mutual effect along with UV exposures, as caffeine exerts its effect by inhibiting pyrimidine dimer excision. The weak mutation induction by UV in *S. fradiae* M1 (Baltz, 1986) was explained with the hypothesis of possessing more than one pathways of error-prone DNA repair system, unusual distribution of adjacent thymine (although it could affect in all *Streptomyces* spp.) and presence of a modified cytosine that precludes efficient mutagenesis at TC dimers (Fix and Bockrath, 1981).

1.5.2 UV mutagenesis to activate cryptic secondary metabolism

Given that streptomycetes genomes contain numerous silent BGCs, random mutagenesis by UV/chemical mutagen might be capable of inducing mutations in the genome which consequently activates the expression of the cryptic biosynthesis pathways. Although chemical mutagens have been used for streptomycete strain improvement, these mutagens have strong bias for specific base-pair transition substitutions. For instance, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) has been reported as a potential mutagen for *Streptomyces* (Baltz and Stonesifer, 1985), but it specifically induces GC to AT transition mutations (Kieser *et al*., 2000). GC to AT transition pathway cannot induce mutations at Phe, Ile, Asn or Lys (prevalent codons in streptomycete), nor can it generate codons for Ala, Gly or Pro (Baltz, 2014). In contrast to chemical mutagens, UV light induces all type of base-pair substitution including transitions, transversions, frameshift mutations and deletions,

either by error-prone repair or by replication of lesions which cannot be repaired by error-free excision repair mechanisms (Baltz, 2014). UV also offers a comparatively safer mutagen compare to other hazardous chemical mutagens.

An early study by Kelner (1949) demonstrated the induction of antibiotic production from poorly expressed or non-expressed antibiotic encoding genes in *S. flaveolus* by UV light (Kelner, 1949). Another study by Devon and Naowarat (2013) revealed the cave actinomycetes as a potential source of antimicrobial compounds by demonstrating the effect of UV light on their antimicrobial activity. Among 176 tested strains, only E1000 showed new activity against *Mycobacterium smegmatis* and *Candida albicans*.

Therefore, this study explores the utility of UV-induced mutation to unlock silent BGCs in *Streptomyces* species. There is to date no comprehensive study regarding the effectiveness of UV to mutagenize *Streptomyces* to switch on the expression of cryptic BGC. Therefore, this study evaluated those aspects. Studies have indicated that the optimum UV short wavelength for mutagenesis is 254 nm (Harold and Hopwood, 1970; Kieser *et al.,* 2000) and this study therefore used UV light of this wavelength for all subsequent mutagenesis experiments.

Once new activity is found, metabolites related to new activity would be detected and identified using analytical chemistry. The next two sections will describe the approaches to detect and de-replication of secondary metabolites from UV-induced mutants (UVMs) of *Streptomyces* species.

1.6 Detection of UV induced new metabolites in streptomycetes

1.6.1 Metabolomics and chemical de-replication

Metabolomics is a high-throughput analytical technique for chemical screening and subsequent detailed comparison of secondary metabolomes, which rapidly reveals the differences among experimental samples (Fiehn, 2002; Zhang *et al.,* 2012). Mass spectrometry (MS) is the dominant analytical technique in metabolomics to determine the elemental composition and structural information of a molecule by providing information such as accurate mass-to-charge ratio (*m/z*), isotope abundance and fragmentation patterns (Strege, 1999; Waridel *et al*., 2001; Shin and van Breemen, 2001). The use of metabolomics for de-replication of known microbial secondary metabolites and strain prioritisation has been demonstrated in previous natural product screening studies (Bitzer *et al.,* 2007; Moss *et al*., 2007; Sashidhara and Rosaiah, 2007; Nielsen *et al.,* 2011; Sarker, 2012; Yuliana *et al.,* 2011; Sheridan *et al.,* 2012; Zhang *et al.,* 2012), and has allowed the discovery of novel scaffolds, such as the microtermolides (Carr *et al.*, 2012), bottromycin D (Hou *et al.*, 2012), and forazoline A (Wyche *et al.*, 2014). Metabolomics has been extensively used in recent years for discovering new natural products from actinomycetes (Paulus *et al*., 2017; Zin *et al.,* 2017). Thus, comparative metabolomics has considered the ideal approach to analyse the secondary metabolites from UV-induced mutants (UVMs) and their corresponding parent strain to detect any new chemical entity resulting the activation of a cryptic BGC.

'Metabolite Detect module', a part of the Metabolite Tools software suite developed by Bruker Daltonics (Germany), is a novel analytical tool that provides sophisticated detection of masses/metabolites from ESI-ion trap mass spectrometry (MS) data (Zurek *et al.,* 2002, 2003). The eXpose™ detection algorithm compares *reference* and test *sample* MS data, and subtracts the reference data from the test. Thus it creates a new dataset, containing only the peaks and spectra of compounds that were exclusively present in the test sample. This new chromatogram is referred to as the "Difference Chromatogram" (Zurek *et al.,* 2002). A new window shows the resulting "detected mass list" with its retention times and *m/z* values. This software also has the provision of designing a new multiple reaction monitoring (MRM) acquisition method (Kondrat *et al.,* 1978) (in esquireControl™), with automatically-generated time segments for a targeted (based on the detected mass list) LC-MS/MS run.

The utility of metabolite detect software was demonstrated by LC-MS and LC-MS/MS characterization of cocaine and other metabolites in plasma samples from rats (Zurek *et al.,* 2002). Plasma samples spiked with cocaine and metabolites, and blank plasma sample were analysed by LC-MS and LC-MS/MS using ESI in the positive mode for ionization. The LC-MS data comparison of two rat plasma samples allowed the detection of compounds exclusively present in the spiked sample. Even norcocaine and cocaine, which do not show detectable peaks in the base peak chromatogram (BPC) were easily recognized by this algorithm.

Therefore, this program can filter trace of metabolites from complex samples. Batch data entry and analysis can speed up large data set analysis. Furthermore, difference chromatogram analysis data can be exported to the Bruker data analysis software, and the molecular formula of detected masses is predicted. Thus, Metabolite Detect is a rapid and sensitive analytical tool which aids in overcoming the time-consuming steps of LC/MS data review. This program was designed for using in metabolism studies and was originally developed to observe very small metabolite differences in patient samples under different conditions (Pelander *et al.,* 2009). To date, this has not been used in natural product antibiotic discovery, but represents an excellent choice in UV mutagenesis study to compare UVMs and their respective parents, and detect any new chemical entity induced by UV irradiation in *Streptomyces* UVMs.

1.7 De-replication of secondary metabolites and molecular networking

A typical LC-MS/MS analysis of any crude extract results in thousands of detectable features characterized by fragmentation pattern, *m/z*, retention time, etc. These features can be employed for database searching in order to achieve de-replication of known compounds present in the extracts. Furthermore, chemically-related molecules can be grouped together in a molecular network and analogues of known compounds can be identified. The Global Natural Products Social Molecular Networking (GNPS) (Wang *et al.,* 2016) platform can serve both purposes. GNPS is the largest public NP database with MS/MS of more than 140,000 natural products (Covington *et al*., 2017). Molecular networking, a component of GNPS's MS/MS data analysis flow allows rapid de-replication of known compounds as well as the

identification of new compounds belonging to a known structural class (Yang *et al.,* 2013; Duncan *et al.,* 2015). The fundamental principle behind this approach is that structurally related NPs are typically characterized by similar MS/MS fragmentation patterns. Thus, the MS/MS structural similarity generates a molecular network wherein unknown related compounds are presented as a cluster (Quinn *et al.*, 2016). The fragmentation patterns observed in the MS/MS spectra in conjunction with molecular networking evaluation can enable the identification of previously unknown close relatives within the same chemical families through spectral correlations (Quinn *et al.,* 2016). Yang *et al.* (2013) demonstrated the usefulness of this approach for natural product discovery by de-replicating 58 natural products from marine and terrestrial microorganisms. Several other studies have also proven its usefulness in NP research (Sidebottom *et al.,* 2013; Moree *et al.,* 2012). This de-replication and molecular networking can detect new metabolites activated in UVMs from streptomycetes in the present study.

1.8 Objectives of the study

This study is aimed at evaluating the potential of UV irradiation to unlock production of cryptic antibiotics in streptomycetes. To address some of the shortcomings of current approaches of cryptic BGC activation, studies were conducted to establish UV induced mutagenesis as a new platform approach for cryptic BCG activation for antibiotic discovery. Since, contemporary antibiotic discovery platforms have failed to generate potential lead compounds against Gram-negative bacteria, this study sought to use this approach to discover new anti-Gram-negative antibiotics. The presented approach involves the deliberate exposure of antibiotic non-producer

strains to UV light to activate their cryptic antibiotic biosynthesis pathways regardless of their genome sequences. The objectives are listed below:

i. Evaluation of the potential of UV mutagenesis to increase streptomycin resistant mutation frequency in genetically distant streptomycetes.
ii. Determination of the lethality of UV in streptomycetes by UV survival analysis.
iii. Evaluate the potential of UV as an activator of new antibacterial activity in different *Streptomyces* species.
iv. Optimization of UV intensity (for a universal UV intensity) to induce mutation in *Streptomyces* leading to the activation of silent BGCs for the discovery of new antibiotics
v. Comparative metabolomics with UV-induced mutants (UVMs) and parent strain to detect any new metabolites/masses
vi. Confirmation of the antibacterial activity of newly detected metabolites/masses (HPLC purification and subsequent bioassay)
vii. Analysis of MS/MS data from UVMs, and molecular networking to perform chemical de-replication of newly expressed antibacterial compounds

As a side project, a fungal contaminant, *Penicillium dipodomyicola* was investigated to identify the Gram-negative antibacterial compound produced by the fungus. (Details in the Chapter Five).

Chapter 2

Materials and Methods

2.1 Strains and growth conditions

Streptomyces strains used in this study (Table 2.1A) were predominantly obtained from the Deutsche Sammlung von Microorganism und Zellkulturen (DSMZ), the Japan Collection of Microorganisms (JCM), and as a generous gift from Dr K. Mcdowall (University of Leeds). Type strains were resuscitated from the freeze-dried stock according to the instructions of the suppliers.

Streptomyces strains were routinely cultured at 28°C for 5-7 days on GYM agar or in GYM broth (same as agar, but omitting calcium carbonate and agar), unless otherwise stated. ISP medium 2 and ISP medium 4 (Difco) were used for phenotypic characterisation, spore stock preparation and secondary metabolite extraction.

Antimicrobial activity screening was performed with indicator bacteria and yeast listed in Table 2.1B. These were routinely cultured on Mueller-Hinton agar (MHA) and in Mueller-Hinton broth (MHB) (Oxoid Ltd, Cambridge, UK) at 37°C for 24 hours. Agar (0.5% [v/v]) was used for agar overlay assay in screening for antimicrobial activity. For this screening assay, GYM, ISP medium 2 and Bennettes agar (according to DSMZ) were used to observe any effect of culture media for the production of antibacterial against indicator organisms.

Table 2.1 A *Streptomyces* strains used in this study

Strain	Description	Medium	Temp	Source
S. mobaraensis	DSM 40847	GYM	28°C	DSMZ
S.niveus	DSM 40088	GYM	28°C	DSMZ
S. lincolnensis	DSM 40355	GYM	28°C	DSMZ
S. virginiae	DSM 40094	GYM	28°C	DSMZ
S. filamentosus	DSM 40022	GYM	28°C	DSMZ
S. paulus	DSM 41646	GYM	28°C	DSMZ
S. griseoluteus	DSM 40392	GYM	28°C	DSMZ
S. tenebrarius	DSM 40477	GYM	28°C	DSMZ
S. ficellus	DSM 930	GYM	28°C	DSMZ
S. prunicolor	DSM 40335	GYM	28°C	DSMZ
S. kanammyceticus	DSM 40500	GYM	28°C	DSMZ
S. chrestomyceticus	DSM 40545	GYM	28°C	DSMZ
S. rifamycinica	DSM 46095	GYM	28°C	DSMZ
S. spectabilis	DSM 40512	GYM	28°C	DSMZ
S. antibioticus	DSM 40234	GYM	28°C	DSMZ
Saccharopolyspora erythraea	NRRL 2338	GYM	28°C	**K. McDowall
S. coelicolor	D132 (SCP1$^+$, SCP2$^+$)	ISP-2	26°C	**K. McDowall
S. avermitilis	-	GYM	28°C, 26°C	**K. McDowall
S. cattleya	NCIMB 11929	ISP-2	26°C	**K. McDowall
S. griseus	ATCC 12475	GYM	28°C, 26°C	**K. McDowall
S. fradiae	NCIMB 8233	ISP-2	26°C	**K. McDowall
S. hygroscopicus	ATCC 14607	ISP-2	28°C	**K. McDowall
S. lividans	TK 54	ISP-2	28°C	**K. McDowall
S. phaeochromogenes	NCIMB 3505	GYM	28°C, 26°C	**K. McDowall
S. rochei	40500	ISP-2	26°C	**K. McDowall
S. sapporensis	-	GYM	28°C	**K. McDowall
Actinoplanes nipponensis	NCIMB 10182	GYM	28°C	**K. McDowall

Strain	Description	Medium	Temp	Source
S. setonii	JCM 4224	ISP-3 (Difco)	28°C	RIKEN
S. agglomeratus	JCM 4888	YSA*	28°C	RIKEN
S. armeniacus	JCM 3070^T	YSA*	28°C	RIKEN
S. atrolaccus	JCM 4892	YSA*	28°C	RIKEN
S. daliensis	JCM 19963	YSA*	28°C	RIKEN
S. yunnanensis	JCM 12115^T	YSA*	28°C	RIKEN
S. fuscoatrus	JCM 4915	YSA*	28°C	RIKEN
S. vulgaris	JCM 4537^T	YSA*	28°C	RIKEN
S. wistariopsis	JCM 4688^T	ISP-4	28°C	RIKEN
S. viridobrunneus	JCM 9096^T	YSA*	28°C	RIKEN
S. bullii	JCM 19671^T	YSA*	28°C	RIKEN
S. drozdowiczii	JCM 13580^T	YSA*	28°C	RIKEN
S. durmitorensis	JCM 16011^T	YSA*	28°C	RIKEN
S. finlayi	JCM 4216^T	YSA*	28°C	RIKEN
S. coelescens	JCM 4739^T	YSA*	28°C	RIKEN
S. fumanus	JCM 4477^T	YSA*	28°C	RIKEN
S. glomeratus	JCM 9091^T	YSA*	28°C	RIKEN
S. graminearus	JCM 6923^T	V-8*	28°C	RIKEN
S. griseoloalbus	JCM 4480^T	ISP4	28°C	RIKEN
S. herbaricolor	JCM 4138^T	YSA*	28°C	RIKEN
S. hiroshimensis	JCM 4083	ISP4	28°C	RIKEN
S. monomycini	JCM 9768^T	V-8*	28°C	RIKEN
S. cidiscabies	JCM 7913^T	YSA*	28°C	RIKEN
S. verne	JCM 4527	ISP4	28°C	RIKEN
S. acidoresistans	JCM 4713^T	ISP-4	28°C	RIKEN

*V-8: V8 Juice Agar; YSA: Yeasts-Starch Agar

**K. McDowall: Dr Kenneth McDowall, University of Leeds.

Table 2.1 B Indicator microorganisms used in this study

Strains	Description	Source/ Reference
Escherichia coli MG1699	Derivative of *E. coli* K12 strain MG1655 and possesses *dhfr1, aph, cat, rpsL^R, sat, tetA, ble, bla, gyrA^R, rpoB^R, aadA, aac, fhuA* Resistant to albomycin, ampicillin, apramycin, bleomycin, chloramphenicol, dibekacin, gentamicin, kanamycin, nalidixic acid, neomycin, netilmicin, rifampin/rifamycin, spectinomycin, streptomycin, streptothricin, tetracycline, tobramycin, trimethoprim.	Cubist, USA
E. coli BW25113	Derivative of *E. coli* K12 strain BD792 and parental strain of Keio collection	(Baba *et al.*, 2006)
E. coli	CTX-M-15, CMY-2, NDM-1	Clinical strain
Enterobacter cloacae	CTX-M-15, ACT/MIR, NDM-1, OXA-48	Clinical strain
Klebsiella pneumoniae	NDM-1	Clinical strain

Strains	**Description**	**Source/ Reference**
Acinetobacter baumannii	NDM-1	Clinical stains
Staphylococcus aureus SH1000	Derivative of strain 8325-4, containing functional *rsbU*	(O'Neill, 2010)
AV13	SH1000 with mutation in *gyrB*	(Vickers *et al.,* 2007)
S. aureus MRSA 252	Clinical MRSA isolate	(Holden *et al.*,2004)
Pseudomonas aeruginosa	PAO1	(Holloway, 1995)
Candida albicans 6	Clinical isolate	(Martel *et al.,* 2010)
Fungal isolate		
Penicillium dipodomyicola	Isolated from air	This study

Penicillium dipodomyicola was isolated from the air in the laboratory (this study). It was routinely sub-cultured on GYM agar at 28°C for 7-10 days, and in GYM broth for metabolite extraction following incubation for 7-10 days at 28°C with aeration (180 rpm). Phenotypic characterization was performed using Czapeck yeast autolysate agar (CYA) (Atlas, 2010), and Yeast extract sucrose agar (YES) (Atlas, 2010). Microscopic observation and DNA extraction were performed after growing the strain on Sabouraud agar (SDA) (Oxoid, UK) at 25°C for 7 days.

2.2 Preparation of spores

Streptomyces spores were prepared by the method of Shepherd *et al*. (2010) with some modification. Briefly, strains were grown in appropriate culture medium for 5-7 days at 28°C. Subsequently, 5 ml of sterile water was added to the plates and spores were recovered in a suspension by mixing vigorously on a vortex mixer. The resulting suspensions were filtered and centrifuged (2000 x *g*, 10 minutes), supernatant removed, and the spore resusepnded in 1 ml of sterile water. A volume of (1 ml) 50 % glycerol (v/v) was added to the spore suspensions, which were then stored at -80 °C.

For generating fungal spores, the isolate was grown on GYM agar for 7-10 days at 28°C. Spores harvested following the same method described above, with the exception that 20% (v/v) glycerol has added ahead of freezing at -80°C.

2.3 Chemicals and reagents

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Poole, UK). Cyclohexane, diethyl ether, and chloroform were from VWR chemicals (Leicestershire, UK). Ethyl acetate, methanol and ethanol were from Thermo Scientific, (Waltham, MA, USA). Phusion® High-Fidelity PCR Kit was from New England Biolabs (Hitchin, UK) and the PCR nucleotide mix from Promega Corporation (Madison, USA).

2.4 Initial evaluation of antimicrobial activity

Streptomyces strains were evaluated initially for their antimicrobial activity against *E. coli* MG1699 by the agar overlay method (Kelner, 1949). Aliquots (3 µl) of spore stock ($1x10^8$ spores/ml) of these strains were inoculated onto GYM agar. Following 5-7 days of incubation, a soft layer (5 ml of 0.5% NA) containing $1x\ 10^8$ cfu/ml of *E. coli* MG1699 was poured onto the surface of the GYM agar plate to overlay the growth and allowed to set prior to incubation overnight at 37°C. Antimicrobial production was detected by the presence of a zone of inhibition of the indicator organism around streptomycetes colonies. Strains with no activity against MG1699 were selected for UV mutagenesis to examine the potential of UV radiation as an activator of cryptic antibacterial activity.

2.5 UV mutagenesis and UV survival in streptomycetes

2.5.1 UV irradiation

Spore suspensions of *Streptomyces* strains were irradiated using a UV-cross linker containing five 254 nm UV bulbs (CL-1000, UVP, Fisher Scientific UK), at different UV intensities range from 0-200,000 µJ/cm^2. Concentrated spore suspensions (10^8-10^{10} cfu/ml) in sterile water were poured into a 90-cm diameter petri plate and placed in the UV cross linker at a standard distance of 10 cm from the UV source. After UV irradiation, spore suspensions were diluted (10^{-5}-10^{-9}) and grown on GYM agar for 5-10 days wrapped with aluminium foil. UV mutagenesis and subsequent incubation were performed in semi-dark conditions to avoid photo-reactivation by visible light (Kelner, 1951; Mortelmans, 1989). UV mutagenesis was performed with spores

wherever possible, but some cases was conducted with mycelial cells prepared according to Baltz (1986).

2.5.2 Mutation frequency study

To evaluate the efficiency of UV-induced mutagenesis in *Streptomyces*, spontaneous and UV-induced mutants (UVMs) were selected for streptomycin resistance. The spore suspension or mycelial culture before and after exposure to different UV intensities (0-200,000 µJ/cm^2) from selected *Streptomyces* strains was spread on GYM agar plates containing streptomycin at 4x the MIC (determined by agar dilution), and plates incubated at 28°C for 7-10 days wrapped with aluminium foil. The frequency of mutation was calculated from the number of resistant mutants and the total viable count, i.e. number of resistant mutant divided by the number of total viable count (O'Neill, Cove and Chopra, 2001). All experiments were performed with three independent cultures, and values expressed as mean ± standard deviation.

2.5.3 UV survival study

To determine the lethal effect of UV radiation, survival of spores after UV treatment was reassured. Spore suspension/mycelial culture exposed at different intensities ranging from 0-200,000 µJ/cm^2 were diluted (10^{-1} to 10^{-9}), and 100 µl of diluted spores (10^{-5} to 10^{-9}) were spread on GYM agar plates and wrapped in aluminium foil for incubation at 28°C for 7-10 days.

Survival percentage was determined by comparing the number of survivors at different UV intensities with total cell count at 0 µJ/cm^2. All experiments were performed with three independent cultures and values are expressed as mean ± standard deviation.

2.6 *In vitro* susceptibility testing

2.6.1 Disc and well diffusion

Antibacterial susceptibility testing of crude extracts and purified compounds was performed by disc and agar well diffusion. Indicator microorganisms (1x10^8 cfu/ml) were first grown as a lawn on the surface of the MHA plates. A volume (20-40 µl) of crude extract or purified compound (dissolved in appropriate solvent; unless otherwise stated, methanol was used as the solvent) has loaded onto a sterile antibiotic disc (6mm, Oxoid). Solvent was also loaded onto separate discs to provide a negative control. The air-dried, impregnated discs were transferred onto the surface of MHA plates and incubated at 37° C for 18-24 hours.

In agar well diffusion (Valgas *et al.*, 2007), as stated As above, MHA plates were first spread with the microbial inoculum (1 x 10^8 cfu/ml), and a well with a diameter of 6 to 8 mm was punched aseptically with a sterile cork borer. A volume (20–100 µL) of culture extract (in methanol, unless otherwise stated) has introduced into the well. The plates were incubated at 37° C for 18-24 hours.

2.6.2 Minimum inhibitory concentration (MIC) determination

Ahead of conducting the UV-induced streptomycin resistance mutation frequency experiment, the agar dilution method was used to determine the MIC of streptomycin. *Streptomyces* spores (1 x 10^8 spores/ml) were spotted onto GYM agar medium containing a double dilution series (0-256 µg/ml) of streptomycin and incubated at 28°C for 5-7 days. The minimum streptomycin concentration able to fully inhibit growth was defined as the MIC.

The broth microdilution method (CLSI, 2012) was used for determining the MIC of the HPLC purified fungal antibiotic at 37°C. Growth control without antibiotic (positive control), broth only (negative control), and solvent only (solvent control) were included in each experiment. Following incubation, the MIC was defined as the lowest concentration of antibiotic that inhibit all visible growth. All susceptibility tests were conducted on a minimum of three independent occasions to ensure reproducibility.

2.7 Microscopy

Microscopy was performed to achieve preliminary identification of the fungus, *P. dipodomyicola*. The fungal culture was grown on SDA for 7 days. A lactophenol cotton blue slide mount was prepared following the method of Leck (1999), and examined by both Phase-contrast microscopy (Olympus BX41) and light microscopy (Olympus BX50) at x 400 and x1000. Light microscopy images were captured with an Olympus Colourview digital microscope camera (by Richard R Barton, LGI, Leeds).

2.8 Molecular biology techniques

2.8.1 Streptomyces colony PCR

To confirm the identity of UV mutants from *S. griseoluteus,* the phenazine biosynthetic SgpD (*sgpD*) gene from *S. griseoluteus* parent strain and the UVMs were amplified using colony PCR. Oligonucleotide primers were designed using Oligo software (Molecular Biology Insights, Inc. Colorado, USA) and synthesized by Eurofins MWG Operon (Ebersburg, Germany).

(Forward *sgp*D 5'-TGCTCAAGGACTTCTGGGGT-3'; Reverse *sgp*D 5'-GACCTGGATGTCGTTGGTGA-3')

Colony PCR was performed according to the Leuven protocol (Leuven iGEM, 2013). For the PCR, the Phusion® High-Fidelity PCR Kit was used. The reaction conditions were as follows; initial denaturation at 95˚C for 6 min, followed by 29 cycles of 30 sec denaturation at 95 ˚C, 30 sec annealing at 65 ˚C, 30 sec/kb extension at 72˚C and a final extension at 72˚C for 10 min.

2.8.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using a standard protocol (Sambrook and Russell 2006). Agarose 0.8% (w/v) (Geneflow LTD, Fradley, UK) was used to prepare the gel with SYBR safe stain (2 µl) (Invitrogen; Paisley, UK) and run in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) using a Bio-Rad electrophoresis cell (Bio-Rad, Hemel Hempstead, UK). Samples were electrophoresed at 90 volts for 30 minutes and the results analysed by capturing the image with the Gene-Genius Bio-Imaging-System (Syngene, Frederick, MD) using the GeneSnap

Program (Syngene). A 1 kb and 100bp molecular weight ladder marker (Bioline) was used in each gel to estimate the molecular weight of PCR products.

2.9 Extraction of secondary metabolites

2.9.1 Extraction from streptomycetes

Small scale agar-based solvent extraction was performed from cultures grown in a 90-mm diameter petri plate. Parent and UV-induced mutants (UVMs) of *Streptomyces antibioticus*, *S. griseoluteus* and *S. ficellus* were grown in specific agar media at 28°C for 7-10 days. *S. antibioticus* and *S. ficellus* were grown in ISP medium 4, and *S. griseoluteus* was grown in ISP medium 2. After incubation, the agar was chopped into small pieces and extracted with ethyl acetate (2 x volumes of agar media), except *S. antibioticus* (extracted with both ethyl acetate and sec-butanol), at 25°C for two hours with shaking (150-200 rpm). The resulting organic layers were separated and filtered through Whatman filter paper (size 240 nm, particle retention 5-13 µm). Filtrates were evaporated using Genevac (EZ2, Genevac Ltd, U.K) and finally dissolved in 1 ml of HPLC-grade methanol. The average concentration of these crude extracts was 1 mg/ml. ISP medium 4 and ISP medium 2 were also extracted in the same manner to provide a media-only control. Large-scale extraction was performed in the same manner described above by growing strains in twenty to thirty 120x120 mm square petri plates. The dissolved extracts were kept in solution at 4°C for a short period, whilst for longer term storage they were dried using the Genevac and kept at 4°C.

2.9.2 Fungal metabolite extraction

Unless otherwise stated, fungal cultures were stored at 4°C until the time of extraction. Initial extraction was performed with ethyl acetate (2x volume of the culture) from the broth culture (GYM broth), and the final extract was dissolved in methanol for further analysis. The method for extraction was then optimized in collaboration with Sannia Farooque (School of Chemistry, University of Leeds, UK) to target the antimicrobial compound from *P. dipodomyicola.* The fungal isolate was grown in 3x1 L of GYM broth at 28°C for 10 days with aeration. The cultures were extracted twice sequentially with equal volumes of cyclohexane, diethyl ether and ethyl acetate. The organic layers were separately dried using a rotary evaporator *in vacuo* until the solvent reached a minimum level. Subsequently, 2-5 ml of solvents (cyclohexane / diethyl ether / ethyl acetate) were added to wash the flask and collected all organic extracts. At this stage, 1 ml extract from each fraction was separated for bioassay before drying extracts *in vacuo* using the Genevac. The dried crude extracts were stored at 4°C.

2.10 New metabolite detection in streptomycetes

Crude extracts from UV-induced mutants (UVMs) were compared with the corresponding parent to link antibacterial activity with specific compounds. This was achieved by comparing high-resolution accurate mass spectrometry (HRMS) data from the crudes of parent and UVMs, as described below.

2.10.1 Liquid chromatography-Mass spectrometry (LC-MS)

High-resolution LC-MS experiments were performed using UHPLC-MS through a reverse phase column (Zorbax Eclipse Plus C18, size 2.1 x 100 mm, particle size 1.8 µm) connected to a Dionex 3000RS UHPLC coupled to Bruker Ultra High Resolution (UHR) Q-TOF MS MaXis mass spectrometer (Bruker Daltonics) with an electrospray source operating in positive ion mode. Sodium formate (10 mM) was used for internal calibration. The crude extracts from forty-six *Streptomyces* strains (UVMs and corresponding parent), ISP2/ISP4 medium control and solvent control were prepared at a concentration of 1.0 mg/mL in methanol. Aliquots (1-2 µl) of samples were injected through the column and acetonitrile-water-0.1 % formic acid gradients were applied for separation where *m/z* = 50 to 1500 scan range was used. LC-MS data was acquired using data analysis viewer, Bruker version 5.0.

2.10.2 Metabolite Tool analysis

Sample LC/MS datasets were submitted to the eXpose™ detection algorithm (Zurek *et al*., 2003) for comparison. The main parameters used were as follows: *Calculate Difference* was used at the *Expose* mode with a ratio of 3, delta time ± 0.20 min, delta mass ± 0.005; *Detect Masses* applied the EIC at the *Base Peak Detection* mode with peak integration, trace width ± 0.40 *m/z*, S/N threshold 3.0 enabled, intensity threshold absolute 1000 enabled; and *Intensity Threshold* was 30%. In this study, the software subtracted the reference sample data (solvent, ISP2/ISP4 medium, parent crude) from the data of corresponding UV mutant. As a result of this comparison, a new, clean dataset was created, containing only the peaks and spectra of compounds that were exclusively present in the crude extract of UV-induced mutant; the result was referred to as the "*Difference Chromatogram*" (Zurek *et al*., 2003).

2.11 De-replication of secondary metabolites

Upon detection of new masses in the crude extracts of UVMs, the samples were further analysed for the presence of previously known compounds (de-replication). High resolution tandem mass spectrometry (HR-MS/MS) data was generated, upload to the Global Natural Products Social Molecular Networking (GNPS) platform (Wang *et al.,* 2016). The GNPS is an automatic de-replication platform which allows searching of a spectral library for known molecules, provided the spectra of these molecules are available in public MS/MS libraries. This platform can also create a network showing the relationship between unknown compounds and structurally-related known compounds.

2.11.1 High resolution tandem mass spectrometry (HRMS/MS)

High-resolution tandem mass spectrometry (HR-MS/MS) data were generated from samples at a concentration of 1.0 mg/mL in methanol. Volumes (2 µl) of the crude extracts and HPLC-purified compounds were injected through a reverse phase column (Zorbax Eclipse Plus C18, size 2.1 x 100 mm, particle size 1.8 µm) connected to a Dionex 3000RS UHPLC coupled to Bruker Ultra High Resolution (UHR) Q-TOF MS MaXis mass spectrometer with an electrospray source operating in positive ion mode using a MS range of *m/z* 50–2000, a MS2 range of *m/z* 200–1500, a MSn range of *m/z* 0–1000 and 30,000 resolution. LC-MS data was acquired using data analysis viewer, Bruker version 5.0.

2.11.2 Molecular networking and database searching

High-resolution tandem mass spectrometry (HR-MS/MS) data were converted to mzXML format using Compass Data Analysis (Bruker Daltonics) and uploaded to the Global Natural Products Social Molecular Networking webserver (http://gnps.ucsd.edu). The LC–MS/MS data for the crude and purified compounds were analysed using the Molecular Networking workflow with the following settings: Parent Mass Tolerance 0.02 Da, Ion Tolerance 0.02 Da, Min Pairs Cos 0.6, Min Matched Peaks 6, Network TopK 10, Minimum Cluster Size 2 and Maximum Connected Component Size 100. A minimum cosine score of 0.7 was selected to reduce the clustering of different compound classes under the same molecular family when visualising the data as a network of nodes connected by edges (nodes correspond to a specific consensus spectrum; edges represent significant pairwise alignment between nodes) (Wang *et al*., 2016).

Molecular networking merged all identical MS and MS/MS spectra, including identical MS/MS spectra of isomers. System impurities were identified by solvent blanks and ISP2/ISP4 media control. The spectra in the network were then searched against GNPS's spectral libraries. Sci-finder (https://scifinder.cas.org/), Pubchem (https://pubchem.ncbi.nlm.nih.gov/) and Chemspider (http://www.chemspider.com/) were searched for the de-replication of new metabolites produced by UVMs using the predicted molecular formula obtained from high-resolution accurate mass.

2.12 Purification and structure elucidation

Purification was performed for new masses detected by Metabolite tools and later analysed by using GNPS platform. The antibiotic from *P. dipodomyicola* was also purified, and the structure was elucidated using X-ray crystallography, NMR and infrared spectroscopy (by Sannia Farooque, Chemistry, University of Leeds).

2.12.1 Mass-guided reverse HPLC

The methanol dissolved crude extracts were fractionated by semi-preparative HPLC via reverse phase column (Kinetex® 5µm EVO C18 100 Å, AXIA Packed LC Column 50 x 21.2 mm, Ea). This column was connected to an Agilent 1260 series with Diode array detector and an Agilent 6100 series single quad mass spectrometer. A gradient of methanol+ 0.1% formic acid ranging from 5% to 95% in water + 0.1% formic acid with a flow rate of 2 mL min-1 was used. Before each experiment was run, an isocratic equilibration using the initial solvent profile was performed for 8 min. Specific mass-based collection was performed over 8-15 min. 50-95% methanol gradient was used for *S. antibioticus*, *S. griseoluteus* and fungal metabolites, and 5-95% methanol gradient was used for *S. ficellus* metabolites. The fractions were collected, pooled, dried and dissolved in methanol for testing activity against *E. coli* MG1699 and other indicator microorganisms.

2.12.2 UV-based preparative reverse-phase HPLC

The optimized purification by UV detection was performed by Sannia from diethyl ether fungal crude**.** The mother liquor was subjected to preparative reverse-phase HPLC (XBridge^{TM} Prep C18 OBD^{TM} 19 × 100 5 µm i.d., 20 mL min^{-1}; gradient: H_2O/MeCN/trifluoroacetic acid from 90:10:0.1 to 20:80:0.1 in 15 min) to give three fractions (F1-F3). F1 was further fractioned using a reverse-phase semi-preparative system (XBridge^{TM} Prep C18, 10 × 50, 5 µm; gradient: H_2O/MeCN/trifluoroacetic acid from 45:5:0.1 to 0:50:0.1 in 15 min) to afford three sub-fractions. F2 was also further fractioned using a reverse-phase semi-preparative system (XBridge^{TM} Prep C18, 10 × 50, 5 µm; gradient: H_2O/MeOH from 95:5 to 5:95 in 15 min) to afford eight sub-fractions.

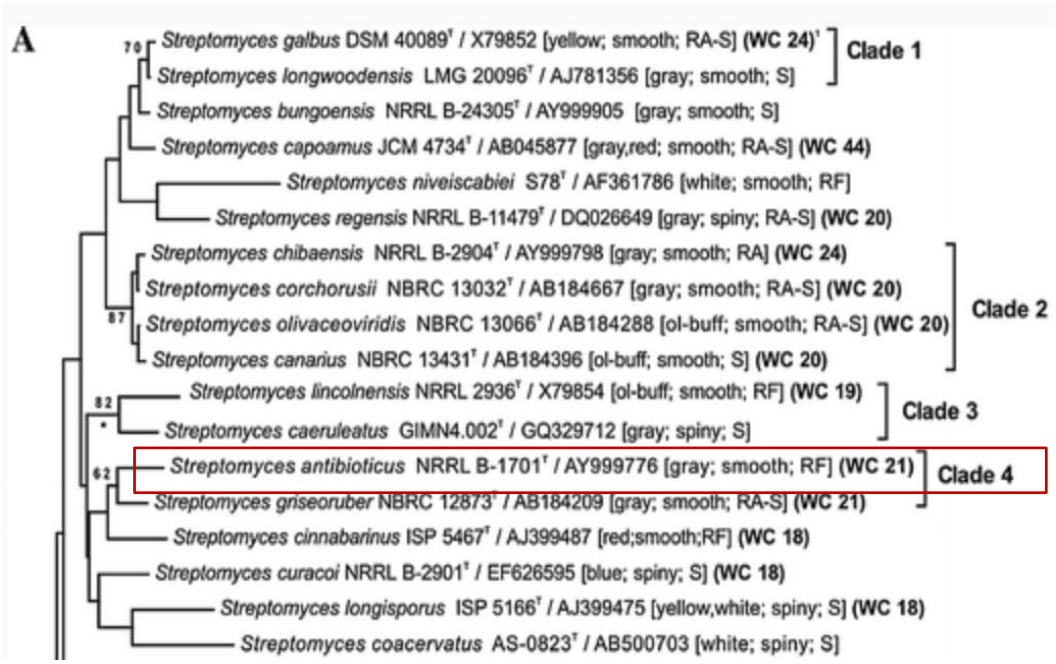
Chapter 3

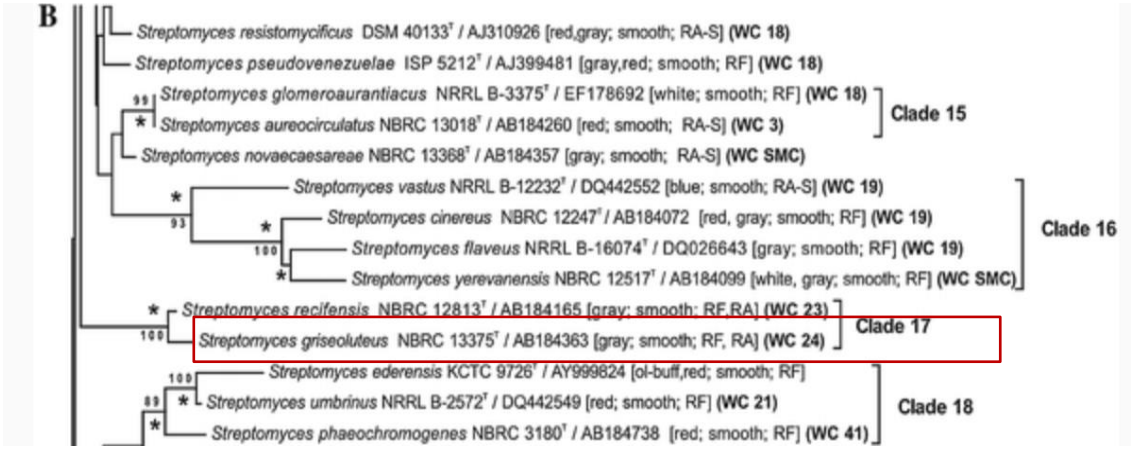
UV mutagenesis to unlock novel antimicrobial activity in streptomycetes

3.1 Mutation frequency induction study and hypothesis of cryptic BGCs activation

As mentioned in the Chapter One, typically the effectiveness of a mutagen was evaluated by selecting mutants for auxotroph or antibiotic resistant, therefore, an experiment was designed to determine the efficiency of UV light as a mean of increasing mutation frequency (MF) against ribosome-acting antibiotic. Review from Baltz (2014) showed that mutation to streptomycin showed all six types of transition mutation in streptomycetes, hence streptomycin resistance induction was used as a marker. For this mutation induction study, four strains were selected randomly based on their phylogenetic relationships (Figure 3.1).

S. kanamyceticus (clade 25-26), *S. grisoluteus* (clade 17), *S. antibioticus* (clade 4), and *S. coelescens* (clade 103) which are well-separated, phylogenetically as they are located in distant clades (Figure 3.2). *S. ficellus* was also included though no information was identified regarding its position in the *Streptomyces* phylogeny. Therefore, it was hypothesized that if UV irradiation can increase the level of streptomycin resistance in phylogenetically distant *Streptomyces* strains, it would be able to increase mutation effect throughout the genome of *Streptomyces*, eventually activating cryptic BGCs.


A
Streptomyces galbus DSM 40089T / X79852 [yellow; smooth; RA-S] (WC 24)T
Clade 1
Streptomyces longwoodensis LMG 20096T / AJ781356 [gray; smooth; S]
Streptomyces bungoensis NRRL B-24305T / AY999905 [gray; smooth; S]
Streptomyces capoamus JCM 4734T / AB045877 [gray,red; smooth; RA-S] (WC 44)
Streptomyces niveiscabiei S78T / AF361786 [white; smooth; RF]
Streptomyces regensis NRRL B-11479T / DQ026649 [gray; spiny; RA-S] (WC 20)
Streptomyces chibaensis NRRL B-2904T / AY999798 [gray; smooth; RA] (WC 24)
Streptomyces corchorusii NBRC 13032T / AB184667 [gray; smooth; RA-S] (WC 20)
Clade 2
Streptomyces olivaceoviridis NBRC 13066T / AB184288 [ol-buff; smooth; RA-S] (WC 20)
Streptomyces canarius NBRC 13431T / AB184396 [ol-buff; smooth; S] (WC 20)
Streptomyces lincolnensis NRRL 2936T / X79854 [ol-buff; smooth; RF] (WC 19)
Clade 3
Streptomyces caeruleatus GIMN4.002T / GQ329712 [gray; spiny; S]
Streptomyces antibioticus NRRL B-1701T / AY999776 [gray; smooth; RF] (WC 21)
Clade 4
Streptomyces griseoruber NBRC 12873T / AB184209 [gray; smooth; RA-S] (WC 21)
Streptomyces cinnabarinus ISP 5467T / AJ399487 [red;smooth;RF] (WC 18)
Streptomyces curacoi NRRL B-2901T / EF626595 [blue; spiny; S] (WC 18)
Streptomyces longisporus ISP 5166T / AJ399475 [yellow,white; spiny; S] (WC 18)
Streptomyces coacervatus AS-0823T / AB500703 [white; spiny; S]


B
Streptomyces resistomycificus DSM 40133T / AJ310926 [red,gray; smooth; RA-S] (WC 18)
Streptomyces pseudovenezuelae ISP 5212T / AJ399481 [gray,red; smooth; RF] (WC 18)
Streptomyces glomeroaurantiacus NRRL B-3375T / EF178692 [white; smooth; RF] (WC 18)
Clade 15
Streptomyces aureocirculatus NBRC 13018T / AB184260 [red; smooth; RA-S] (WC 3)
Streptomyces novaecaesareae NBRC 13368T / AB184357 [gray; smooth; RA-S] (WC SMC)
Streptomyces vastus NRRL B-12232T / DQ442552 [blue; smooth; RA-S] (WC 19)
Streptomyces cinereus NBRC 12247T / AB184072 [red, gray; smooth; RF] (WC 19)
Clade 16
Streptomyces flaveus NRRL B-16074T / DQ026643 [gray; smooth; RF] (WC 19)
Streptomyces yerevanensis NBRC 12517T / AB184099 [white, gray; smooth; RF] (WC SMC)
Streptomyces recifensis NBRC 12813T / AB184165 [gray; smooth; RF,RA] (WC 23)
Streptomyces griseoluteus NBRC 13375T / AB184363 [gray; smooth; RF, RA] (WC 24)
Clade 17
Streptomyces ederensis KCTC 9726T / AY999824 [ol-buff,red; smooth; RF]
Streptomyces umbrinus NRRL B-2572T / DQ442549 [red; smooth; RF] (WC 21)
Clade 18
Streptomyces phaeochromogenes NBRC 3180T / AB184738 [red; smooth; RF] (WC 41)


Streptomyces torulosus LMG 20305T / AJ781367 [red, gray; knobby; RA-S]
Streptomyces hygroscopicus subsp. ossamyceticus CGMCC 4.1866T / AY999876 [gray; smooth; S]
Clade 23
Streptomyces neyagawaensis ATCC 27449T / D63869 [gray; smooth; S] (WC 18)
Streptomyces decoyinicus NRRL 2666T / EU170127 [gray; rugose; S]
Streptomyces bottropensis ATCC 25435T / AB026217 [gray; smooth; S] (WC 19)
Clade 24
Streptomyces stelliscabiei CFBP 4521T / AJ007429 [gray; smooth; S]
Streptomyces diastatochromogenes ATCC 12309T / AB026218 [gray; smooth; S] (WC 19)
Streptomyces deccanensis KCTC 19241T / EF219459 [gray; hairy; RF]
Clade 25
Streptomyces europaeiscabiei CFBP 4497T / AJ00742 [gray; smooth; S]
Streptomyces scabiei ATCC 49173T / D63862 [gray; smooth; S] (WC 3)
Streptomyces atriruber NRRL B-24165T / EU812169 [gray; smooth; RF]
Streptomyces kanamyceticus NRRL B-2535T / DQ442511 [white, yellow; smooth; RF] (WC 42)
Streptomyces rectiviolaceus NRRL B-16374T / DQ026660 [violet; smooth; RF]
Streptomyces aureus B7319T / AY094368 [gray; smooth; RF]
Streptomyces longisporoflavus NBRC 12886T / AB184220 [yellow; smooth; RA-S]
Clade 26
Streptomyces durmitorensis MS405T / DQ067287 [ol-buff; smooth; RF]
Streptomyces beijiangensis YIM 6T / AF385681 [white; smooth; RF]
Streptomyces drozdowiczii NBRC 101007T / AB249957 [gray; smooth; RF]

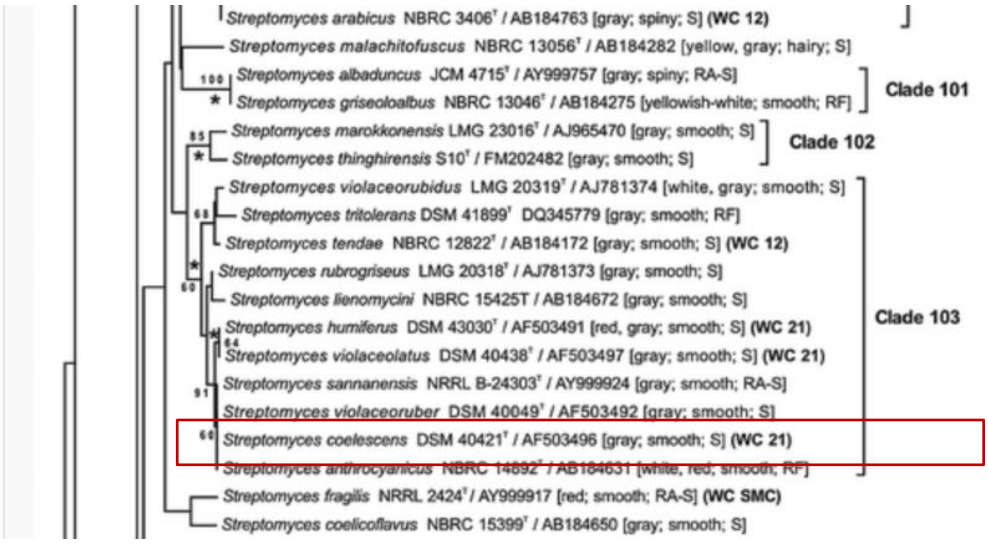


Figure 3.1 Phylogenetic relationship of selected four strains, *S. antibioticus* (clade 4), *S. grisoluteus* (clade 17), *S. kanamyceticus* (clade 25-26), and *S. coelescens* (clade 103) (Adapted from Labeda *et al*., 2012).

3.2 Biological de-replication of new antimicrobial activity

This research was conducted to address the failure of contemporary antibiotic discovery platforms to generate potential lead compound against Gram-negative bacteria from microbial secondary metabolites. However, natural product antimicrobial discovery faces major disadvantage by the lack of early de-replication thus discovering known chemical scaffolds (Wright, 2017). Moreover, since each streptomycete can produce several bioactive compounds the next consideration (first consideration was to choose UV mutagenesis over the chemical) was how to evaluate the induction of new antimicrobial activity and how to screen for the novel activity.

Studies have been on going to generate a platform for biological de-replication of NPs. One recent study has developed an antibiotic resistance platform (ARP), a cell-based array of mechanistically distinct individual resistance elements in Gram-negative *Escherichia coli* for antibiotic de-replication (Cox *et al.,* 2017). Unfortunately, this newly developed de-replication platform cannot address the permeability issue for the compounds in Gram-negative bacteria. The main limitation is that the developed *E. coli* strain series are hyperpermeable and efflux deficient which would allow the entrance of antibacterial compounds but in practical, those compounds would not cross the intact membrane in Gram-negative bacteria. A resistant strain named *E. coli* MG1699 (details in the chapter two), a decedent from the parent *E. coli* MG1655 was developed by Cubist pharmaceuticals which confers resistance to seven classes of antibiotics including synthetic trimethoprim (in-house name of the strain is *E. coli* CM400) (Gullo *et al.,* 2006). The competitive advantage of MG1699 is its intact membrane and efflux system. Therefore, it would reduce false positive results and only give hits for those compounds which are able to pass the barrier of Gram-negative membrane and exert their effects inside the cell. This study has taken the advantage of using MG1699 strain as the indicator of new antibacterial activity.

3.3 Aims and objectives

The primary aim of the study was to investigate whether UV mutagenesis is capable of inducing the expression of cryptic antibiotic biosynthetic gene clusters (BGCs) in streptomycetes. Fifty-two strains of *Streptomyces* spp. were first screened for antimicrobial activity against MDR *Escherichia coli* MG1699, and twelve species with no known antibacterial activity were selected for UV mutagenesis experiments. Therefore, the objectives of the study were as follows:

(i) Evaluation of the potential of UV irradiation to increase mutation frequency to streptomycin resistance (Str^R), and survival studies in phylogenetically distant *Streptomyces* species.

(ii) Trial experiment for induction of new antibacterial activity against *E. coli* MG1699 in different *Streptomyces* species.

(iii) Optimum UV intensity determination to unlock cryptic BGCs across *Streptomyces* species using two selective UV intensities and screening for antibacterial activity among UV survivors.

(iv) Characterization of UV induced mutants (UVMs) according to their cultural characteristics and their spectrum of antimicrobial activity against different indicator microorganisms.

3.4 Results

3.4.1 Initial screening for antibacterial activity against MDR *E. coli* MG1699

Since the aim of the UV mutagenesis approach was to induce new antibacterial activity, *Streptomyces* strains were initially screened for pre-existing antibacterial activity against MG1699 by agar overlay assay. Of these fifty-two tested strains, twenty-four (from RIKEN) have no known antibiotics, and rest of the strains are known to produce various antibiotics (Table 3.1). Although some of these known antibiotics are broad-spectrum, most of them are active particularly against Gram-positive bacteria.

After overlay assay, twenty-five strains demonstrated no activity against MG1699 (Table 3.1). It is worth to note that the diameter of zone of inhibition was not determined since the aim of the experiment was to screen for any pre-existing activity, therefore, if there was any detectable activity even minute, it was considered as positive activity. Since media components have a major effect on the production of secondary metabolites in streptomycetes, different media was used to rule out any false negative results. All these strains were grown in three different media; GYM, ISP medium 2, and Bennett's agar. These three media were chosen according to literatures for the growth and secondary metabolite production of *Streptomyces*. For examples, if a strain showed activity in one of three media, it was scored as having activity against MG1699. Some representative results are shown in Figure 3.2.

Table 3.1 Antibacterial activity of streptomycetes against *E. coli* MG1699 by agar overlay method.

Strain	known metabolites	Activity against *E. coli* MG1699
S. mobaraensis	Bleomycin, detoxin, piericidins A, B	+
S.niveus	Novobiocin, isonovobiocin, Leukotriene B4 antagonist.	+
S. lincolnensis	Lincomycin.	+
S. virginiae	Actithiazic acid, cycloserine.	+
S. filamentosus	Caryomycin, Antibiotic SF 1961 A, Dioxolamycin	+/-
S. paulus	Paulomycins	-
S. griseoluteus	Gryleolutin acid, griseolutin A and B, O-methyl cinnamide, antibiotic 539A	-
S. tenebrarius	nebramycin (= tobramycin)	-
S. ficellus	Antibiotic U-47, 929, feldamycin, nojirimycin	-
S. prunicolor	Benthocyanin, napthgeranine A, pironetin	-
S. kanammyceticus	Kanamycin A, B and C	-
S. chrestomyceticus	Aminocidin, neomycin E, neomycin E, F (=paromomycin I, II).	+
Amycolatopsis *rifamycinica*	rifamycin SV	+
S. spectabilis	Actinospectacin, prodigiosine, streptovaricin complex.	+
S. antibioticus	Actinomycin X (B), clavam antibiotic, boromycin, chlorothricin, cinerubins, esmeraldine A, furanone, indnaomycin, multhiomycin, oleandomycin, rubromycin	-
Saccharopolyspora erythraea	Erythromycin A, B, and C	+
S. coelicolor	Actinorhodin, Undecylprodigiosin, Calcium dependant antibiotic, Clorobiocin,	-
S. avermitilis	Avermectin	-
S. cattleya	Thienamycin penicillin N, and cephamycin C	+
S. griseus	Streptomycin	+
S. fradiae	Neomycin, fradicin	+
S. hygroscopicus	Glebomycin (antibiotic S-438)	+
S. lividans	Small amount of actinorhodin and undecylprodigiosin	+
S. phaeochromogenes	Angucycline, ikarugamycin, kikumycin	+
S. rochei	borrelidin	+
S. sapporensis	Bicozamycin	+

Actinoplanes nipponensis	Lipopeptide antibiotic 41,012	+
S. setonii	-	-
S. agglomeratus	-	+
S. armeniacus	-	-
S. atrolaccus	-	+
S. daliensis	-	-
S. yunnanensis	-	-
S. fuscoatrus	-	-
S. vulgaris	-	+
S. wistariopsis	-	+
S. viridobrunneus	-	-
S. bullii	-	-
S. drozdowiczii	-	-
S. durmitorensis	-	-
S. finlayi	-	+
S. coelescens	-	-
S. fumanus	-	-
S. glomeratus	-	-
S. graminearus	-	+
S. griseoloalbus	-	-
S. herbaricolor	-	-
S. hiroshimensis	-	+
S. monomycini	-	
S. verne	-	+
S. cidiscabies	-	-
S. acidoresistans	-	-

(+): zone of inhibition present; (-): zone of inhibition absent

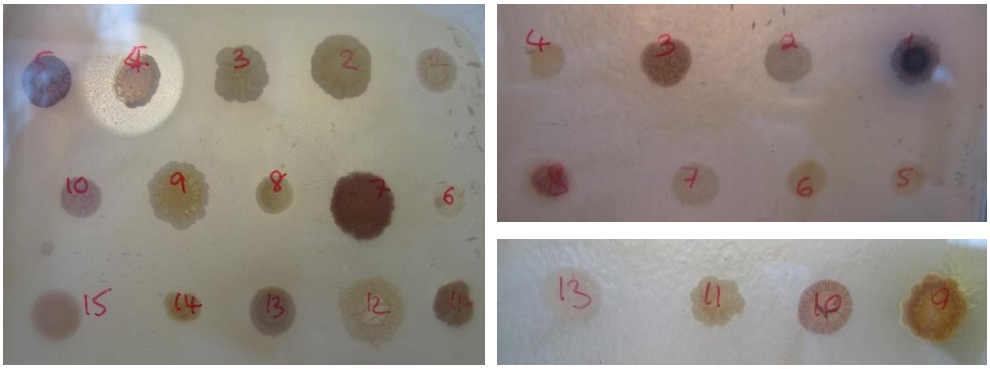


Figure 3.2 Antibacterial activity screening against *E. coli* MG1699 in *Streptomyces* spp. Strains were grown on GYM, ISP2 and Bennett agar media, and zone of inhibition was observed after overnight incubation at 37°C. Strains were numbered for experimental convenience. 4 (*S. mobaraensis)* showed a zone of inhibition on GYM agar medium.

3.4.2 UV induced mutation to streptomycin resistance and survival study

The selected five strains for mutation induction study, had no prior antibacterial activity against *E. coli* MG1699. Although numerous protocols for UV irradiation exist in the literature, there is no universal protocol. Therefore, initial experiments were performed to determine whether spores or mycelia should be employed for UV mutagenesis studies, and to determine the optimum concentration of spores for UV exposure (data not shown). Spores with a concentration of 10^8 spores/ml were found to be optimal for UV mutagenesis studies. Several trials of mutation induction were performed to optimize the concentration of streptomycin at which mutants can be recovered (data not shown). Later, UV induced Str^R of these five strains were determined by exposing their spores to UV intensities ranges between 10,000-300,000 µJ/cm^2 (Figure 3.3). The MF of Str^R in tested *Streptomyces* strains gradually increased with proportion to the UV intensity until it reached a point where no UV mutants survived. Among all these strains, *S. kanamyceticus* showed highest increase of MF at 60,000 µJ/cm^2

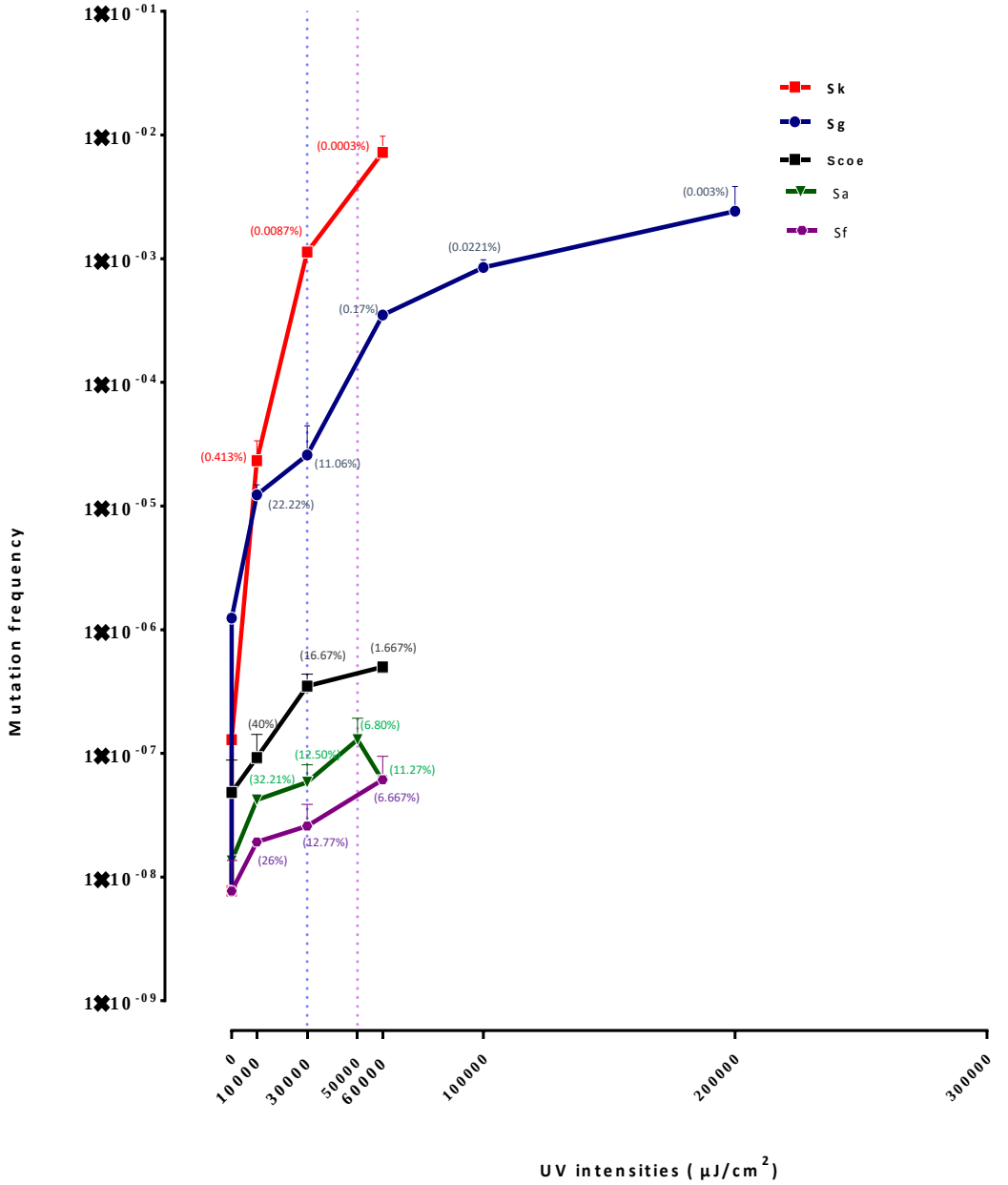


Figure 3.3 Induction of streptomycin resistance (Str^R) and survival in five *Streptomyces* strains exposed to increasing UV intensities (µJ/cm^2). The survival percentage shown in brackets where survival percentage at 0 µJ/cm^2 was 100%. Two UV intensities (30,000 and 50,000 µJ/cm^2) were highlighted as dotted lines, blue line showing the star of increased Str^R in these strains with reasonable survival percentage. The purple line showing the MF increase in Sa at 50,000 µJ/cm^2 (only done with Sa to determine the actual point of increased MF). Sk: *S. kanamyceticus*; Sg: *S. griseoluteus*; Scoe: *S. coelescens*; Sa: *S. antibioticus*; Sf: *S. ficellus*. Values shown are the means from three independent experiments. Error bars represent standard deviations from the mean. The graph is normalized at the position (X, 0.0; Y, 7.71×10^{-9}. The complete data is shown in Appendix I, Table 1.

S. grisoluteus showed an increase of MF until 200,000 µJ/cm^2 where it reached three magnitudes of increase in Str^R MF. The other three strains *S. antibioticus*, *S. ficellus* and *S. coelescens* showed 4.5-, 8-, and 10-fold increases in MF at 60, 000 µJ/cm^2, respectively. After having only 4.5-fold of increase in MF in *S. antibioticus* at 60,000 µJ/cm^2, the effect of lower UV intensity was also investigated, and a 9.5-fold increase in MF was observed at 50,000 µJ/cm^2.

For any mutagen, the absolute number of viable mutants in the population will increase with dose up to a certain level, and but will fall as killing overtakes the induction of new mutants (Keiser *et al.*, 2000). From past studies, it has been stated that the optimal mutagenesis occurs when the level of bacteria that survive exposure to UV is ~1% (Keiser *et al.,* 2000).

To determine the lethal effect of UV, survival after UV exposure was performed in parallel to the mutation induction study. The antibiotic-free plates were used to calculate total survivor count at different UV intensities similar to mutation induction study. The survival percentages are shown in the same graph (Figure 3.3). The percentage of UV killing in tested strains increased in proportion to UV intensity except in the case of Sa (survival data in Appendix I, Table 1). The reason behind this survival variability in Sa perhaps is unclear. Except for some variations in *S. antibioticus*, Str^R mutation frequency reached at the peak in these strains when UV lethal effect was maximal. *S. kanamyceticus* showed maximum increase in MF when only 0.0003% of the population survived. However, *S. griseoluteus* was less sensitive to UV compared to *S. kanamyeticus* where the maximum MF was observed in the 0.003% of survivors.

3.4.3 UV mutagenesis and survival studies in *S. kanamyceticus* with mycelial culture

To evaluate any differences of UV induced mutation between spore and mycelia upon UV treatment, the mycelia of *S. kanamyceticus* was used to conduct a separate UV induced Str^R mutation frequency experiment (Figure 3.4). This time three order of magnitude increases in MF was achieved at 4, 00,000 µJ/cm^2 (0.91% of total survivors). It required very high UV intensity ~6.5× more than spore treatment to induce maximum MF. It is apparent that mycelia are less sensitive to UV compared to spores.

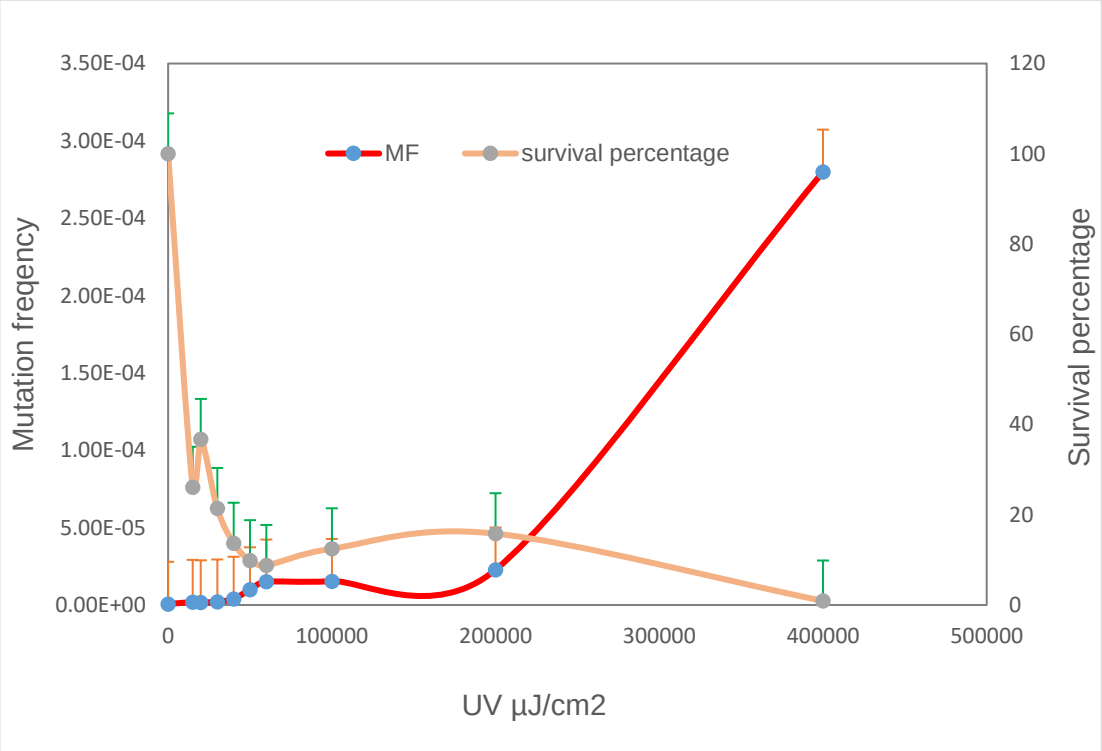


Figure 3.4 Induction of streptomycin resistance (Str^R) and survival of *S. kanmyceticus* with mycelial culture. Values shown are the means from three independent experiments. Error bars represent standard deviations from the mean. The complete data is shown in Appendix I, Table 2.

3.4.4 Effect of caffeine on UV induced MF and survival

Caffeine is known as an inhibitor of pyrimidine dimer excision (UV induced lesion), and the presence of caffeine has shown to increase mutation rate in *Streptomyces clavuligerus* (Saunders and Holt, 1982). In the presence of caffeine less damage is repaired by excision repair system and more damage eludes the error-prone repair system (Saunders and Holt, 1982), as a consequence, the chances of mutation increases. Therefore, an experiment was conducted with *S. kanamyceticus* by adding caffeine into the culture medium in order to maximize the mutation frequency to Str^R, if possible. Figure 3.5 and 3.6 shows the effect of the caffeine on survival and induction of mutation to Str^R, respectively. Caffeine reduced the survival of *S. kanamyceticus* in the control experiment by >5%. In UV-treated cells, survival percentages were reduced by 2.5-3% when caffeine was added. Two-tailed t-test was performed with QuickCalcs (online) and the P value was determined. At 95% confidence level, P value was 0.1412 which is statistically not significant. As expected, mutational hit to lethal hit (ratio of mutation and lethal induction) was increased, but only a 10-fold increase at 1000 $\mu J/cm^2$. Unfortunately, at 10,000 $\mu J/cm^2$ this increase in mutation and survival in the presence of caffeine was lost. Two-tailed t-test was also performed for mutation frequency comparison and the P value was determined. At 95% confidence level, P value was 0.1995 which is statistically insignificant. Further investigations were carried out to observe the effect of caffeine at higher UV intensities (15,000-60,000 $\mu J/cm^2$). However, the results showed only negligible changes in mutation induction to Str^R in the presence of caffeine (data not shown).

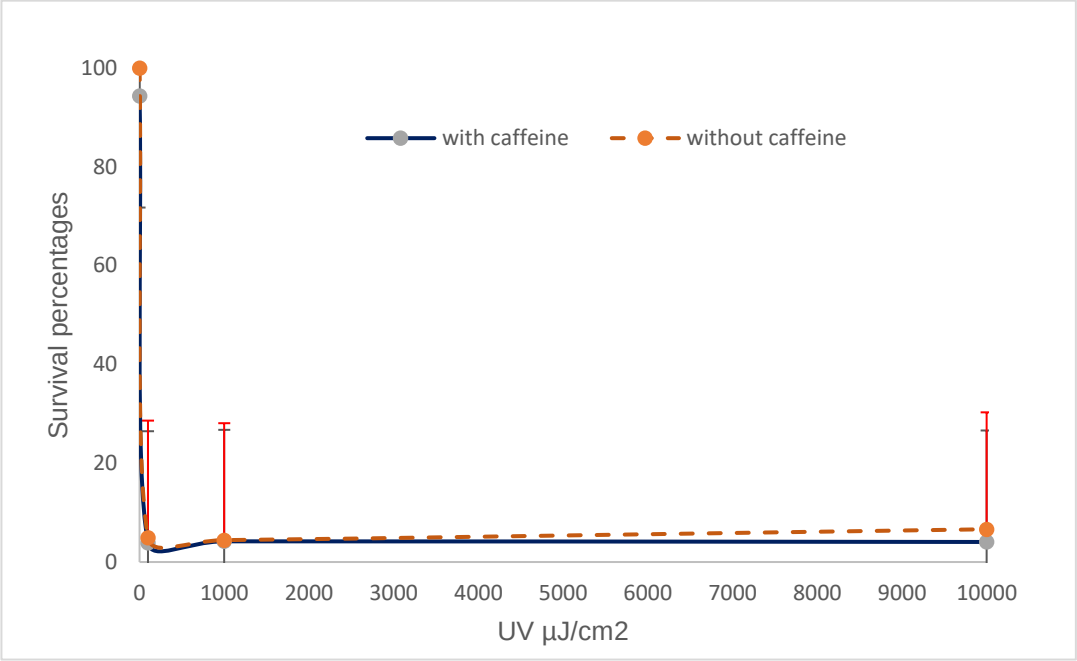


Figure 3.5 Effect of caffeine on survival of UV treated *S. kanamyceticus.* Experiments were conducted with three individual biological replicates. Error bars represent standard deviations from the mean. The two-tailed P value equals 0. 1412, this difference is not statistically significant.

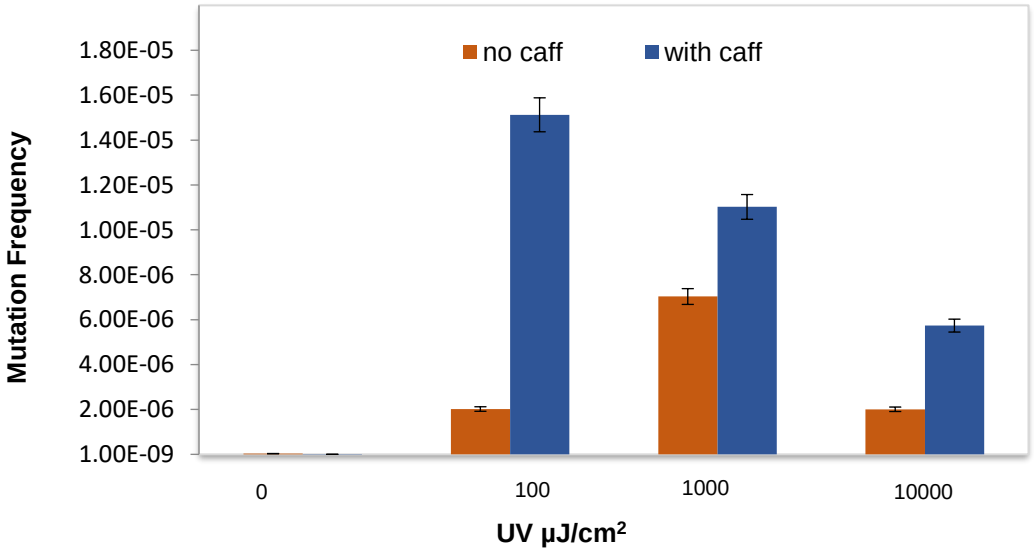


Figure 3.6 Effect of caffeine in UV induced mutation frequency to streptomycin resistance in *S. kanamyceticus*. Experiments were conducted with three individual biological replicates. Error bars represent standard deviations from the mean. The two-tailed P value equals 0.1995, this difference is not statistically significant.

3.4.5 Trial of UV mutagenesis for awakening cryptic BGC activity

Once it was evident that UV light can increase mutation frequency in streptomycetes, an investigation was conducted to test whether UV has an effect on the induction of silent BGC expression in streptomycetes. Eleven strains (*S. filamentosus*, *S. ficellus*, *S. kanamyceticus*, *S. tenebrarius*, *S. coelicolor* D132, *S. griseoluteus*, *S. avermitilis*, *S. yunnensis*, *S. prunicolor, S. niveus, and S. antibioticus*) having no prior antibacterial activity against MG1699 were selected for the UV mutagenesis trial experiment. The trial UV experiments aimed to achieve 99.90-99.99% killing rate because optimal mutagenesis has been reported to occur when the level of bacteria that survive exposure to UV is ~ 1% (Keiser *et al.* 2000). This was observed to hold true in the MF study to Str^R (Figure 3.3).

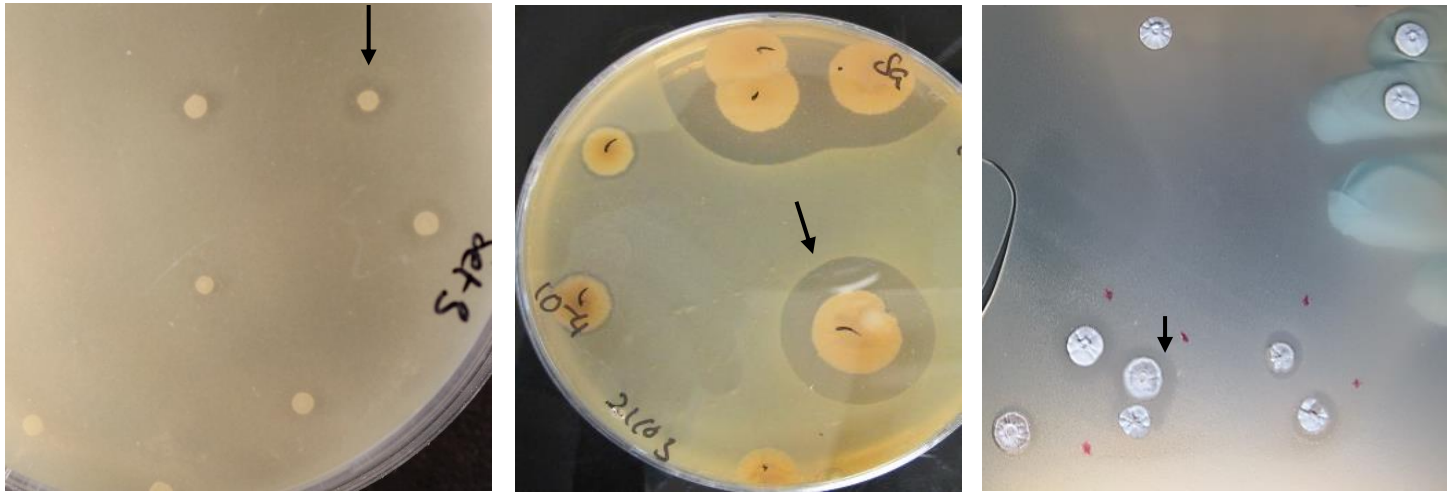
An initial experiment was conducted with *S. filamentosus, S. ficellus, S. kanamyceticus, and S. coelicolor* D132. The UV intensity range (10,000 – 50,000 µJ/cm^2) was chosen on the basis of previous experiments (Figure 3.3). The results are summarized in Table 3.2. The desired lethal effect was observed from 40,000 µJ/cm^2 in all the strains, except for *S. ficellus*. All the survivors were screened for new activity against MG1699. Survivors from *S. ficellus* and *S. kanamyceticus* (Figure 3.7) exhibited antibacterial activity against MG1699.

Table 3.2 Induction of antibacterial activity following UV mutagenesis in four different *Streptomyces* species (using spore suspension of 10^5 cfu/ml). The results show the lethality of UV light, raw number of survivors and mutant with new antibacterial activity in streptomycetes against *E. coli* MG1699 after UV treatment.

Strains	***S. fillamentosus***			***S. ficellus***		
UV µJ/cm²	% lethality	Survivors	Mutants	% lethality	Survivors	Mutants
30,000	98.80	400	0	99.99	5	2
40,000	99.96	4	0	99.99	9	0
	No new activity			**New activity**		

Strains	***S. kanamyceticus***			***S. coelicolor* D132**		
UV µJ/cm²	% lethality	Survivors	Mutants	% lethality	Survivors	Mutants
30,000	97.50	30	0	98.50	200	0
40,000	99.96	13	0	99.96	100	0
	No new activity			New activity		

(Data presented for 30,000-40,000 µJ/cm² only, where expected killing percentage ~ 99.99% was achieved, only three plates were screened)



S. kanamyceticus mutants

S. antibioticus mutants

S. griseoluteus mutants

Figure 3.7 Examples of UV-induced mutants that show antibacterial activity against *E. coli* MG1699. Zones of inhibitions are shown with arrows.

Table 3.3 UV mutagenesis of seven different *Streptomyces* species (using spore suspension of 10^5 -10^7 cfu/ml) using two UV intensities. The results show the lethality of UV light and percentage of new antibacterial activity in streptomycetes against *E. coli* MG1699 after UV treatment.

Strains	***S. grisoluteus***		***S. avermitilis***		***S. yunnensis***		***S. prunicolor***		***S. niveus***		***S. antibioticus***		***S. tenebrarius***	
UV µJ/cm^2	% lethality	% new activity	% lethality	% new activity	% lethality	% new activity	% lethality	% new activity	% lethality	% new activity	% lethality	% new activity	% lethality	% new activity
30,000	99.97	100	100	0	98.90	0	99.97	0	99.99	50	98.40	75	99.99	0
40,000	99.99	100	100	0	99.60	25	99.99	100	99.99	0	99.1	88.88	100	0
	New activity		No new activity		**New activity**		**New activity**		**New activity**		**New activity**		No new activity	

(These data were generated by employing two UV intensity (30,000 and 40,000 µJ/cm^2) with seven streptomycetes, Minimum of 10 colonies of survivors and 2 colonies of mutants were observed)

It was speculated that 30,000-40,000 µJ/cm^2 could be the desired range for UV mutagenesis. Therefore, the next experiment was performed with another seven strains using 30,000-40,000 µJ/cm^2 and new antibacterial activity was observed in five of them (Table 3.3). Both 30,000 and 40,000 µJ/cm^2 showed the potential to awaken silent antibiotic BGC. However, active mutant from *S. yunnanensis* and *S. prunicolor* did not survive upon subculture; it seems that the mutation(s) induced were not favourable for their survival. Also, the lethal effect of UV was high in *S. avermitilis* and *S. tenebrarius*.

3.4.6 Optimisation of UV intensity

This study was performed to optimise a universal UV intensity to activate silent BGCs across *Streptomyces* species. From the Figure 3.3, it was obvious that the rise of MF showed a relationship to increasing UV dose, but (except for *S. kanamyceticus* and *S. griseoluteus*) the MF dropped to zero above 60,000 µJ/cm^2. Although the MF jumped from 1.29×10^{-07} to 2.33×10^{-05} at 10,000 µJ/cm^2 for *S. kanamyceticus*, the remaining four strains had MF increases from 30,000 µJ/cm^2. Moreover, *S. antibioticus* exhibited a higher MF at 50,000 µJ/cm^2 compared to that at 60,000 µJ/cm^2. These results were similar to those seen in the UV mutagenesis trial experiment, as both 30,000 and 40,000 µJ/cm^2 showed the ability to induce new antimicrobial activity in tested strains. Therefore, two UV intensities (30,000 µJ/cm^2 and 50,000 µJ/cm^2) were used as a universal optimum. Five strains used in the MF experiment were exposed to these two UV intensity and colonies were screened for new antibacterial activity against MG1699.

A minimum of 200 UV survivors and untreated colonies were screened. That the antibacterial activity observed was UV-induced was established by screening colonies for spontaneous mutation (at 0 µJ/cm^2) from the same spore suspension used for UV treatment. All the strains gained new activity against MG1699 in both UV conditions, except *S. coelescens*. The UV intensity at 50,000 µJ/cm^2 induced higher percentage of new activity (Figure 3.8). Figure 3.9 shows representative plates of survivors exhibiting new antimicrobial activity.

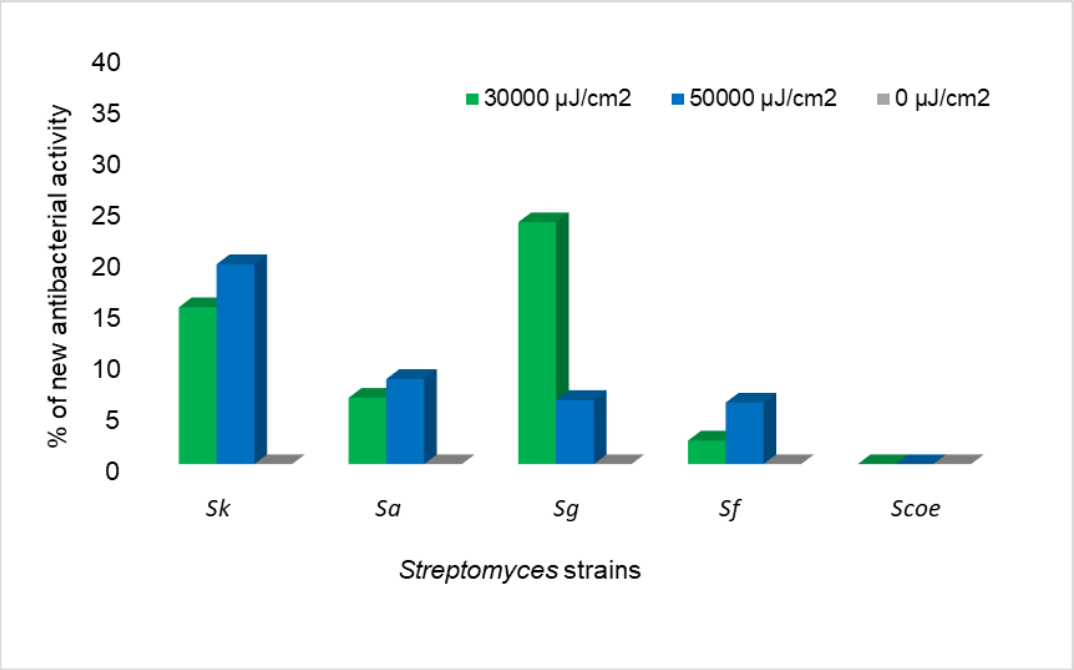


Figure 3.8 Percentage of new antibacterial activity against MDR *E. coli* MG1699 in five *Streptomyces* spp. after UV exposures. Values represent the percentage of new activity at 30,000 and 50, 0000 µJ/cm^2 relative to untreated controls (0 µJ/cm^2). Minimum 200 - maximum 2000 UV survivors for both of UV intensities and control colonies were screened for new activity. Sk: *S. kanamyceticus*; Sg: *S. griseoluteus*; Scoe: *S. coelescens*; Sa: *S. antibioticus*; Sf: *S. ficellus*.

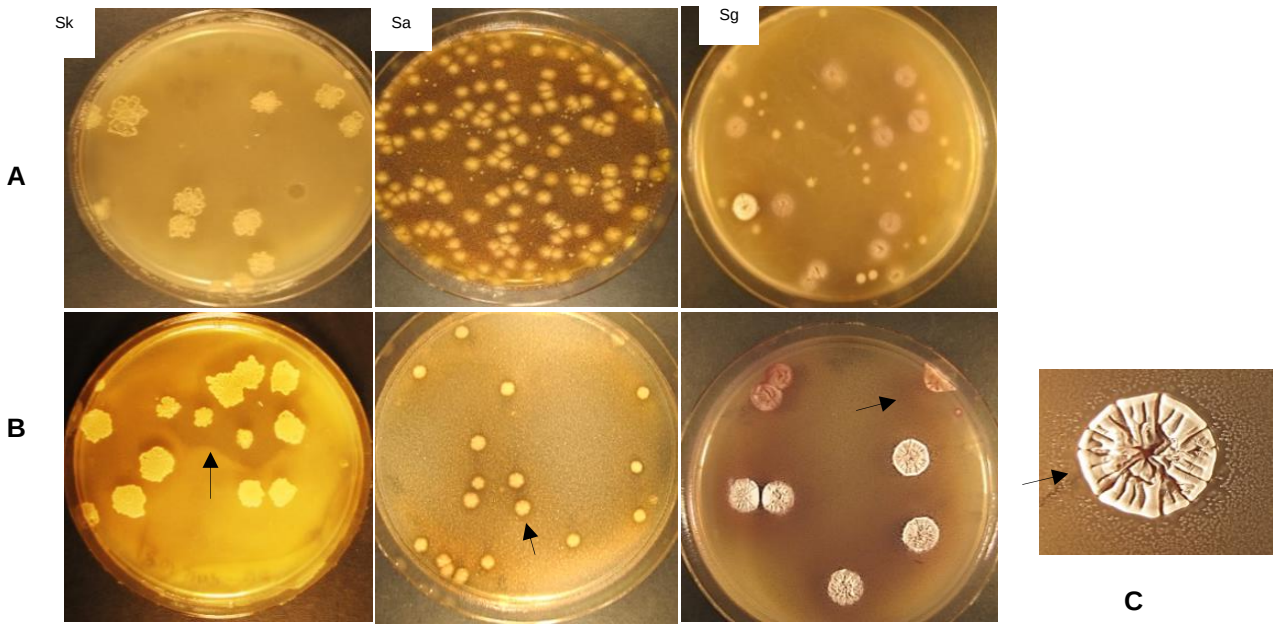


Figure 3.9 Activation of new antimicrobial activity in tested *Streptomyces* strains showing zone of inhibition (shown with arrow) against MDR *E. coli* MG1699. Sk: *S. kanamyceticus*; Sg: *S. griseoluteus*; Scoe: *S. coelescens*; Sa: *S. antibioticus*. (A) Control plates of corresponding strains, these are non-exposed colonies (0 µJ/cm^2). (B) A typical plate of gaining new antibacterial activity in UV survivors exposed at 30,000 µJ/cm^2. (C) A particular Sg mutant colony was zoomed for better resolution of inhibition around it

3.4.7 Characterization of selected UVMs

3.4.7.1 Phenotypic characteristics of UVMs

UVMs were characterized according to their phenotypic changes and antimicrobial spectrum was determined. This was performed to investigate whether UV irradiation can induce mutation that leads to different consequences in UVMs for a given strain. In this study, the morphological characteristics involving colour of the aerial and reverse colony, presence and absence of soluble pigments, colony size, colony morphology, ability to produce spores were considered. The number of mutants was reduced later due to the inability of some mutants to grow in fresh medium during sub-culturing stage. The results are summarized in the Table 3.4.

Table 3.4 Total number of active mutants and type of colony types from *Streptomyces species* recovered from UV mutagenesis.

Strain	Number of active mutants (initial hits)	Morphological types
S. kanamyceticus	9	4
S. antibioticus	33	11
S. griseoluteus	33	7
S. ficellus	17	6

Mutants from all four parent strains presented variability in their cultural characteristics, especially the colour of the aerial/mycelial growth, and their ability to sporulate. UV induced at least four types of colonial variants in streptomycetes (in *S. kanamyceticus*) (Figure 3.10), with the maximum colony diversity observed amongst Sa mutants (11 types) (Figure 3.11). The UVMs from *S. antibioticus* and *S. griseoluteus* showed pigments (Figure 3.11) which were new compared to their respective parent strains. In some instances, mutants had lost their ability to produce spores (especially in SfM) (Figure 3.13) and/or showed more wrinkles in their colonies. Furthermore, UVMS from *S. griseoluteus* were confirmed as decedents from *S. griseoluteus* parent strain by PCR amplification of a gene from phenazine gene clusters (*sgp*D). A 245 bp PCR product was generated by colony PCR (not shown). This was done to make sure that UVMs were not contaminants.

3.4.7.2 Antimicrobial spectrum of UVMs

Spectrum of the antibacterial activity of UVMs was determined by screening against indicator microorganisms (listed in Chapter two). Failure to inhibit the growth of *C. albicans* was scored as possessing specific antibacterial activity. The results were presented in Table 3.5-3.7, and representative bioassay photos are shown in Appendix II, Figure 1.

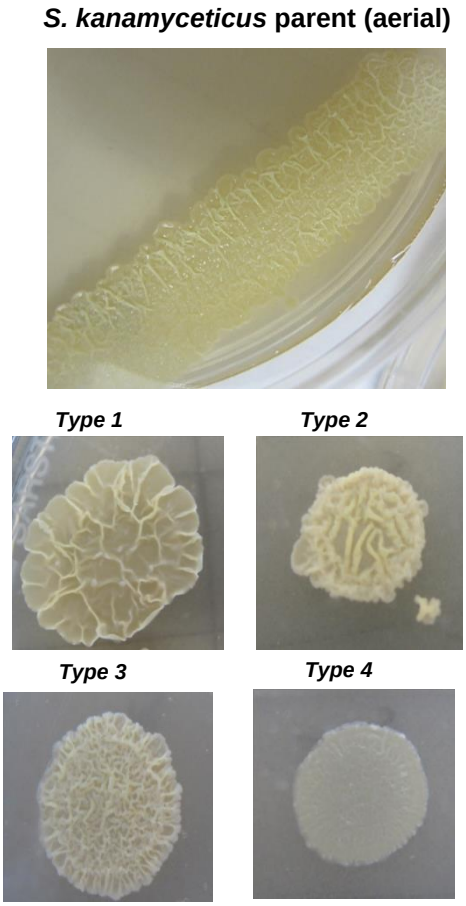


Figure 3.10 Different types of colony morphology (aerial view) in *S. kanamyceticus* UV mutants (below) compared to the parent strain (above). All strains were grown in GYM agar for 5 days at 28°C.

Type 1: Aerial: lemon yellow, Reverse: light brown, Soluble pigment: none, Colony size/morphology:medium/wrinkled
Type 2: Aerial: dark beige, Reverse: light beige, Soluble pigment: none, Colony size/morphology: small/less wrinkled
Type 3: Aerial: beige, Reverse: beige, Soluble pigment: none, Colony size/morphology: medium/compact wrinkled
Type 3: Aerial: light beige, Reverse: light beige, Soluble pigment: none, Colony size/morphology: poorly wrinkled

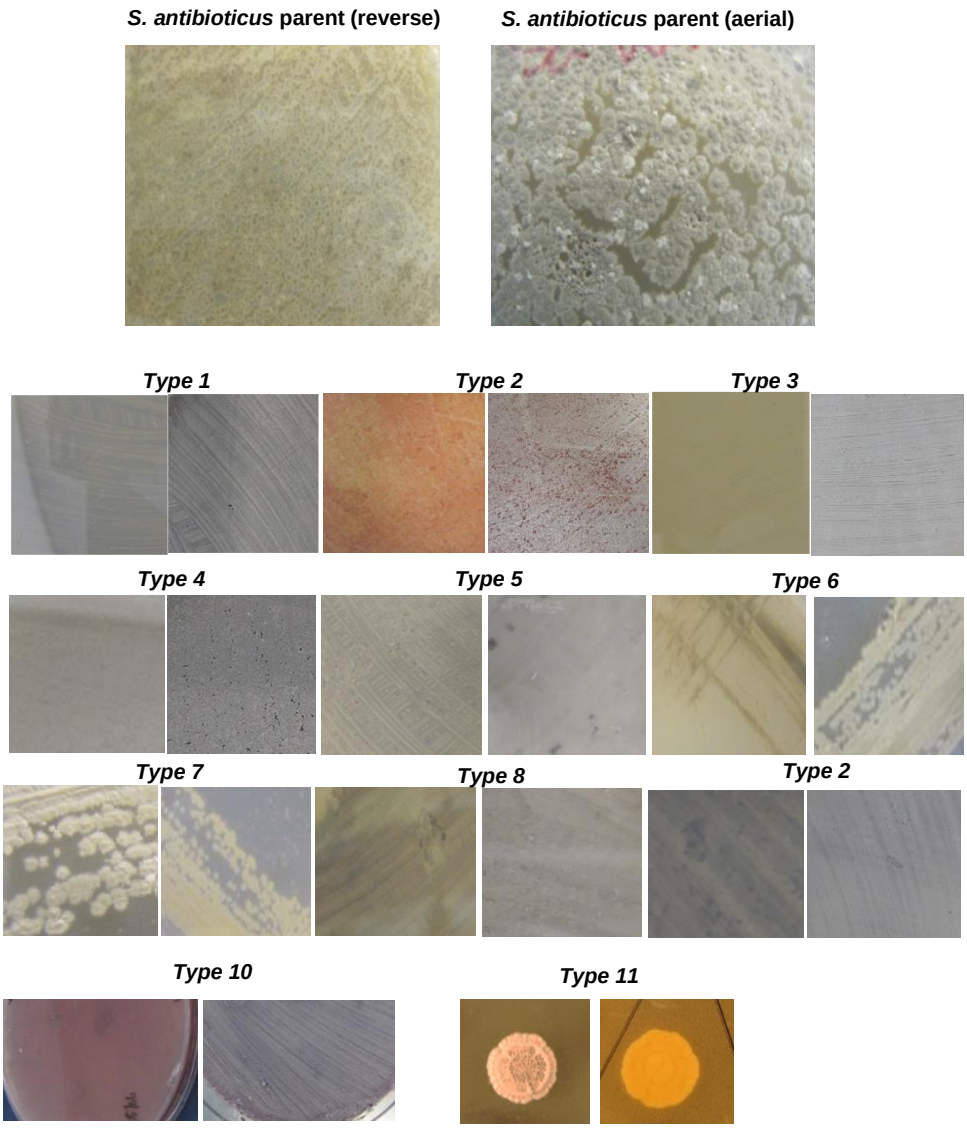


Figure 3.11 Different types of colony morphology in *S. antibioticus* UV mutants (below) compared to the parent strain (above). All strains were grown in ISP medium 4 agar for 5-7 days at 28°C.

Type 1: Aerial: Grey, Reverse: Dark grey, Soluble pigment: none, Sporulation: sporulating; **Type 2**: Aerial: White, Reverse: orange to red, Soluble pigment: orange to red, Sporulation: sporulating; **Type 3**: Aerial: grey white, Reverse: Dark beige to light yellow, Soluble pigment: none, Sporulation: sporulating; **Type 4**: Aerial: Grey, Reverse: grey, Soluble pigment: none, Sporulation: non-smooth sporulating; **Type 5**: Aerial: light grey, Reverse: light grey, Soluble pigment: none, Sporulation: sporulating; **Type 6**: Aerial: white, Reverse: black grey, Soluble pigment: none, Sporulation: sporulating; **Type 7**: Aerial: white, Reverse: light yellow to grey, Soluble pigment: none, Sporulation: non-smooth sporulating, **Type 8**: Aerial: Grey, Reverse: black grey, Soluble pigment: none, Sporulation: sporulating; **Type 9**: Aerial: white to Grey, Reverse: beige to grey, Soluble pigment: none, Sporulation: non-smooth sporulating; **Type 10**: Aerial: white purple, Reverse: pink to purple, Soluble pigment: none, Sporulation: sporulating; **Type 11**: Aerial: white to faint pink, Reverse: white, Soluble pigment: none ± faint pink, Sporulation: sporulating.

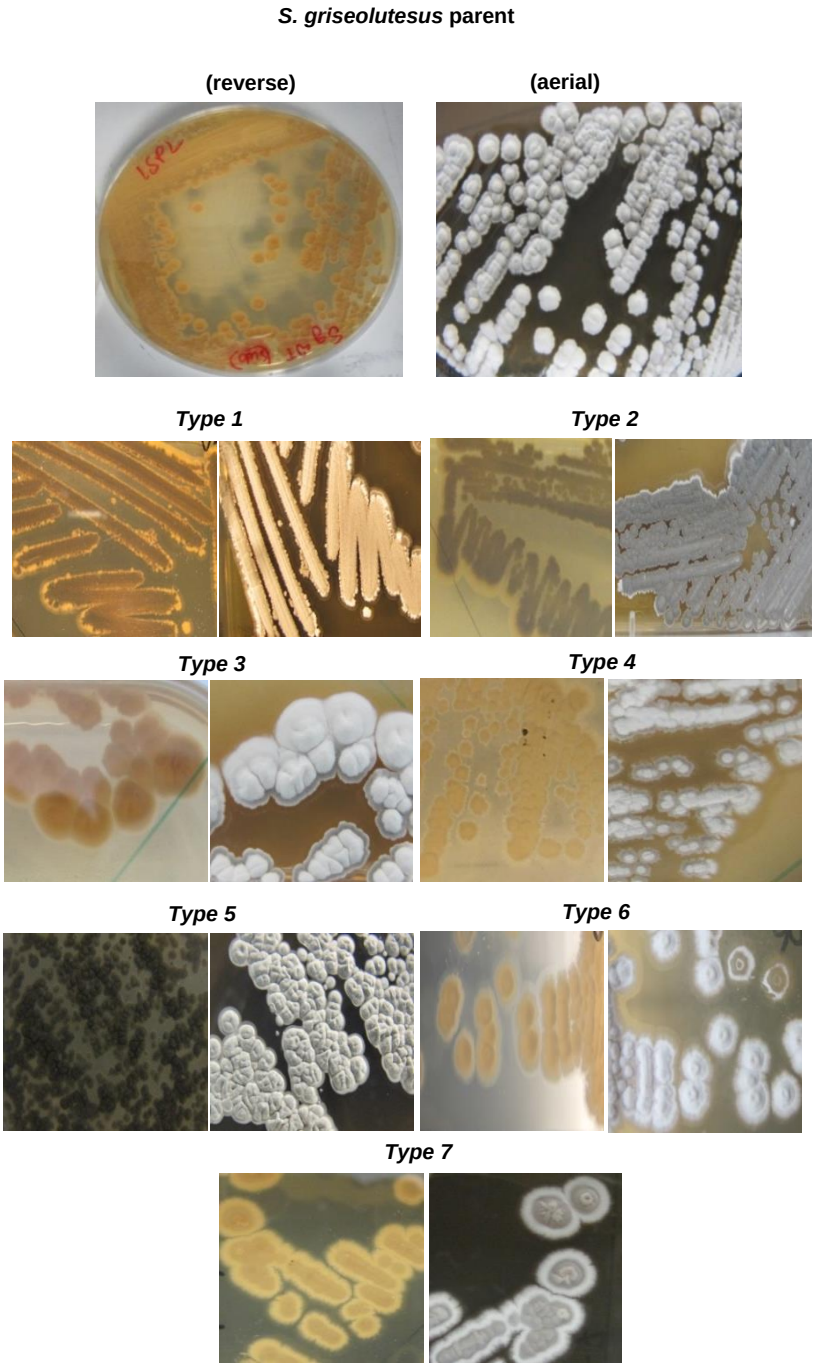


Figure 3.12 Different types of colony morphology in *S. griseoluteus* UV mutants compared to the parent strain. All strains were grown in ISP medium 2 agar for 5-7 days at 28°C.

Type 1: Aerial: White and grey, Reverse: light brown, Soluble pigment: light brown/none, Sporulation: sporulating; **Type 2**: Aerial: darkgrey±White boarder, Reverse: dark brown, Soluble pigment: light greenish brown, Sporulation: sporulating; **Type 3**: Aerial: white with grey border, Reverse: yellow to light brown, Soluble pigment: none, Sporulation: sporulating; **Type 4**: Aerial: white with grey border, Reverse: yellow to light brown, Soluble pigment: none, Sporulation: non-smooth sporulating; **Type 5**: Aerial: white and grey, Reverse: chocolate/dark purple/black, Soluble pigment: chocolate/dark purple/black, Sporulation: sporulating; **Type 6**: Aerial: white with distinct centre, Reverse: light brown, Soluble pigment: none, Sporulation: non-smooth sporulating; **Type 7**: Aerial: light grey with distinct centre, Reverse: yellow to brown, Soluble pigment: light greenish brown, Sporulation: poorly sporulating t edges of the colony.

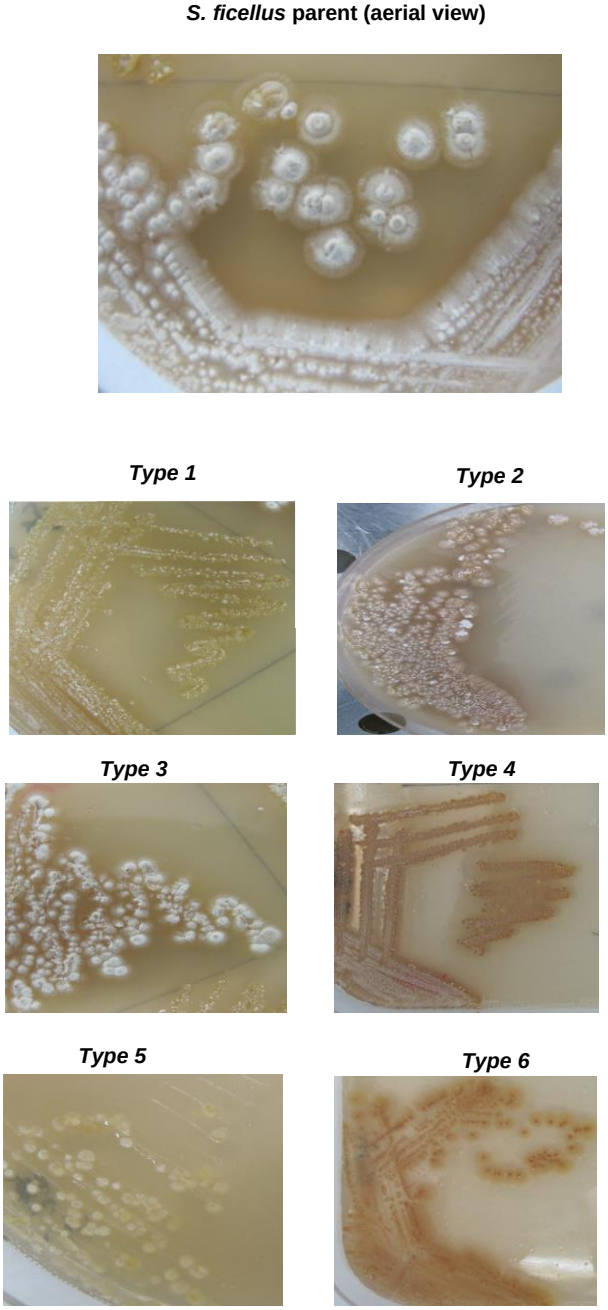


Figure 3.13 Different types of colony morphology in *S. ficellus UV* mutants (below) compared to the parent strain (above). All strains were grown in ISP medium 4 agar for 5-7 days at 28°C.

Type 1: Aerial: light yellow, Reverse: light yellow, Soluble pigment: none, Sporulation: poorly sporulating; **Type 2**: Aerial: brown beige, Reverse: dark brown, Soluble pigment: brown, Sporulation: moderately sporulating; **Type 3**: Aerial: white, Reverse: light reddish brown, Soluble pigment: light brown/none, Sporulation: sporulating; **Type 4**: Aerial: reddish brown, Reverse: reddish brown, Soluble pigment: none, Sporulation: poorly sporulating; **Type 5**: Aerial: beige, Reverse: beige, Soluble pigment: none, Sporulation: non-sporulating; **Type 6**: Aerial: brown, Reverse: brown, Soluble pigment: none, Sporulation: non-sporulating.

UVMs from *S. kanamyceticus* showed non-specific activity since all the mutants were active against CA6 (Table 3.5). UVMs from *S. antibioticus* exhibited variability in their antimicrobial activity (Table 3.6). The parental *S. antibioticus* strain showed activity against yeast and Gram-positive bacteria only. Interestingly, exception with the SaM9 and SaM18, all other *S. antibioticus* mutants lost their activity against the Gram-positive strain, *S. aureus* SH1000. SaM21, 37 and 40 exhibited specific antibacterial activity (also active against *A. baummannii*). Moreover, SaM3 had shown to be active against *K. pneumoniae*. The parent strains of *S. grisoluteus* and *S. ficellus* did not show antimicrobial activity against CA6; however, five of the twenty-two *S. griseoluteus* mutants, and one of seventeen *S. ficellus* mutants showed activity against CA6.

The outstanding question was whether the new activities detected result from newly activated gene clusters and/or new compounds, or from the overproduction of compounds known to be produced by *S. kanamyceticus*. It was not possible at this stage to say that these are from all new expression of cryptic BGCs. Since *E. coli* MG 1699 harbour a gene encoding resistance to kanamycin (kanamycin MIC is >256 µg/ml in this strain), mutants from *S. kanamyceticus* could be the result from the overproduction of this antibiotic. To resolve this issue, another *E. coli* (Kan^R) strain conferring kanamycin resistance only (MIC value of 256 µg/ml) was used. UV induced mutants (UVMs) from *S. kanamyceticus* were screened for the antibacterial activity against this *E. coli*. The bioassay results showed increased activity (larger zone of inhibition) in *E. coli* (Kan^R) compare to that of against *E. coli* MG1699 (data not shown). It indicates the presence of more than one compounds excluding kanamycin in these UVMs

Table 3.5 Spectrum of antimicrobial activity of *S. kanamyceticus* parent and UVMs against a range of indicator microorganisms

Strains	Colony Type	*E. coli* MG1699	*Candida albicans* C-*6*	*E. coli* *Kan*^{R*}
Sk parent	Parent	-	+	+
SkM1	1	+	+	++
SkM2	1	+	+	++
SkM3	2	+	+	++
SkM4	3	+	+	+
SkM5	1	+	+	+
SkM6,7	4,3	+	+	+

(+) antimicrobial activity, (-) No antimicrobial activity,ND: Not determined
*KanR: Kanamycin resistant strain (MIC 256 µg/ml)

Table 3.6 Spectrum of antimicrobial activity of *S. antibioticus* parent and UVMs against a range of indicator microorganisms.

Strains	Colony type	*E. coli* MG1699	*S. aureus* SH1000	*P. aeruginosa* PAO1	*A. baumannii*	*C. albicans* 6	*K. pneumoniae*
Sa Parent	Parent	-	+	-	-	++	-
SaM1	1	+/-	-	-	-	-	ND
SaM9,18	2	+	+	-	-	+	ND
SaM20	4	+	-	+	-	+/-	ND
SaM21,37,40	1,6,7	+	-	+	+	-	ND
SaM25	5	+	-	+	+/-	+/-	ND
SaM41	8	+/-	-	+	+/-	+/-	ND
SaM43	9	+/-	-	-	-	+/-	ND
SaM3	11	+++	-	ND	ND	+	+

(+) antimicrobial activity, (-) No antimicrobial activity, ND: Not determined, (+/-): variable

Table 3.7 Spectrum of antimicrobial activity of *S. griseoluteus and S. ficellus* parent, and their UVMs against a range of indicator microorganisms.

Strains	Colony type	*E. coli* MG1699	*C. albicans* 6
Sg Parent	Parent	-	-
Sg M1-2	1	+	+
Sg M3-4,18	2	+	-
Sg M5	3	+	-
Sg M11	5	+	-
Sg M12	5	+	+
Sg M14,17	3	+	+
Sg M15-16 19-22, 25	1	+	-
Sg M23,33	6	+	-
Sg M32	7	+	-
Sf Parent	Parent	-	-
SfM14,15,23	1	+	-
Sf M3	2	+	+/-
Sf M5	2	+	-
Sf M12,24,27	3	+	-
Sf M4,6,11	4	+	-
Sf M9,10,22	5	+	-
Sf M7,8,13	6	+	-

(+) antimicrobial activity, (-) No antimicrobial activity, (+/-): variable

3.5 Discussion

UV mutagenesis was used in this study to establish a novel approach for discovering new antibiotics by activating cryptic biosynthetic gene clusters (BGCs) in streptomycetes. UV-induced streptomycin resistance (Str^R) mutation frequency (MF) determination in phylogenetically distant *Streptomyces* species revealed up to a ~50,000 fold the increase. This finding is similar to the results of UV induced mutation study in *S. coelicolor* (Clarke and Hopwood, 1976) and *S. clavuligerus* (Saunders *et al*., 1982; Holt and Saunders, 1985), where UV induced MF were high for auxotrophy and resistance to rifampicin, respectively. Parallel UV survival study revealed that MF to Str^R hit its maximum level when the survivors were in the range of 0.0003-6.667%.

Since the aim of the study was to evaluate the potential of UV light to induce mutation, there was no further study carried out to investigate UV induced damage and repair system in any mutant. However, results can be correlated and explained in the light of previous detailed studies by Saunders *et al*. (1982, 1985). UV light has shown its potential to induce mutation in *S. kanamyceticus* and *S. griseoluteus* as the proportion of mutants amongst survivors increased even at higher lethal effects. This hypermutable characteristic (where mutation frequency reaches a peak and then declines) has also been shown in *S. clavuligerus* Uvs (UV light sensitive mutant) CL105 and CL89 (Saunders *et al*., 1982), which were excision (error-free, pre-replication repair) deficient mutants. Coleman & Holt (unpublished, see Saunders *et al.,* 1982), also confirmed the presence of certain *uvs* loci that confer hypermutability in a *S. coelicolor* strain. Low induction of Str^R MF in *S. ficellus*, *S. antibioticus* and *S. coelescens* suggested that these strains might have either faulty error-prone DNA repair systems (post-replication repair) or have both error-free and error- prone DNA repair systems (Stonesifer and Baltz, 1985). One-order of

magnitude increase in S. antibioticus was observed at lower UV intensity (50,000 µJ/cm^2). *S. clavuligerus* CL90 had shown the same low dose effect of UV, i.e. low dose was more mutagenic than the high dose (Saunders *et al*., 1982).

Although UV induces wider variety of mutations, Batlz considered UV light too weak to be useful in actinomycetes as its mutational rate was lower compared to the chemical mutagen MNNG in *S. fradiae* (Baltz, 1985; 1999). However, this statement is based on UV mutation induction study with *S. fradiae* M1 and its MNNG-derived mutants. *S. fradiae* M1 itself is a spontaneous mutant which is deficient in aerial mycelium and aerial spores, and incapable of producing tylosin (Baltz 1978). Therefore, Baltz used mycelial culture instead of a spore suspension for his UV mutagenesis studies. Use of mycelia could make a considerable difference in UV treatment, because spores provide easiest access to a large population of unigenomic haploid cells compare to multinucleate mycelial cells. Spores produce mutants with a minimum of segregational delay (Kieser *et al.,* 2000); segregation delay can retard phenotypic expression of an induced mutation in multinucleate cells (Auerbach, 2013). It was speculated by Coyne *et al.* (1984) that the UV damage repair system might differ between spores and vegetative cells in streptomycetes. To evaluate any differences of UV induced mutation between spore and mycelia upon UV treatment, the mycelia of *S. kanamyceticus* was used to conduct a separate UV induced Str^R mutation frequency experiment. It is apparent that mycelia are less sensitive to UV compared to spores.

Furthermore, it is now known that sporulation-deficient mutants of *Streptomyces spp.* are *bld* (comes from bald, lacking characteristics fuzzy morphology) mutants of wild type strains (Salerno *et al*., 2009). Several *bld* genes have been characterized (Leskiw *et al.,* 1991; Nguyen *et al*., 2003; Takano *et al*., 2003; Chater and Chandra,

2008) and some of them play a role as transcriptional regulators and are involved in different regulatory pathways. For instance, BldD is essential for morphological development and antibiotic production (Elliot *et al.,* 1998). Identification of its regulon (encompasses ~167 transcriptional units) using ChIP-chip revealed that 42 BldD target genes (~25% of the regulon) encode regulatory proteins, emphasising the pleiotropic role of BldD in *Streptomyces coelicolor* (Hengst *et al.,* 2010). Therefore, it is understandable that failure to achieve effective UV mutation in *S. fradiae* M1 (bld mutant as defective in aerial mycelium and aerial spore production) was not only a consequence of weak mutagenesis by UV light.

The appearance of new antibacterial activity against Gram-negative MDR *E. coli* MG1699 has been demonstrated by UV mutagenesis in two-thirds of the tested strains. Activation of new antibacterial activity confirmed that UV light has potential to induce mutation leading to the induction of silent antibiotic BGCs among different species of streptomycetes. Both of the chosen UV intensities (30,000 µJ/cm^2 and 50,000 µJ/cm^2) generated new antibacterial activity in four out of five tested strains. A 2%-23% induction of new antibacterial activity against MDR *E. coli* MG1699 was observed in all the tested strains except for *S. coelescens*. Even though there was only a low increase in Str^R MF in *S. ficellus* and *S. antibioticus* (Figure 3.2), bioassay with survivors from both strains revealed new antibacterial activity. Consequently, mutation frequency determinations do not necessarily reflect the ability of a UV mutagen to induce secondary metabolism diversity in streptomycetes. A study by Baltz speculated that a large number of genetic targets may influence the mutability of antibiotic production (Baltz, 1986), and the total number of genes affecting the production of antibiotic may total more than 100 genes in *Streptomyces* (McCabe, 1990). Furthermore, Xu *et al.* (2017) have shown that about more than two-third of 354 genes were responsible for the altered RED production in *S. coelicolor*

(described in chapter one). These studies can be correlated with activation of new antibacterial activity in UVMs from *S. antibioticus* and *S. ficellus*. In short, the results presented here reveal the efficiency of UV mutagenesis as a novel approach to access unlock new secondary metabolites encoded by cryptic BGCs in streptomycetes.

In an early study, Chakraborty (1969) showed the ability of UV radiation to generate both morphological and biochemical mutants in S*. indicus.* He also observed noticeable variation in the antibiotic activity of these mutants. Likewise, morphological changes due to UV mutagenesis have resulted in increased yield of valuable secondary metabolites in previous studies (Klanova *et al.,* 1977; Blumauerova *et al.,* 1978). *Streptomyces fradiae* NRRL-2702, a tylosin antibiotic producer, exhibited six altered type of colony morphology upon exposure to UV radiation, and one of these altered mutants had shown 2.7±0.22-fold increase of tylosin yield compare to the wild type strain (Khaliq *et al.,* 2009).

An investigation was conducted with UVMs from *S. niveus*. A novobiocin resistant S*. aureus* AV-13 (Vickers *et al.,* 2007) for which novobiocin has an MIC of 16 µg/ml and its novobiocin-susceptible strain SH1000, are similarly used to screen for overproduction of novobiocin in *S. niveus* mutants. These results suggested overexpression of novobiocin as the diameter of the zone of inhibition was reduced in AV-13 compare to that of SH1000 (data not shown).

In present study, UVMs from *S. kanamyceticus*, *S. antibioticus*, and *S. ficellus* exhibited different colony characteristics, and UVMs were categorised according to their cultural characteristics. Antimicrobial activity against different indicator microorganisms has shown some variations in their spectrum of activity, even from the same type of colony. This emphasises the importance of exploring every single mutant, if possible. Nevertheless, detailed metabolomics is essential to conclude whether these morphological variants are producing diverse active metabolites.

UV mutagenesis to activate silent BGCs is a flexible, rapid and simple plate-based technique, and it does not require any prior genetic information or knowledge of factors affecting secondary metabolites production of the tested strain. Since a large number of *Streptomyces* species (over 550) have been reported so far, genome-based approaches would take a while to activate all the metabolites encoded by silent BGCs. Additionally, there is no way to screen for antimicrobial activity of metabolites encoded by these silent BGCs, unless they are expressed in a host. UV induced mutagenesis could be an alternative way of unlocking BGCs in much shorter period and lead to crucial discoveries. This new approach has advantages over other existing approaches, which also do not require genomic information, such as OSMAC and ribosome engineering. OSMAC relies on manipulation of growth conditions, and ribosome engineering induces mutation in ribosome and RNA polymerase genes. Neither of these approaches accelerates the rate of evolution in genome level that would induce significant diversity among secondary metabolisms in *Streptomyces* spp. Conversely, UV induces genome-wide DNA damages by chance, and in principle, UV mutagenesis should be able to switch all of them on.

A limitation of this approach would be perhaps the inability to induce effective mutations in *Streptomyces* strain where defective error-prone DNA repair system or efficient error-free DNA repair mechanisms exists. Moreover, UV hypersensitive strains would result in low mutation to lethal induction ratio. In some instances, instability of UV mutants (Blumauerová *et al*., 1976) and reduced yield of antibiotic (Khaliq *et al.,* 2009) have been demonstrated. It is worth noting that *Streptomyces* are the only prokaryotes which can undergo frequent and extreme DNA amplification without any selection (Baltz, 1986). Moreover, they have evolved more efficient error avoidance mechanisms than those commonly observed in single-celled eubacteria (Stonisifer and Baltz, 1985). These properties could have an underlying effect on UV mutagenesis in streptomycetes. Nevertheless, this is the first report of utilising UV mutagenesis as a novel approach to unlock cryptic BGCs with antibacterial activity against Gram-negative MDR pathogens.

Chapter 4
Identification and preliminary characterization of novel antibiotics from UV-induced mutants of *Streptomyces spp.*

4.1 Rational of the study

From the previous chapter, it is apparent that UV mutagenesis has the potential to induce new antimicrobial activity in streptomycetes. However, there are two outstanding questions; (i) does UV mutagenesis allow the recovery of multiple new compounds from a given strain (or do all the mutants produce the same compounds)? and (ii) does it induce the production of chemically-novel antibiotics? The first question could effectively be addressed by metabolomics; analysing the secondary metabolites from the mutants and comparing them with those of their corresponding parent strain. The second could be addressed by chemical de-replication of known compounds (Beutler *et al.,* 1990) and identification of new compounds with existing chemical scaffolds by 'molecular networking' (grouping of chemically similar compounds) (Garg *et al.,* 2015). Thus, all the known chemical scaffolds will be filtered out and unknown compounds (if any) could be further investigated. Therefore, the work presented in this chapter focused on comparative metabolomics and chemical de-replication (and molecular networking) to examine the antibacterial compounds induced by UV mutagenesis in the previous chapter.

4.2 Aims and objectives

The aim of this chapter was to resolve the outstanding questions regarding validation of the UV mutagenesis approach to access novel antibiotics encoded by cryptic BGCs. The objectives of the study were as follows;

i. Detection of new metabolites in UV induced mutants (UVMs)
ii. Confirmation of the antimicrobial activity of newly detected mass(s)
iii. Chemical de-replication of new mass(s) by MS/MS fragmentation pattern analysis and online database searching

4.3 Results

Results from the previous chapter established that the antibiotic(s) produced by all UVMs of *S. kanamyceticus* lacked bacterial selectivity (exhibited activity against *Candida albicans* 6). Thus, the subsequent studies focused on only UVMs from the other three *Streptomyces* species *S. antibioticus*, *S. griseoluteus and S. ficellus.*

4.3.1 Initial studies involving manual data analysis of SaMs

Initial investigation with UVMs from *S. antibioticus* was performed before the utilization of metabolite tool and GNPS platform in this research work. Crude extracts from SaMs mutants (except SaM3, since it was recovered later) with antibacterial activity against MG1699 were further analysed via comparative metabolomics profiling alongside the Sa parent strain. LC-MS and HPLC chromatograms were analysed manually to observe new peaks and/or loss of peaks in these mutants (Figure 4.1 A-B). Several new peaks were observed in SaM9 and SaM21 compared to the crude extract from the parent strain.

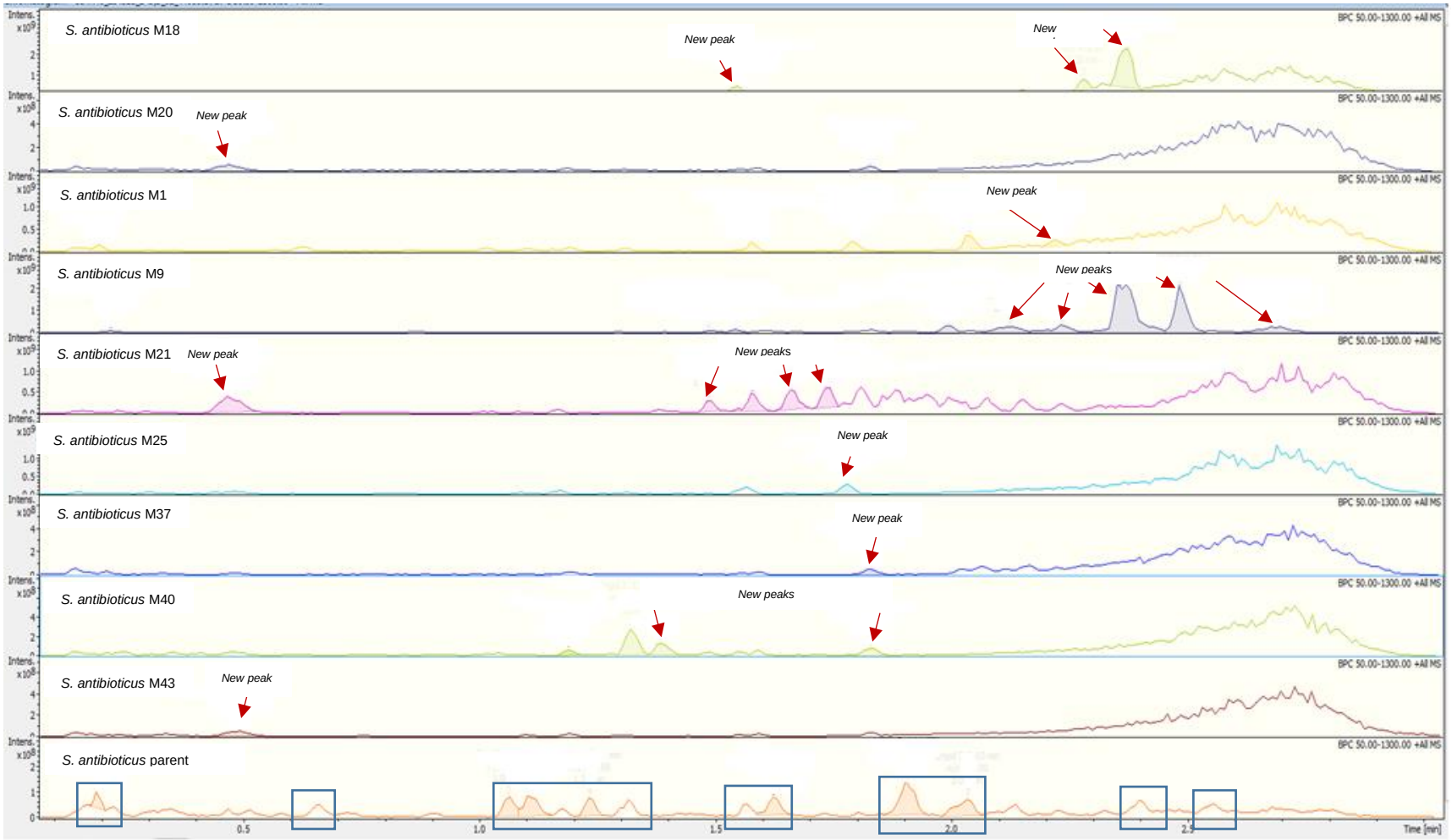


Figure 4.1 A LC-MS chromatogram shows the comparison of metabolites from the mutants and the parent of *S. antibioticus*. The blue square boxes show peaks from parent strains. Red arrows show the presence of new peaks in UV mutants compared to the parent *S. antibioticus*.

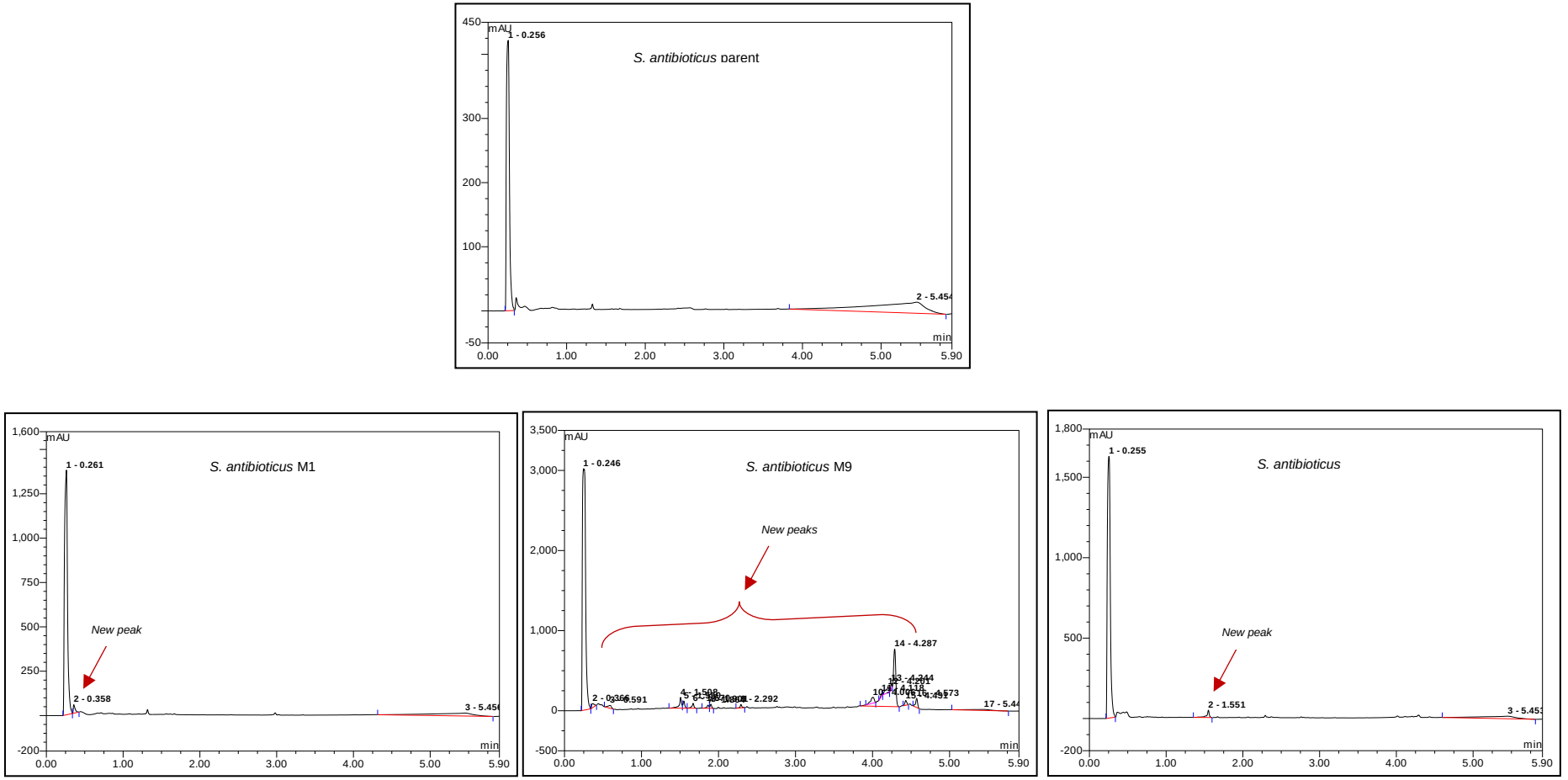


Figure 4.1 B HPLC (analytical) chromatogram shows the comparison of metabolites from the mutants and the parent of S. antibioticus. Red arrows show the presence of new peaks in UV mutants compared to the parent S. antibibioticus. This experiment was performed by Martin Huscroft (School of Chemistry).

SaM9 exhibited altered colony characteristics with red pigment production compared to the parent strain. Thus, it was chosen to investigate if the altered colony screening could serve as a viable approach of UVM selection with diverse metabolite producing capability. Time-based bioactivity-guided HPLC fractionation generated 17 fractions. Twelve of these fractions exhibited bioactivity against *E. coli* MG1699. After high resolution LC-MS analysis of active masses, several mass patterns were observed (Appendix II, Table 4). Three predominant masses $(M+H)^+$(393, 465, and 478) were chosen for mass-directed fractionation because these masses were new compared to *S. antibioticus* parent, and also because they failed to match reported metabolites from S. *antibioticus* (Appendix II, Table 1). These semi-pure mass fractions were tested against MG1699 and other indicator strains (Table 4.1). Although these mass fractions exhibited activity against MG1699, they were also found to possess antifungal activity. Given the lack of selectivity of masses $(M+H)^+$ (393, 478, 465), these were not pursued further.

Table 4.1 Bioassay of HPLC purified mass fractions from *S. antibioticus* mutants (SaMs). (agar diffusion assay)

Mass	*E. coli* MG1699	*C. albicans* 6	*S. aureus* SH1000	*P. aeruginosa* PAO1	*A. baumannii*
393 $(M+H)^+$	+	+++	growth reduced	++	+/-
479 $(M+H)^+$	+	+	++	++	-
465$(M+H)^+$	+	+	++	++	-

(+) antimicrobial activity, (-) No antimicrobial activity, (+/-) variable activity

4.3.2 New active masses in UV mutants (UVMs)

4.3.2.1 Detection of new masses by Metabolite tool

A total of forty-six UVM crude extracts generated from three strains (*S. antibioticus*, *S. griseoluteus* and *S. ficellus)* were analysed by metabolite detect software and new peaks were identified in UVM crude extracts as expected. Figure 4.2 shows a typical results window for analysis by metabolite tool (here *S. griseoluteus* parent Vs *S. griseoluteus* M18 crude extracts). Table 4.2 shows all the new masses detected in UVMs. These new masses were subject to an initial de-replication step by cross-checking their mass against published metabolite masses from the respective *Streptomyces* strains (Appendix II, Table 1-3) before further investigation.

4.3.2.2 Purification and preliminary biological characterization of new masses

A total of six UVMs (SaM3, SgM11, SgM18, SfM3, SfM15 and SfM27) were further investigated to confirm their antibacterial activity in crude extracts. These UVMs were selected because they showed a larger zone of inhibition compared to other UVMs, and possessed at least two new masses detected by the metabolite detect software. These crude extracts exhibited antibacterial activity against MG1699. Some representative pictures are shown in Figure 4.3.

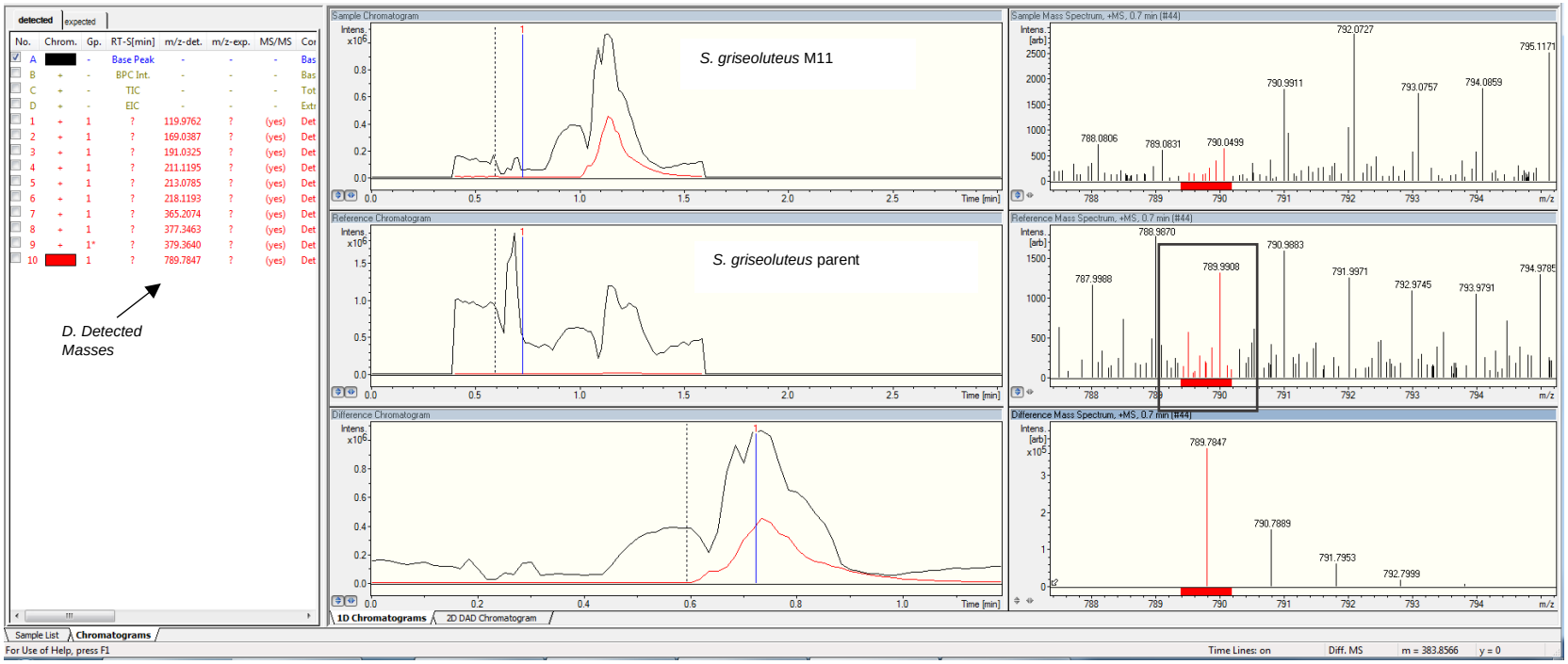


Figure 4.2 Comparison of *S. griseoluteus* parent crude extract versus *S. griseoluteus* M11 crude extract. A) S. griseoluteus M11, B) Reference (*S. griseoluteus* parent), C) Comparison, D) Detected mass list. On the right, mass spectrum are displayed.

Table 4.2 Detection of new masses (*m/z*) from UVMs in comparison to the parent strain using the Metabolite Detection tool. Highlighted UVMs exhibited the greatest number of new masses.

UVMs	Mass detected by metabolite tool from high-resolution accurate mass spectrometry HRMS data (*m/z*)
SaMs (*S. antibioticus* mutants)	
SaM1	337.24,767.48
SaM3	266.13, 536.18, 796.30
SaM9	392.27,477.22, 463.21,465.25, 630.50,509.84,479.22
SaM18	478.25, 767.49
SaM20,25	767.50
SaM21	389.25, 447.82, 495.26, 545.42, 563.59, 603.54, 621.33, 767.48
SgMs (*S. griseoluteus* mutants)	
SgM1	339.25
SgM2,15,25	191.06
SgM3	450.16
SgM4, SgM12 (colored)	546.48, 551.26
SgM5,21	307.06
SgM11 (colored)	789.38, 743.37, 379.0
SgM14	none

Table 4.2 Detection of new masses (*m/z*) from UVMs in comparison to the parent strain using the Metabolite Detection tool. Highlighted UVMs exhibited the greatest number of new masses. **(Continued)**

UVMs	**Mass detected by metabolite tool from high-resolution accurate mass spectrometry HRMS data (*m/z*)**
SgM16	332.32
SgM17,24,32	365.26
SgM18	450.15, 359.14
SgM19	299.07, 277.09
SgM20,22	326.37
SgM23	908.42
SgM31	267.10
SgM33	702.86. 365.26
SfMs (*S. ficellus* mutants)	
SfM3	126.05, 394.31, 441.29, 661.53
SfM4,6,12,14,22-24	441.29
SfM5	365.26, 396.32, 407.27, 681.51
SfM7,11	407.28
SfM8,9,10,13	381.26
SfM15	288.92, 441.30
SfM27	154.08, 327.00, 348.98, 381.26, 441.29

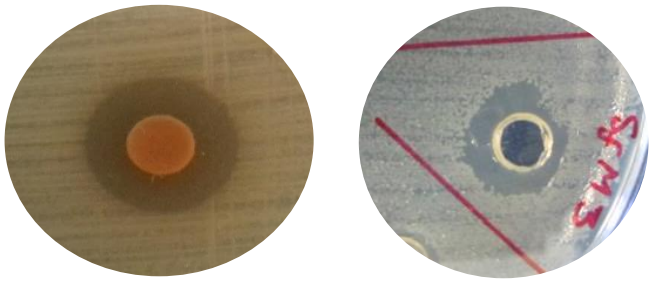


Figure 4.3 Bioassay of crude extracts from selected UVMs against MDR *E. coli* MG1699. Bioassay was performed by both agar diffusion and disc diffusion assay. SaM18: *S. griseoluteus* M18; SfM3:*S. ficellus* M3.

Mass-directed HPLC purification of these six UVMs was conducted. The compiled results are shown in Table 4.3. Both masses from SaM3 were found to be inactive against MG1699; potentially, these compounds were produced in too low an amount to provide a detectable antibacterial effect, or were not the active compounds. All the other tested new masses from *S. griseoluteus* mutants exhibited specific antibacterial activity against MG1699. Three of the purified mass fractions from *S. ficellus* mutants exhibited activity against MG1699. This resolves the first outstanding question of the potential of the UV mutagenesis approach to recover multiple new chemical entities from a given strain. Some representative pictures are shown in the Figure 4.4.

Table 4.3 Antimicrobial activity of HPLC purified masses from UVMs.

UVMs	Masses *m/z*	MG1699	CA6
SaM3	266	-	ND
SaM3	536	-	ND
SgM11	789	++	-
SgM11	379	+	-
SgM18	450	+	-
SgM18	359	++	-
SfM27	327	+	ND
SfM27	381	-	ND
SfM3	394	+	ND
SfM3,14,27	441	+	ND

ND: Not determined because the yield of purified fraction was low

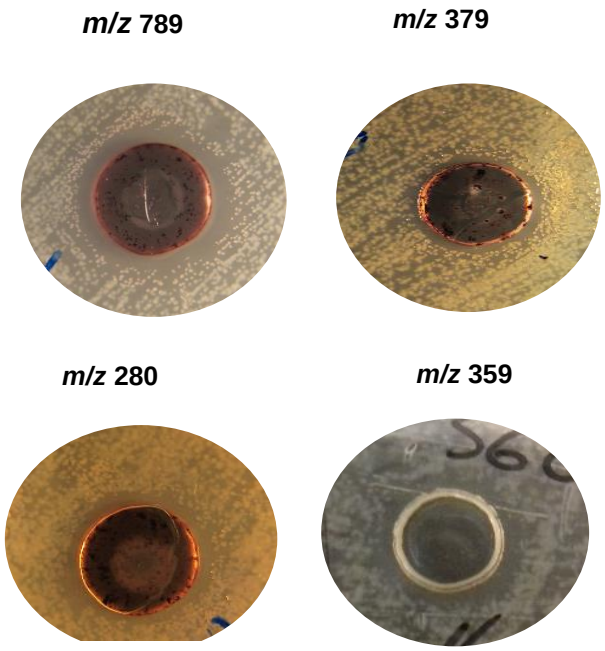


Figure 4.4 Antibacterial activity of semi-purified compounds from UVMs against MDR *E. coli* MG1699. Bioassay was performed by agar diffusion assay.

4.3.3 De-replication of the compounds

To achieve chemical de-replication, crude extracts of twenty UVMs and three parent strains (and available HPLC purified compounds including *m/z* 789, 379, 359, 450, 536,327, and 394) were subjected to high-resolution tandem mass spectrometry (HR-MS/MS) to obtain fragmentation patterns of the compounds present in the crude extracts. HR-MS/MS data was uploaded to the GNPS under the online molecular networking workflow. After low hit recovery in initial experiments, the settings were changed to lower stringency conditions (parent Mass Tolerance 2.0 Da, Ion Tolerance 0.5 Da) to capture the maximum number of hits. Molecular networking merged all identical MS and MS/MS spectra (including identical MS/MS spectra of isomers) and these spectra were then searched against GNPS's spectral libraries. System impurities were identified by solvent blanks and ISP2/ISP4 media control. The results are summarized in Tables 4.4-4.6.

4.3.3.1 De-replication of SaMs

The *S. antibioticus* parent strain and two mutants SgM9 and SgM3 were initially chosen for this study. As a result of molecular networking, forty-two parent ions as node, both clustered in spectral family and as individual ions, were obtained. The results are summarized in Table 4.4.

A search of each MS/MS spectrum against GNPS's spectral libraries resulted in the de-replication of two compounds from SaM9 including streptorubin (*m/z* 392) and 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (16:0 Lyso PE), (*m/z* 477, data matched from *Mus musculus*). Streptorubin has antimicrobial and cytotoxic

activity (Bycroft 1987), though a literature search did not find any previous report on the production of streptorubin from *S. antibioticus*. Phosphatidylethanolamines (PE) are the phospholipids present both in bacterial (Dowhan, 1997) and eukaryotic membranes (van Meer ***et al.,*** 2008). The remaining active mass of *m/z* 465 returned neither database hits nor any predicted clustering in molecular network.

PE was also observed amongst the metabolites from SaM3; the other new masses from SaM3 had no match to compounds in the database. A molecular network was created for mass 536 with related unknown chemical entities (Figure 4.5). Although the 536.44 mass has been reported before from *S. antibioticus* and assigned to rubromycin, an antibiotic with Gram-positive antimicrobial activity, the crude extract from SaM3 was not active against the Gram-positive bacterium, *S. aureus* SH1000 (data not shown). The GNPS database returned additional hits for 5–methylthioadenosine (*m/z* 298.09) and undecylprodiginine (394.28) from SaM9, and Ile-Pro-Ile (*m/z* 342) from SaM3. All of these compounds have cytotoxic activity (Kol *et al.*, 2010; Song *et al*., 2015; Rahfeld *et al.,* 1991). These masses were not purified to test their activity because these were not detected as new masses exclusively present in the SaMs and they are likely to be cytotoxic.

Table 4.4 De-replication of UV-induced mutants from *S. antibioticus* using GNPS database

UVMs (Colony type)	**Mass detected by metabolite tool from HRLC-MS data (*m/z*)**	**Hits/Molecular network in GNPS**	**Known activity**
SaM9 (type2)	392.27	Streptorubin	Antibiotic, cytotoxic
	477.22	1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	-
	463.24,465.25, 630.50	No hits/ no molecular networking	-
	479.22	Related to PC(17:0/0:0); [M+H]+ C25H53N1O7P1	-
		Additional hits: Methylthioadenosine (*m/z* 298.09); Undecylprodiginine (*m/z* 394.285)	Cytotoxic; antimalarial and cytotoxic
SaM3 (type11)	266.13, 796.30	No hits/ no molecular networking	
	53618	No hits/ molecular networking without parent	-
		Additional hits: Ile-Pro-Ile (342.305001)	Cytotoxic

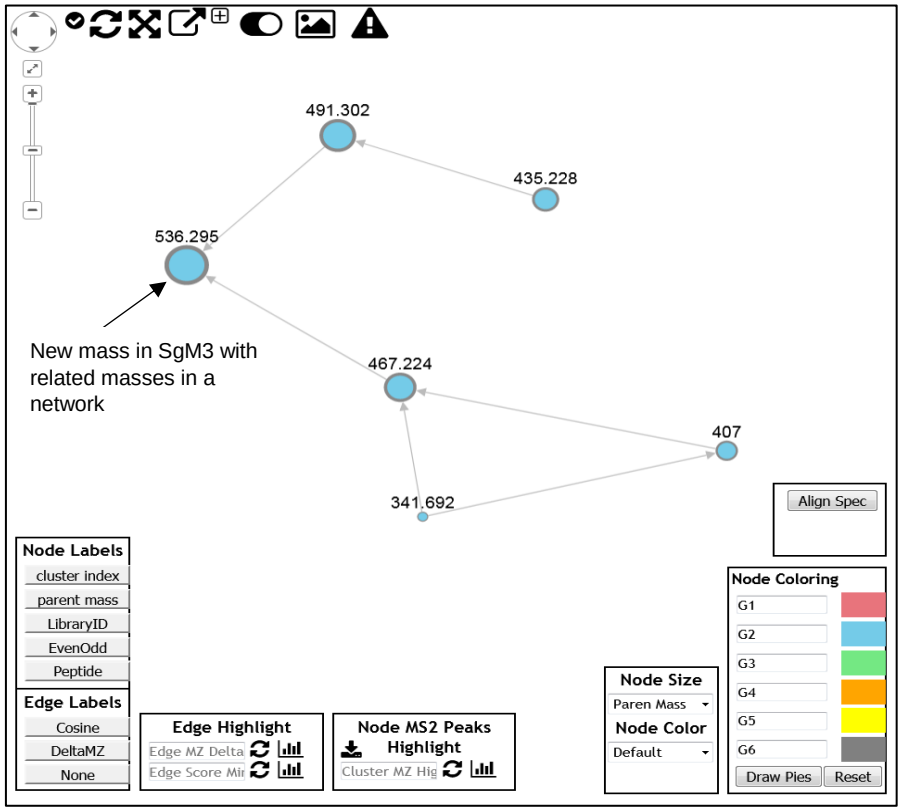


Figure 4.5 Molecular networking for the new mass *m/z* 536 from SaM3. UV induced new mass of *m/z* 536 had no match to GNPS database but molecular networking found related chemical entities for this mass. The size of the node indicates the number of spectra in each precursor mass. G1: Sa parent, G2: Sa M9 and SaMnew3, G3: ISP medium 4, G4: Methanol blank.

4.3.3.2 De-replication of SgMs

S. griseoluteus parent and eleven mutants were chosen for analysis. As a result of molecular networking, 128 parent ions parent ions as node, both clustered in spectral family and as individual ions, were obtained. The results are summarized in Table 4.5.

A search of each MS/MS spectrum against GNPS's spectral libraries did not return a match for any of the active masses from SgMs. A molecular network was created for the active mass *m/z* 359.14 (Figure 4.6). However, a mass from SgM11 (*m/z* 743.37) was found linked to a known spectral family of diadzin (*m/z* 417.11) and genistin (*m/z* 433.11) in the database. This suggests the presence of additional analogues amongst these isoflavone compound classes. These compounds are ubiquitous in plants, where they provide protection against UV radiation (Widyarini *et al*., 2001). They have also been isolated from several *Streptomyces* species (Huang *et al*., 2013; Liu *et al.,* 2009).

The GNPS database returned additional hits for Cyclo (Tyr-Leu)/8-(1,2-dihydroxy-3-methylbut-3-enyl)-7-methoxychromen-2-one (*m/z* 277.09) from SgM19. Cyclo(Tyr-Leu) is an antifungal compound (Kumar *et al*., 2013). This mass was purified; however, the purified fraction of this mass had no activity against MG1699.

Table 4.5 De-replication of UV-induced mutants from *S. griseoluteus* using the GNPS database

Mutants/Type	**Mass detected by metabolite tool from HRLC-MS data *m/z***	**Hits/molecular network by GNPS**	**Known activity**
SgM1(type 1)	339.24	No hits/molecular network with parent	-
SgM3 (type 2)	450.15	No hits/ no molecular networking	-
SgM4 (type 2)	546.48	No hits/ no molecular networking	-
	551.26	No hits/ molecular networking without parent	-
SgM11(type 5)	789.38, 379.0	No hits/ no molecular networking	-
	743.37	No hits/ molecular networking showed similarity to Diadzin (*m/z* 417.117) and Genistin (*m/z* 433.111)	Genistin, a tyrosine kinase inhibitor
SgM15 (type 1)	191.06	NF	-
SgM18 (type 2)	359.14	No hits/ molecular networking with parent	-
	450.15, 280.88	No hits/ no molecular networking	-
		***Additional hits**: 8-(1,2-dihydroxy-3-methylbut-3-enyl)-7-methoxychromen-2-one (278.09500);	related compound has antibiotic activity
SgM19 (type 1)	299.07	NF	-
	277.09	Cyclo(Tyr-Leu)/8-(1,2-dihydroxy-3-methylbut-3-enyl)-7-methoxychromen-2-one	Antifungal/ related compound has antibiotic activity
SgM20 (type 1)	326.37	No hits/molecular network with parent	-
SgM23 (type 6)	908.42	No hits/molecular network with parent	-
SgM31(type 4)	267.10	No hits/ molecular networking without parent	-
SgM33 (type 7)	365.26	No hits/ no molecular networking	-
	702.86	NF	-

NF: not found

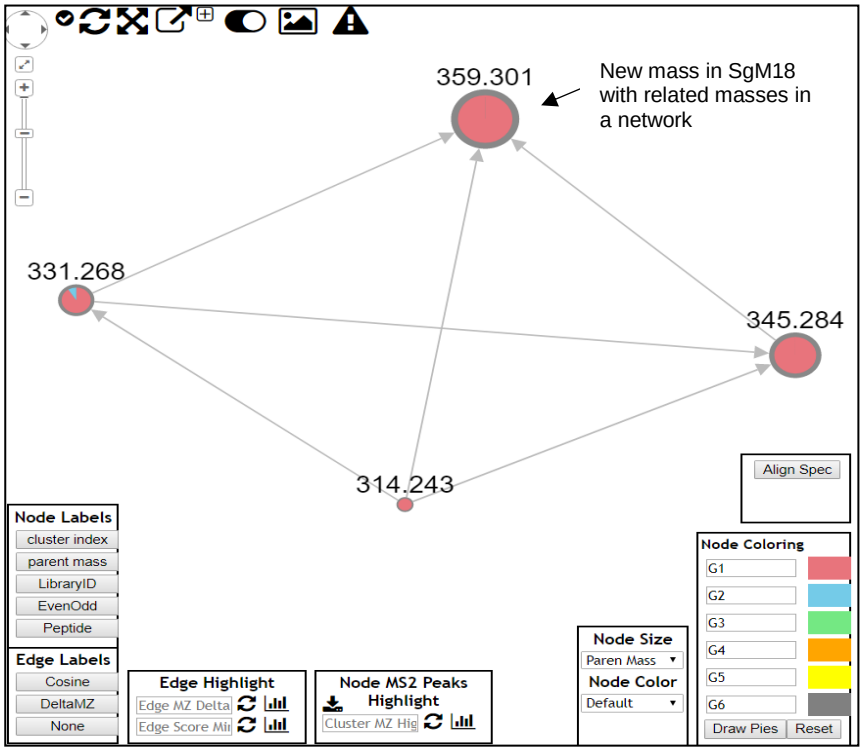


Figure 4.6 Molecular network for the new mass *m/z* 359 from SgM18 mutant. UV induced new mass of *m/z* 359 had no match to GNPS database but molecular networking found related chemical entities for this mass. The size of the node indicates the number of spectra in each precursor mass. G1: Sg mutant parent, G2: Sa parent, G3: Methanol blank 4, G4: ISP medium.

4.3.3.3 De-replication of SfMs

MS/MS data analysis from *S. ficellus* parent and 7 SfMs led to create 72 parent ions as node, both clustered in spectral family and as individual ions. The results are summarized in Table 4.6. None of the new active masses from SfMs returned matches to the database. A molecular network created for active mass *m/z* 441.30 linked to *m/z* 551.39 (Figure 4.7).

The GNPS database returned additional hits for drimentine F (*m/z* 504.36) in SfM27. This compound has been found in *Streptomyces* and has cytotoxic and antifungal activity (Netz and Opatz, 2015). However, this mass was not purified to test its activity because it was not detected as a new mass exclusively present in the SfMs and is likely to be cytotoxic.

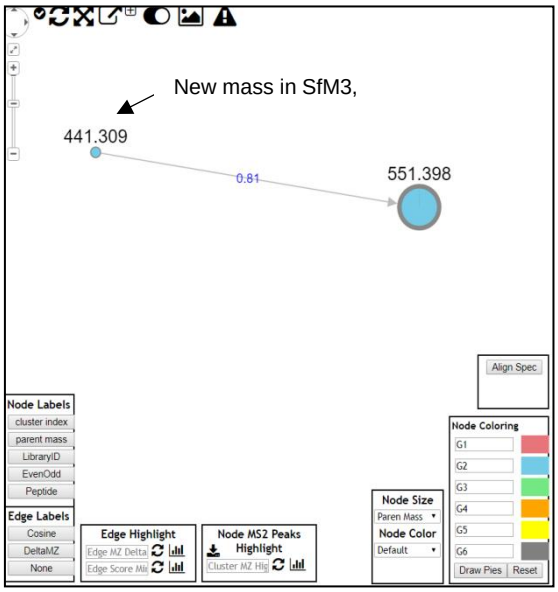


Figure 4.7 Molecular network for the new mass *m/z* 441.30 from SfMs. UV induced new mass of *m/z* 441.30 had no match to GNPS database but molecular networking found related chemical entities for this mass.. The size of the node indicates the number of spectra in each precursor mass. G1: Sf parent, G2: Sf mutants G3: ISP medium 4, G4: Methanol blank.

Table 4.6 De-replication of UV-induced mutants from *S. ficellus* using the GNPS database

Mutants/Type	Mass detected by metabolite tool from HRLC-MS data *m/z*	Hits/molecular network by GNPS	Known activity
SfM3 (Type 2)	126.05	NF	-
	394.31	No hits/ no molecular network	-
	661.52	No hits/ no molecular network	
	441.29	No hits/ molecular networking without parent	-
SfM4 (Type 4)	441.29	No hits/ molecular networking without parent	-
SfM5 (Type 2)	365.26, 396.32	No hits/ no molecular network	-
	407.27	NF	-
	681.51	No hits/ molecular networking with ISP4	-
SfM11 (Type 4)	407.27	No hits/ molecular networking with parent	-
SfM14 (Type 1)	441.29	No hits/ molecular networking without parent	-
SfM15 (Type 1)	288.92	NF	-
	441.29	No hits/ molecular networking	-
SfM27 (Type 3)	381.26	No hits/ no molecular network without parent	-
	154.08, 348.98	NF	-
	327.00	No hits/ molecular networking with parent	-
	441.29	No hits/ molecular networking without parent	-
		Additional hits: Drimentine F(504.36);	Antifungal, cytotoxic

NF: not found

4.4 Discussion

This study aimed to further evaluate UV mutagenesis as a new approach to access new antibacterial activity by activating cryptic BGCs in streptomycetes. Although UV irradiation demonstrated its potential as an activator of cryptic BGCs (in Chapter three), there were several outstanding questions to answer to establish UV mutagenesis approach as a platform for antibiotic discovery. The first question to resolve was whether UV mutagenesis allows the recovery of multiple new compounds from a given strain.

Results presented this chapter demonstrate the induction of multiple new masses/metabolites within a given strain. For instance, 19 new masses were detected from *S. antibioticus* mutants compared to its parent strain. Similarly, 18 and 11 new masses were detected from *S. griseoluteus* and *S. ficellus* mutants, respectively, compared to their respective parent strains. Although the distribution of some masses/metabolites (*m/z* 441.29 and 381.26) produced by SfMs were found to be identical in several mutants, SaMs and SgMs showed substantial diversity in their UV induced new masses. In some instances, individual UVMs produced more than two new metabolites. This data can be related to the OSMAC approach, where changing growth conditions or addition of chemicals induced production of six new compounds from a given strain of *S. flaveolus* (Qu *et al*., 2011). Bioassay with semi-purified compounds demonstrated that 7 out of 10 new masses were active against MG1699. Four of these active masses belongs to *S. griseoluteus* mutants and three of active masses belongs to *S. ficellus* mutants. These data suggested that UV mutagenesis is capable of inducing production of multiple antibiotics from a given strain. It is worth noting that UV induced changes in colony morphology and/or pigmentation were associated with different masses compared to UVMs that were morphologically dissimilar to their parent strain. For

instance, SaM9 and SaM3 presented different colony characteristics; SaM9 produces red orange to dark pink soluble pigments and SaM3 exhibited light pink colony. These morphological differences were associated with induction of different metabolites. Similarly, SgM11 and SgM12 produce dark purple to blue-black pigmented colonies, and were demonstrated to produce different masses/metabolites. From the literature, the common phenotypic changes in *Streptomyces* strains induced by genetic changes include variations in growth rate, colony size, shape and colour, lack of sporulation, and ability to produce pigments and/or antibiotics (Leblond and Decaris, 1994). Furthermore, they are known to have co-relation between colony morphology and antibiotic activity (Birch, Häusler and Hütter, 1990). The greatest variation was observed in SaMs regarding their spectrum of antimicrobial activity. SaM21, 37 and 40 exhibited selected antibacterial activity against clinical *A. baummannii* strain; whereas the parental strain and most of the other SaMs did not. Therefore, detection of multiple new masses, UV induced morphological changes and antimicrobial spectrum of activity in UVMs within a particular strain provides evidence that UV mutagenesis offers the recovery of UVMs with multiple antibiotics from a single *Streptomyces* strain.

The remaining question to address was whether all these ‘new’ antibiotics are structurally related to known compounds or are novel scaffolds. Although accurate masses or the predicted molecular formula of extracted compounds can be used to search databases of known natural products, this approach lacks de-replicating power because of the extensive number of potential isomers for a given mass/ elemental composition (Rojas-Chertó *et al.,* 2011). However, matching MS/MS fragmentation pattern similarity to the database offers a rapid and sensitive approach of de-replication. MS/MS data from selected UVMs were analysed and

searched for any match in the GNPS database. Except for SaM9, other new active masses from UVMs detected by Metabolite Tools were not found to match known compounds with in the database. Molecular networking, a data analysis platform offered by GNPS is useful complimentary analytical tool to reveal structural uniqueness and relatedness in large datasets. In this study, several new masses, for example, *m/z* 536 (SaM3), *m/z* 359 (SgM18), *m/z* 441 (SfM5/14) were grouped in their individual network with related chemical ions. These results could aid structural elucidation of these new masses. Although molecular networking develops cluster of similar compounds, sometimes it can group ions together having side chain similarities instead of core structure (for instance, similarities in alkyl side chains (Nguyen *et al.,* 2017), which allows to identify a part of a novel compound.

In this study comparative metabolomics permitted the detection of new chemical entities/metabolites in UV irradiated strains by comparing with their respective parent stains, and chemical de-replication revealed activation of silent BGCs that encode apparently novel antibiotics.

Chapter 5

Dimeric hydroxanthone with Gram-negative antibacterial activity from *Penicillium dipodomyicola*

5.1 Background to the Study

The main purpose of the PhD research was to access cryptic secondary metabolites in streptomycetes through UV mutagenesis approach. While working on *Streptomyces*, a fungal contaminant was found to be active against MDR *E. coli* MG1699. Later that fungal contaminant was tested for antifungal activity, and interestingly it showed a lack of activity against *C. albicans* 6. This was promising because there are very few clinically relevant antibacterial drugs from fungi so far (Albeti *et al.,* 2017). Among them only some semi-synthetic derivatives of β-lactams (penicillin and cephalosporin) are active against enterobacteriaceae (Susić, 2004). Another antibacterial compound pleuromutlin is active against some Gram-negative pathogens, but ineffective in *E. coli* (Brown and Dawson, 2015). The interesting fungal contaminant was further investigated, and bioassay results against enterobacteriaceae possessing different β-lactamases including ceftazidime-hydrolysing CTX-M-15 (Pitout *et al.,* 2005), AmpC like β-lactamase CMY-2 (Jacoby, 2009), and New Delhi metallo- β-lactamase (NDM-1) (Hammerum *et al*., 2010) indicted apparent absence of any β-lactam antibiotics. It was also found to be active against methicillin resistant *S. aureus* (MRSA) (Deresinski, 2005). Therefore, identification and structure elucidation of the antibacterial compound from this fungus was conducted alongside the unlocking of cryptic secondary metabolites in streptomycetes, and this is independent from the UV mutagenesis study.

The primary aim of this study was to characterize and elucidate the structure of the antibacterial compound from the fungal contaminant. Microscopy and 18s rRNA gene sequencing were employed to identify the fungus, first (done by Dr Richard Barton, Principal Clinical Scientist, Mycology Reference Centre, LGI, Leeds). Then solvent-solvent extraction was performed with ethyl acetate for large-scale extraction of secondary metabolites. Through LC-MS data analysis of the crude extract, target masses were chosen for further purification to assess their *in vitro* antibacterial potential. To achieve this, mass-directed HPLC was utilized and several masses were purified. Since LC-MS/MS offers a sensitive approach of chemical de-replication of natural products (Allard *et al*., 2016), this approach was employed to de-replicate the active metabolites in the HPLC purified fractions. NMR spectroscopy, X-ray crystallography, and Infrared spectroscopy (IR) were utilized to elucidate the structure of active compound(s) in collaboration with Dr. Chris Rayner, Department of Chemistry, University of Leeds. (all experiments and analysis done by Sannia Farooque). Purification of any targeted compound can be optimized by using different solvents depending on the polarity of the target compound (Eskilsson and Björklund, 2000). Therefore, significant effort went into optimization of extraction and purification of the active compound under guidance of Dr. Chris Rayner.

5.2 Results

5.2.1 Isolation and morphological characterization of the fungus

In the course of screening of UV mutants from *Streptomyces kanamyceticus* by agar overlay method for activity against MDR *E. coli* MG1699, an apparently airborne fungal contaminant was detected to possess antibacterial activity against MG1699. The fungus showed white to yellowish mycelia, with droplets of yellow exudates on its surface (Figure 5.1 A) and a brown colour on its underside (not shown). The fungal isolate was subsequently tested for its antifungal activity against *C. albicans* 6; interestingly it exhibited a lack of antifungal activity (Figure 5.1 B).

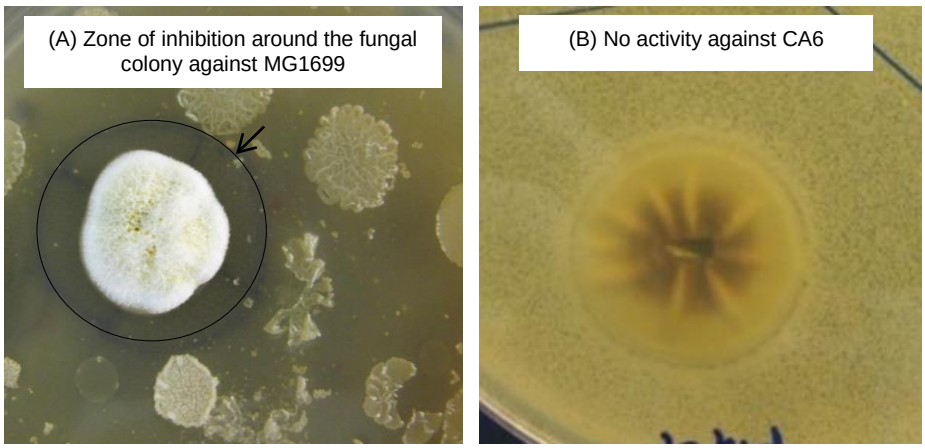


Figure 5.1 Antibacterial activity of fungal contaminant by agar overlay assay. (A) Detection of antimicrobial activity against MDR *E. coli* MG1699. (B) Lack of antifungal activity against *C. albicans* 6. The zone of inhibition was observed after 16-24 hours of incubation at 37°C.

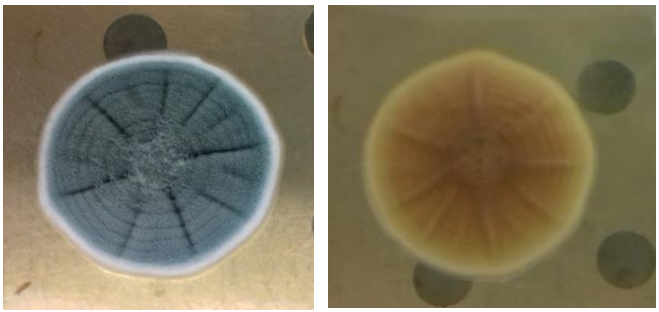


Figure 5.2 Cultural characteristics of the fungal isolate on Sabouraud dextrose (SD) agar medium after 3-5 days of incubation at 28°C. Left: aerial view, Right: reverse view.

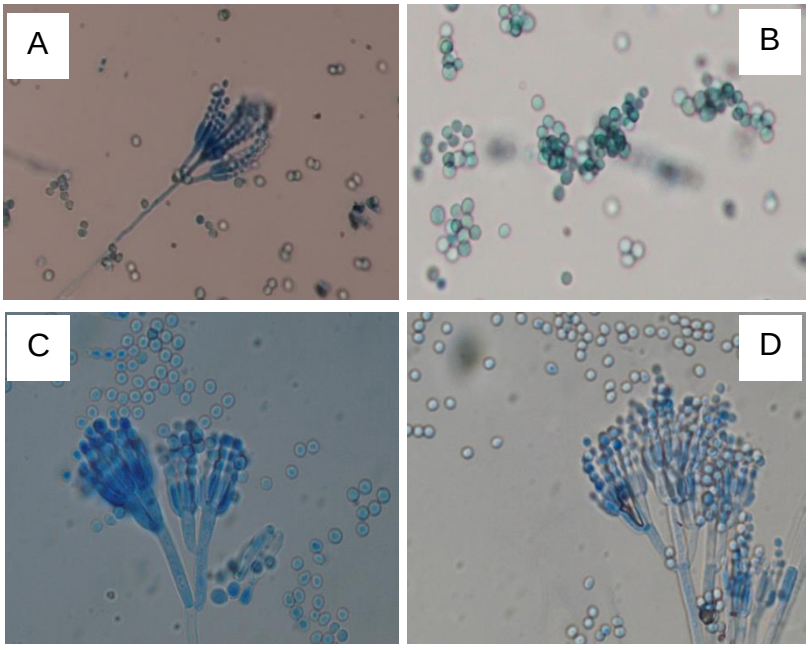


Figure 5.3 Microscopic observations of fungal isolates after growing on Sabouraud dextrose (SD) agar medium for 7 days. (A). Biverticilliate banching pattern (×400) under Phase-contrast microscopy. (B) Smoothed round conidia (×1000) under Phase-contrast microscopy. (C) Terverticillate (×400) branching pattern under light microscope. (D) Biverticillate (×1000) branching pattern under light microscope. Light microscopy was performed by Dr Richard Barton, LGI.

The fungal contaminant was later subcultured on Sabouraud Dextrose (SD) agar for observing its cultural characteristics. After 3-5 days of incubation at 28°C, the following colony characteristics were observed: velvety and dark grey green conidium, brown on the reverse, and absence of diffusible pigments and exudates (Figure 5.2). The microscopic observation of fungal isolate by Phase-contrast (Figure 5.3 A-B) and light microscope (Figure 5.3 C-D) showed biverticillate and terverticillate branching pattern, cylindrical phialides with a distinct neck and chains of smooth walled conidia. The light microscopy and primary identification of the isolate as *Penicillium* spp. were conducted by Dr Richard Barton (Principal Clinical Scientist, Mycology Reference Centre, LGI, Leeds).

5.2.2 Identification of *Penicillium* spp.

Genomic identification by 18s rRNA gene sequencing was also conducted by R. Barton by applying panfungal primers to amplify a region of the rRNA genes and spacers (Heidemann *et al*., 2010). After BLAST analysis, 99.8% similarity was found for *P. dipodomyicola* with a score of 829/831. However, 99.5% similarity with a score of 827/831 was matched for *P. griseofulvum*. Both species are closely related and they represent unique species without very close relationships with other species (Frisvad and Samson, 2004). However, both species are distinct and can be distinguished by the branching patterns of their conidiophores (a specialized hyphal branch of fungi that produces conidia).

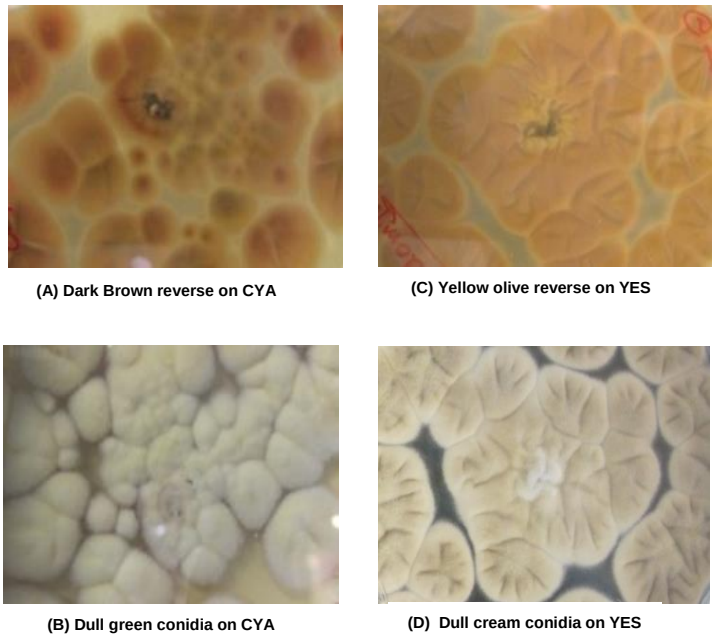


Figure 5.4 Colony characteristics of *Penicillium* spp. on CYA (A, B) and YES agar (C, D) medium. Observation was made after incubation at 28° C for 7 days.

P. dipodomyicola produce predominantly bi- to rarely ter-verticillate structures while *P. griseofulvum* has ter- to quarter-verticillate structures (Samson *et al*., 2004). Also on CYA agar *P. dipodomyicola* produces dull green conidia and a darker brown reverse than *P. griseofulvum* (Samson *et al*., 2004). Since genomic identification showed a high degree of relatedness to *P. griseofulvum*, macroscopic observation was performed to distinguish between them. It exhibited dull green conidium colour and dark brown reverse on CYA (Figure 5.4 A-B), and yellow olive reverse on YES medium was observed (Figure 5.4 C-D). This analysis confirmed the fungal isolate as *P. dipodomyicola*. Although this fungal species was first isolated from cheek pouches of kangaroo rats (*Dipodomys spectabilis*) (Frisvad *et al*., 1987), it was later also found in other habitats such as mounds, chicken feeds, and soil under sagebush (Frisvad and Samson, 2004); stem of the mangrove plant (Li *et al*., 2014) and even in human gut (Gouba *et. al*., 2013).

5.2.3 Antimicrobial spectrum of *P. dipodomyicola*

In an agar overlay assay, *P. dipodomyicola* exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria (Table 5.1). This species is closely related to *P. griseofulvum*, which is known to produce penicillin (Frisvad and Samson, 2004). However, bioassay against β-lactamase possessing microorganisms still showed the inhibition of growth, i.e. the absence of penicillin. It was active against Gram-negative *E. coli* irrespective of the presence of β-lactamases such as ceftazidime-hydrolysing CX-M-15, ampC like β-lactamase CMY-2, and New Delhi metallo- β-lactamase (NDM-1). It was also active against Gram-positive laboratory *S. aureus* (susceptible) and methicillin resistant clinical *S. aureus* MRSA 252.

Table 5.1 Spectrum of activity of *P. dipodomyicola* against a range of indicator microorganisms by agar overlay assay.

Pathogen	***P. dipodomyicola***
E. coli MG1699	+
E. coli BW2511	+
E. coli (CTX-M-15, CMY-2, NDM-1)	+
S.aureus SH1000	+
S. aureus MRSA 252	+
P. aeruginosa PAO1	-
K. pneumoniae (NDM-1)	+
A. baumannii (NDM-1)	-
E. cloacae (CTX-M-15, ACT/MIR, NDM-1, OXA-48)	-
Candida albicans C-6	-

(+) Activity (zone of inhibition); (-) No Activity (no zone of inhibition)

5.2.4 *In vitro* susceptibility and LC-MS analysis of crude extract

Broth culture of *P. dipodomyicola* appeared yellow after 7-10 days incubation at 28°C (Figure 5.5 A). After extraction with ethyl acetate, the crude extract was partially dissolved in methanol. Because of this solubility issue, different solvents were used to dissolve the extract (Figure 5.5 B). All these fractions were tested against a range of indicator organisms (data not shown); however, only the methanol dissolved brown fraction showed the specific antibacterial activity against Gram-positive and Gram-negative bacteria (i.e. no antifungal activity was observed) (Table 5.2). The yellow fraction dissolved in ethyl acetate and the white fraction dissolved in acetone also exhibited antibacterial activity against Gram-positive bacteria (data not shown); however, the aim of this work was to identify the Gram-negative antibacterial activity observed against MG1699, therefore only the brown fraction was taken forward for further investigation.

The LC-MS (positive mode) analysis of the brown crude fraction showed a cluster of three major peaks (639, 657 and 675) along with some other minor peaks (Appendix III Figure 1-2). Ethyl acetate dissolved yellow crude showed the presence of *m/z* 639 (Appendix III Figure 3-4). None of these major compounds (*m/z* 639, 657, 675) matched to any of metabolites reported from different strains of *P. dipodomyicola* (Appendix III Table 1). However, the extracted ion chromatogram showed the masses corresponding to cyclopiazonic acid (*m/z* 337-339), patulin (*m/z* 155-157) and 2, 4-dihydroxy-5-methylacetiphenone (*m/z* 210-211).

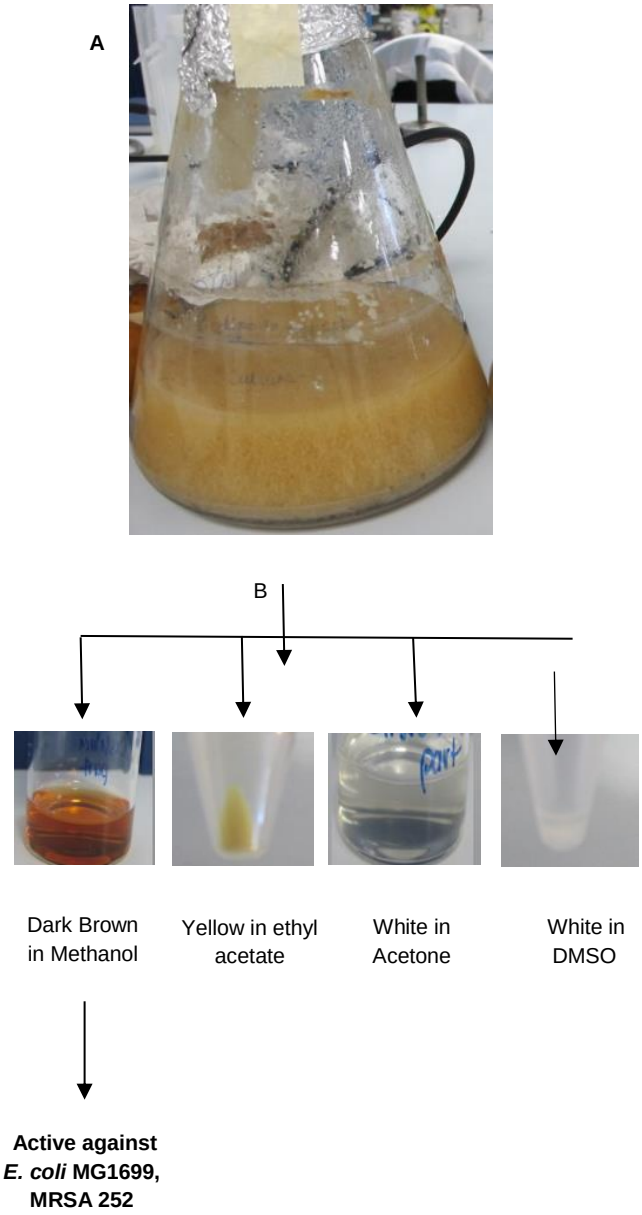


Figure 5.5 Growth and extraction of *P. dipodomyicola*. (A) Yellow pigments in GYM broth after 10 days incubation at 28°C (B) extracted crude in different solvents.

Table 5.2 Spectrum of activity of *P. dipodomyicola* methanol dissolved crude extract against a range of indicator microorganisms.

Pathogen	Crude extract in Methanol
E. coli MG1699	+
E. coli BW2511	+
E. coli E. coli (CTX-M-15, CMY-2, NDM-1)	+
S.aureus SH1000	+
S. aureus MRSA 252	+
P. aeruginosa PAO1	+/-
K. pneumoniae (NDM-1)	+
A. baumannii (NDM-1)	-
E. cloacae (CTX-M-15, ACT/MIR, NDM-1, OXA-48)	-
Candida albicans C-6	-

(+) Antibacterial activity; (-) no antibacterial activity; (+/-) variable

5.2.5 HPLC semi- purification and MS/MS based partial de-replication

Mass-directed, bioactivity-guided fractionation of the brown crude extract was performed to purify three major peaks (*m/z* 639, 657 and 675), and three minor peaks corresponding to molecular mass of cyclopiazonic acid, patulin and 2, 4-dihydroxy-5-methylacetiphenone. After HPLC purification, fractions were concentrated and dissolved in methanol; and bioassays were performed to test their activity (Table 5.3). Semi-purified putative new compounds (*m/z* 639, 657 and 675) exhibited specific antibacterial activity against MG1699. Among others *m/z*639 was

considered as a potential candidate for further investigation based on their broad spectrum antibacterial activity.

Table 5.3 Spectrum of activity of HPLC purified masses against a range of indicator microorganisms.

Purified Mass $(M+H)^+$	**MG1699**	**PAO1**	**MRSA 252**	***K. pneumoniae***	***A. baumannii***	**CA6**
640	+	+*	+	+*	+/-	-
658	+	+*	+	-	+/-	-
676	+	+*	+	-	-	-
156	-	ND	+	ND	ND	ND
212	+*	ND	+	ND	ND	ND
336	+*	ND	+*	ND	ND	ND

(+) antibacreial activity; (-) no antibacterial activity; * low activity; ND not determined due to low yield. MG1699: *E. coli* MG1699, PAO1: *P. aeruginosa* POA1, MRSA 252: methicillin resistant *S. aureus* 252, CA6: *C. albicans* 6

To achieve partial chemical de-replication, LC-MS/MS data of purified compound 640 $(M+H)^+$ was analysed by Global Natural Product Social Molecular Networking (GNPS) (Wang *et al.*, 2016) and this gave hits for a compound assigned as 130088 (MW 639.17) from Cichewicz fungi collection. 130088 is a dimeric tetrahydroxanthones (Bräse *et al.,* 2009) and structurally similar to Blennolide I. Blennolide I is the heterodimers of monotetrahydroxanthones or hemisecalonic acids (El-Ellimat *et al*., 2014). Thus 130088 is related to secalonic acid, however, sacalonic acids are homodimers (Wezeman *et al*., 2015).

A molecular network was created for 130088 related compounds where *m/z* 657 and 675 were linked to 130088 (Appendix III Figure 5). Although no bioassay information about 130088 was found, closely match Blennolide I was reported to have a MIC of > 70 µg/ml in Gram-positive, Gram negative and yeasts (El-Elimat *et al*., 2015). The MIC of this semi-purified compound 640 $(M+H)^+$ from *P. dipodomyicola* was determined which resulted 0.5 µg/ml and 1.0 µg/ml against *E. coli* MG1699 and *S. aureus* SH1000, respectively (not shown here). Another structurally close compound secalonic acid (SAD) reported to have activity mostly against Gram-positive bacteria (Andersen *et al*., 1977). Repeated purification and MS/MS analysis found 130008 and SAD as a match, but compounds were not identical i.e. cosine value was in the range of compound from 0.78 to 0.88. The semi-purified fraction of 640 $(M+H)^+$ was sent to the department of Chemistry for structural analysis (work done by Sannia Farooque, School of Chemistry, University of Leeds).

The 1-D (^1H, ^{13}C) and 2D NMR revealed a low concentration of SAD in the purified material but there were no conclusive results for the active compound. However, IR spectroscopy showed some functional groups that did not match SAD. After consulting with Prof Chris Rayner (Chemistry, University of Leeds), a sequential extraction of fungal metabolites was performed with diethyl ether and ethyl acetate. From this crude extract (yellow and not dissolved in methanol) SAD (**1**) was purified and characterized by 1-D and 2-D NMR spectroscopy (Figure 5.6), X-ray crystallography, and high-resolution mass spectrometry. *In vitro* susceptibility test was conducted by purified SAD but it was neither active against MG1699 nor

SH1000. From the results, it was apparent that SAD was not the compound responsible for the antibacterial activity. The brown fraction dissolved in methanol was further purified by normal phase preparative thin layer chromatography (TLC) and four fractions of yellow to brown coloured were obtained. Unfortunately, none of them were active against MG1699.

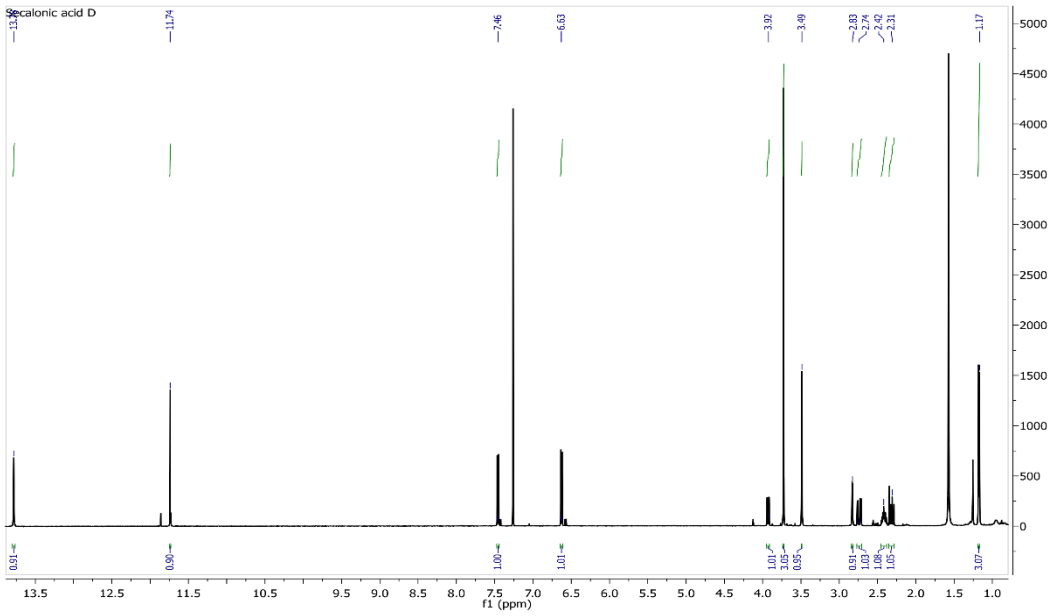


Figure 5.6 NMR H^1 for Selalonic acid (SAD).

5.2.6 Optimized purification and structure elucidation

This time extraction of metabolites was performed sequentially with cyclohexane, diethyl ether and ethyl acetate to optimize the purification of the active compound. The diethyl ether fraction showed the expected specific activity against *E*. *coli* MG1699 (Figure 5.7). The active diethyl ether fraction was also subjected to MS/MS experiment and data was analysed. Several hits with different molecular weight, along with the previously identified hits were observed by database searching. However, no new compound assigned for *m/z* 639 or related masses.

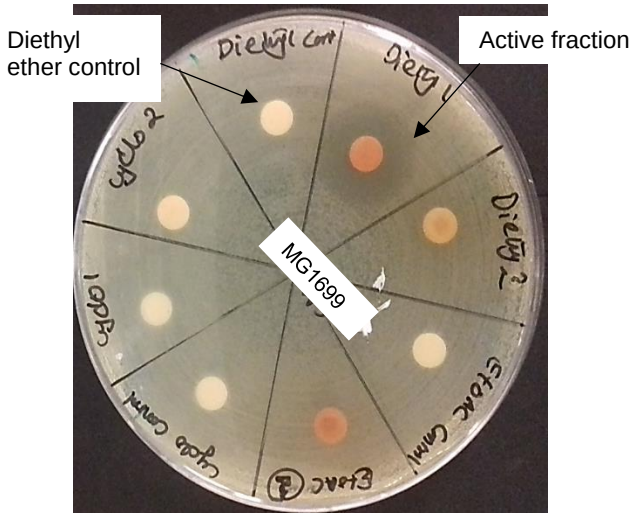


Figure 5.7 Antibacterial activity of diethyl ether fraction against MDR *E. coli* MG1699 by disc diffusion assay. Zone of inhibition was observed after 18-20 hours of incubation at 37°C. Diethyl ether control was also performed which showed no activity.

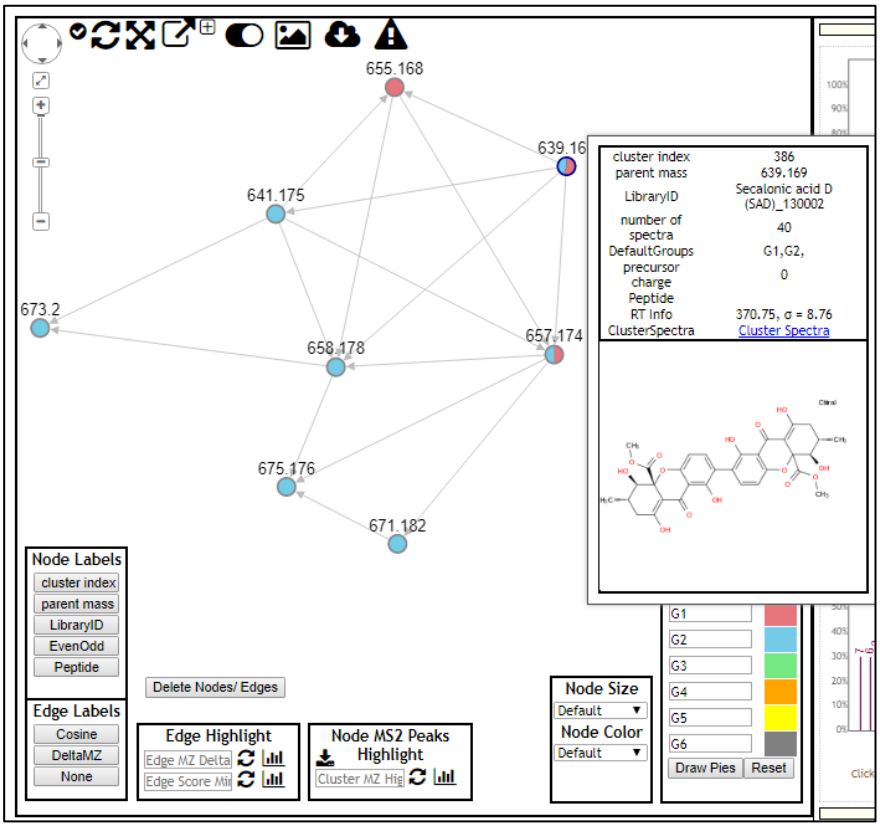


Figure 5.8 The molecular networking for Secalonic acid D (SAD). This shows the link between SAD and other two analogues (*m/z* 657 and 675). This network was created from the active diethyl ether fraction denoted as red (G1) and semi-purified 639 (active) denoted as blue (G2). Although the purification was done for *m/z* 639, other two analogues were also present.

All this information was provided to the Chemistry collaborator to aid the identification process. Since, secalonic acid D is ionised easily, it was detected in the MS/MS fractions analysed by GNPS even if present in very low concentration. GNPS created a molecular network of SAD related compounds (Figure 5.8) when compared with active diethyl ether crude and purified 639 fractions, but no conclusive results for *m/z* 335 obtained. It indicated initially the breakdown of dimer into monomeric form.

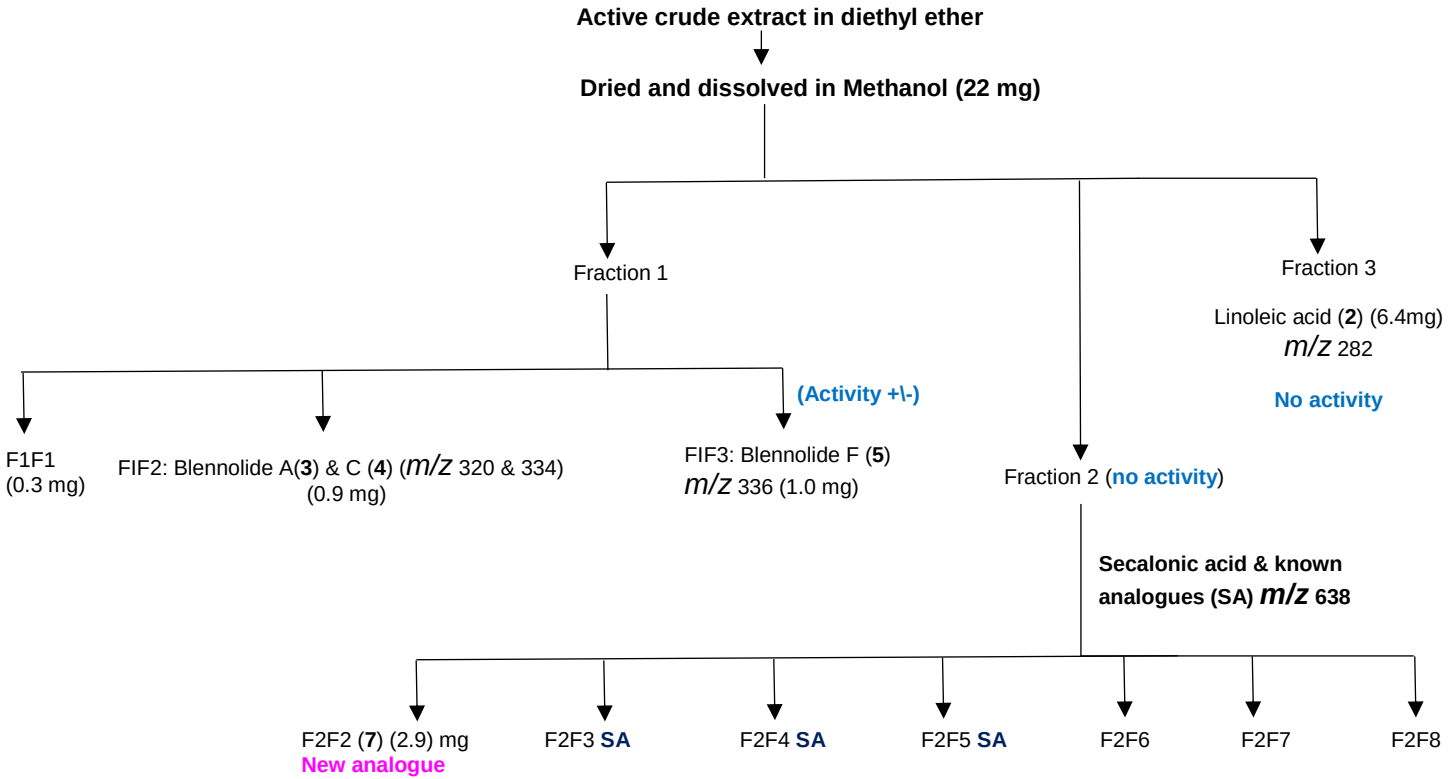
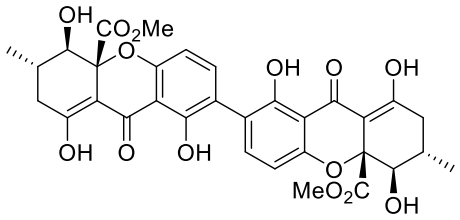


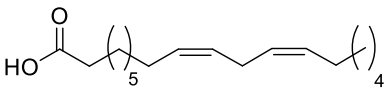
Figure 5.9 UV-based HPLC fractionation of active crude extract and the bioactivity of these fractions obtained from *P. dipodomyicola* dissolved in methanol. *m/z* values were generated by HRESIMS.

UV-based preparative reverse-phase HPLC was performed by Sannia to purify all compounds present in this diethyl ether crude. Total three fractions were collected, and two of these fractions were further divided into several fractions. Bioassay of all these purified fractions are summarised in the Figure 5.9. **F3** (6.4 mg) was identified to be linoleic acid (**2)**. **F1** was further fractioned and these sub-fractions were tentatively assigned as blennolides A (**3)** (0.5 mg), C (**4)** (0.4 mg) and F (**5)** (1.0 mg) based on ^{1}H NMR spectroscopy, accurate mass spectrometry and the literature data. The fraction **FIF3** (**5**) exhibited very low activity (tiny zone of inhibition) against MG1699. Unfortunately, due to small amount of this fraction, the bioassay could not be repeated.

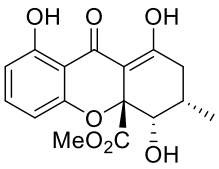
F2 was also further fractioned to afford eight sub-fractions. F2F2 (yellow gum, 2.9 mg) was characterised using ^{1}H NMR and mass spectroscopy; *m/z* of 656 suggests there is presence of an extra hydroxyl group in position 8 (Figure 5.10). Furthermore, there was a characteristic peak at 4.60 ppm in the ^{1}H NMR spectrum which is indicative of presence of an ester. There were four sets of aromatic signals which confirm hetero-nature of the two halves. Therefore compound **7** was tentatively assigned to be a novel isomer of SAD/blennolide. NMR spectrometry is shown in Figure 5.11. However further work is required to establish the connectivity and stereochemistry of the molecule. In general, the sample sizes were too small to perform complete analysis and determine the absolute stereochemistry of these compounds. Further work needs to be done in order to fully elucidate the structures.



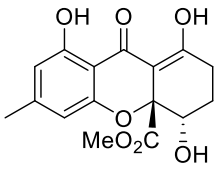
Secalonic Acid D **1**



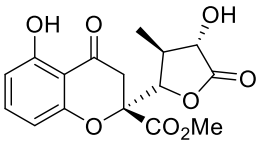
Linoleic acid **2**



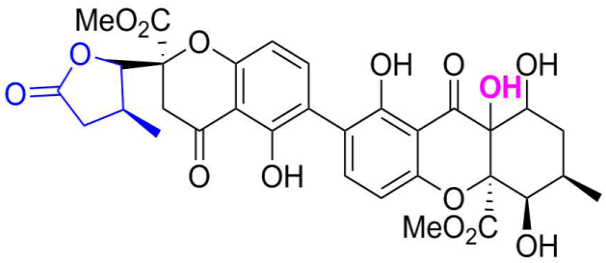
Blennolide A **3**



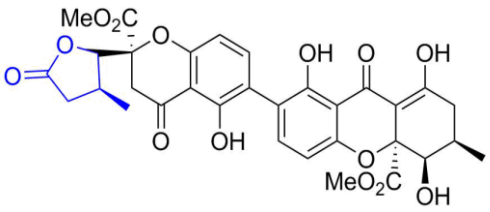
Blennolide C **4**



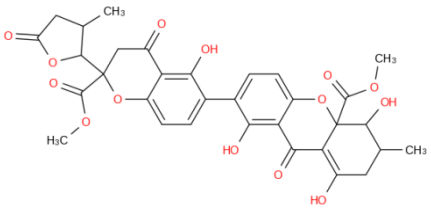
Blennolide F **5**



New analogue **7**
Exact Mass: 656.17



Blennolide I
Exact Mass: 638.16



130088
Exact Mass: 638.164

Figure 5.10 Compounds identified from the HPLC purified fraction of the active crude extracts of *P. dipodomyicola.* Blennolide I and Compound 130088 (MS/MS database match) are also listed to show the similarity with the new analogue.

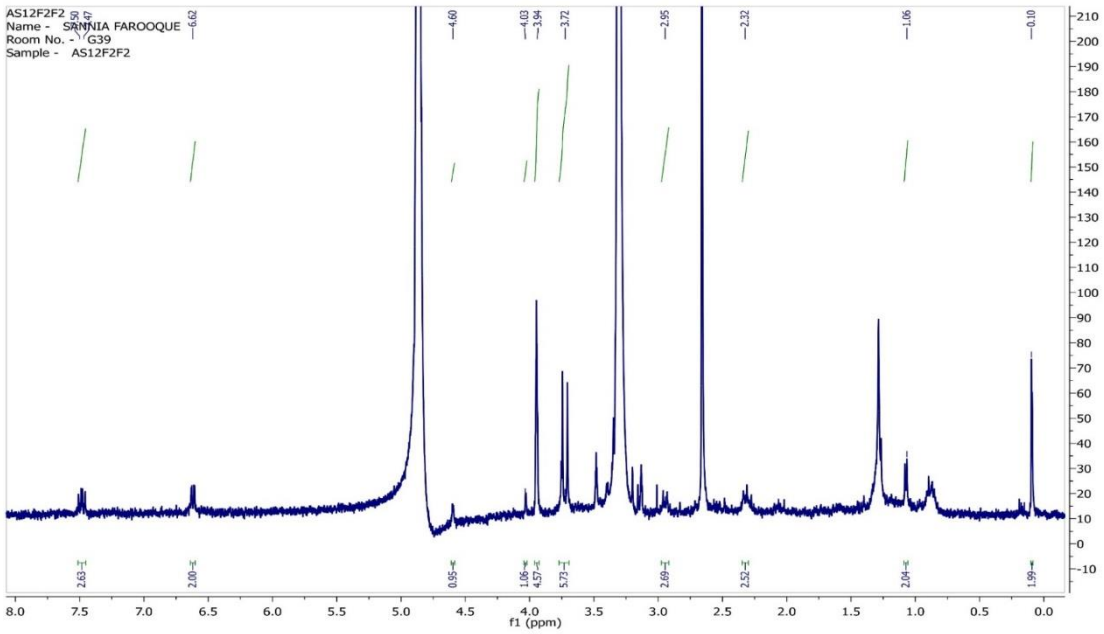


Figure 5.11 H^1 NMR spectroscopy for compound **7. Sample size was too small for COSY.**

5.3 Discussion

The present study demonstrates new antibacterial potential of *Penicillium dipodomyicola* against multidrug resistant *Escherichia coli.* This fungal strain also exhibits strong antibacterial activity to Gram-positive *Staphylococcus aureus* (susceptible strain) and methicillin-resistant *S. aureus* (MRSA). Chemical analysis has revealed the structure of active compounds is related to the members of ergochrome and xanthone family, more specifically to hemisecalonic acids (monomer) and secalonic acids (dimer).

Both ergochrome and xanthones are pigmented polyketides (Wezeman *et al*., 2015). Fungal ergochromes are mainly mycotoxins presenting either red, red orange or pale yellow coloured appearance. Xanthones are yellow pigmented compounds and diverse in their chemical structures in monomeric, dimeric and tetrameric forms (Wezeman and Masters, 2015). They also have diversity in their biological activity such as antibacterial (Stewart *et al*., 2004; Shim et al., 2011), antifungal (Chutrakul *et al*., 2009), antitumor (Wang *et al*., 2010), etc. The discovery of novel and new xanthone analogues (moneric and dimeric) are reported regularly with biological activity. However, most of them are cytotoxic (Lee *et al.,* 2010) and none of these reported compounds exhibit specific Gram-negative antibacterial activity. These are mostly active against Gram-positive, except a few compounds. For instances, two hemisecalonic acids, blennolides A and B (monomeric) exhibited antibacterial activity against *E. coli* as well as antifungal and antialgal activity (Zhang *et al.*, 2008). In another example, crude fraction of *Talaromyces stipititus* GgCr22.1b exhibited Gram-negative antibacterial activity against *E. coli* but none of the characterized compounds has Gram-negative activity (da Silva *et al.,* 2017). However, xanthones are also reported to exhibit activity against MRSA, for example γ-mangostin has shown antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) at MICs ~12.5 µg/ml (Dharmaratne, 2013).

Initial semi-purification of Gram-negative active fraction [*m/z* 640$(M+H)^+$] from *P. dipodomyicola* shows close structural relation to blenolides and secalonic acid D (SAD). SAD has teratogenic and cytotoxic activity along with Gram-positive antibacterial activity (Mislivec and Tuite, 1970). However, SAD from marine *Penicillium* spp. SCSGAF is the only exception that has moderate activity against *E. coli* JVC1228 and *B. subtilis* (MIC 24.4 µg/ml for both). Although the molecular mass

of the purified compound is in the same range of SAD (639.17), MS/MS fragmentation analysis of the compound reveals the presence of other masses (*m/z* 657.17, 671.182 and 675.176) which indicate the possibility of synergistic effects from structural analogues of SAD. These masses mostly present together which makes their separation difficult; nevertheless semi-purified fractions [*m/z* 658$(M+H)^+$ and 676 $(M+H)^+$] also exhibited activity against MG1699. The purification and characterization of SAD from crude extract, and subsequent bioassay excluded the role of SAD for the antibacterial activity; instead, closely related analogues were the source of the antibacterial activity.

It was speculated that *m/z* 335/336 corresponded to the mass of cyclopiazonic acid. However, blennolide A and F possess similar molecular weight (*m/z* 334-336). Low activity of *m/z* 335 (mentioned in the Table 5.5) might be due to the blennolide F, nevertheless, these results need to be confirmed by bioassay with purified blennolide F because no antibacterial activity has been reported from this compound yet. Also, initial semi-purified *m/z* 639 (now identified as SAD **1**) exhibited activity, it could be either due to the presence of *m/z* 335 (blennolides) or the presence of small amount of two other analogous (*m/z* 657 and 675). Unfortunately, this time *m/z* 657 (**7**) did not exhibit any activity; absence of bioactivity could be related to the solubility issue of this fraction (was not dissolved in the methanol, partially dissolved in chloroform-ethanol (2:1). Due to low yield of the metabolites, experiments could not be repeated. This study, therefore, reports first time the specific antibacterial activity of monomer and dimeric members of secalonic acid family. NMR and HRESIMS data supported the isolation of new analogues from *P. dipodomyicola*.

Although dimeric xanthones are reported frequently, most of the compounds are Gram-positive specific, this study revealed the potential of secalonic acid analogue as specific antibacterial Gram-negative bacteria. This finding is the similar to other studies; new mono and dimeric members (blennolides A-G) of secalonic family along with known secalonic acid B was discovered from *Blennoria* sp. (Zhang *et al*., 2008). Paecillin D, a yellow needles crystal was discovered from endophytic *Talaromyces* along with secalonic acid A, blennolide G and other known compounds (da Silva *et al.*, 2017). Versixanthones A–F (xanthone–chromanone) dimers from the marine-derived Fungus *Aspergillus versicolor* HDN1009 were discovered along with SAD (Wu *et al.*, 2017). Sixteen polyketides belonging to diverse structural classes, including monomeric/dimeric tetrahydroxanthones were isolated from fungal culture *Setophoma terrestris* (MSX45109) using bioactivity-directed fractionation (El-Elimat *et al.,* 2015). Most of these new analogous exhibits new activity compared to known secalonic acids.

To conclude, the study presented here contributes to the knowledge of dimeric xanthones with novel Gram-negative antibacterial activity. This research highlights on the re-exploration of fungal NPs for discovering novel activity. Fungi have a privilege of producing rich and complex chemical scaffolds because of their eukaryotic origin (Kumar and Rzhetsky, 1996), large size of their genomes (Mohanta and Bae, 2015), ubiquitous presence in terrestrial and marine environments (Moore and Frazer, 2002), and diverse biochemical evolution (Wisecaver *et al*., 2014). Since, there is a paucity of Gram-negative antibacterial compound in the discovery pipeline, fungal NPs can be the source of novel chemistry that would deliver potential anti Gram-negative candidates. Due to time constriction the re-investigation of purified analogues is not presented here.

6. General discussion and future direction

6.1 General discussion

The antibiotic resistance crisis is driving the need for novel antibacterial agents to fight multi-drug resistant (MDR) bacterial infection. Antibiotic discovery strategies employed by large pharmaceutical companies typically focus on modification of previous antibiotic scaffolds and target-based antibacterial development, approaches which have failed to tackle the resistance problem. Re-exploration of natural products with innovative strategies could increase the number of potential antibiotic drug candidates in the clinical pipeline. The work described in this thesis was aimed to validate and establish UV mutagenesis as a new platform approach for unlocking cryptic antibiotic biosynthetic gene clusters (BGCs) from streptomycetes.

Streptomyces, the largest genus of Actinobacteria, is the major source of clinically used natural product antibiotics (Genilloud, 2017). As mentioned in Chapter 1, whole genome sequencing of model species *S. coelicolor* (Bentley *et al*., 2002) revealed the presence of more than 20 BGCs, which encode enzymes to produce secondary metabolites. Until then only four-five such metabolites were known to be produced from this species. The presence of additional putative BGCs that remain silent in typical laboratory conditions indicated the hidden treasure of natural products. Indeed, subsequent studies identified new secondary metabolites from these silent BGCs (Pawlik et al., 2007; Yang *et al*., 2009; Gottelt et al., 2010; Schäberle *et al*., 2014). Although different approaches have been employed to activate these cryptic

BGCs, none of the approaches has addressed the urgent need for discovering antibiotics, especially for MDR Gram-negative pathogens. Furthermore, the challenges associated with activation of these BGCs remain significant, as large-scale and comprehensive activation of cryptic BGCs demands establishment of a novel approach for antibiotic discovery.

This thesis contains the first report of employing UV radiation as a new approach to activate cryptic biosynthesis pathways with evidence of new secondary metabolite production from streptomycetes. The work presented in Chapter Three has made a significant contribution to the knowledge of activating cryptic BGCs from streptomycetes; it evaluates the potential of UV mutagenesis to induce the expression of new antimicrobial activity against Gram-negative bacteria. To validate the claim of inducing new antibacterial activity, Chapter Four examines evidence generated by comparative metabolomics and analytical chemistry. This study therefore presents a novel platform (Figure 6.1) of a UV mutagenesis approach to discover new secondary metabolites from a given strain without prior genomic knowledge. It is hoped that this approach can be employed across different species of *Streptomyces* and would be useful to discover novel antibiotics from this genus.

The regulation of antibiotic production in streptomycetes involves multiple factors (both intra and extracellular) and regulatory cascades that together create a complex network (Bibb, 1996). This network of regulation is controlled at two main levels. At the lower level, pathway-specific regulators can modulate the antibiotic biosynthetic genes of a particular cluster (Takano *et al.*, 1992; Arias *et al*., 1999; Ryding *et al.,* 2002). Often the onset of production of more than one antibiotics is coordinately controlled by pleiotropic or global regulatory genes that constitutive the upper level regulation of antibiotic biosynthesis pathway (Chakraburtty and Bibb

1997, Elliot *et al*., 1998; Mascher *et al*., 2006; Som *et al*., 2017). In this study, two strains *S. kanamyceticus* and *S. griseoluteus* have shown the highest increases in mutation frequencies to streptomycin resistance after UV irradiation, which reflects highest percentage of new activity against *E. coli* MG1699 in their respective UV-induced mutants (UVMs). Except *S. kanamyceticus*, *S. antibioticus*, and *S. griseoluteus*, no genomic sequence is available for other tested strains. AntiSMASH analysis showed the presence of 84, 26 and 35 BGCs in *S. kanamyceticus* NRRL B-2535 (Labeda, 2016), *S. antibioticus* DSM 40234 (Ju *et al*., 2015) and *S. griseoluteus* NRRL ISP-5360 (Ju *et al.,* 2015), respectively. Among 84 clusters in *S. kanamyceticus*, 43 are annotated as putative. This implies that the UV mutagenesis might have been activated more than one BGCs with in *S. kanamyceticus*.

Furthermore, chemical de-replication of a new mass from SgM11 (mutant from *S. griseoluteus*) shows chemical relatedness with daidzein and genistein. Both of these compounds are new to its respective *S. griseoluteus* parent strain but soil streptomycetes are known to produce daidzein and genistein (Matseliukh *et al.,* 2005). Interestingly, Matseliukh *et al.* (2005) showed that *Streptomyces globisporus* 1912 derivative mutant defective in biosynthesis of antibiotic restored the synthesis of landomycin E by addition of genistein to the medium. It was also demonstrated that genistein increased the production of polyketide antibiotics landomycines A and E, tetracenomycin and chlortetracycline by 11-42%. Hence, these two isoflavones might work as an inducer of global regulation of antibiotic production in SgMs. It can be speculated that UV has induced mutation to change in DNA sequence corresponding to a global regulator (and/or pathway-specific regulators) that consequently responsible for activating different chemical entities/metabolites.

On the contrary, low induction of Str^R MF in *S. antibioticus* (Sa) and S*. ficellus* (Sf) does not reflect their ability to produce new antimicrobial activity. In this study, UVMs from *S. antibioticus* have exhibited diverse antimicrobial activity; some of these SaMs have even shown specific Gram-negative antibacterial activity against *P. aeruginosa* and *A. baumannii.*

In addition, this thesis has made a contribution to the discovery of new fungal analogue exhibiting Gram-negative antibacterial activity. This study is the first report of discovering a dimeric xanthone which possesses antibacterial activity against Gram-negative MDR pathogens. it has been shown that even closely related compounds from different fungal species can exhibit novel antimicrobial activity. Activity against MDR *E. coli* MG1699 and other clinical Gram-negative pathogens has signified the outcome of this research. Due to time constriction, the complete characterization and mode of action of the active antibacterial compounds remain outstanding.

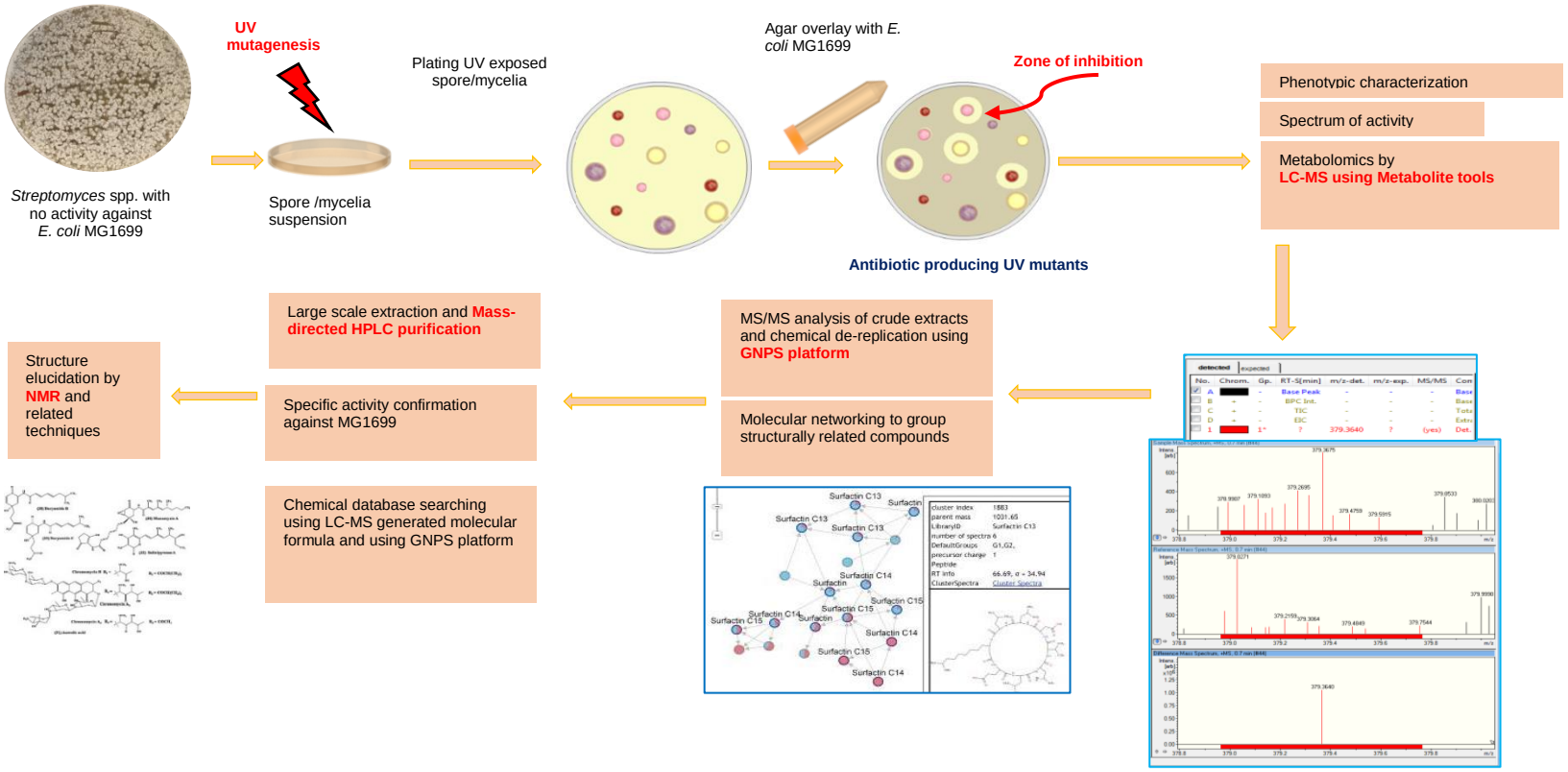


Figure 6.1 UV mutagenesis approach combined with metabolomics to access novel antibiotics from ordinarily silent BGCs in *Streptomyces* species.

6.2 Concluding remarks and Future direction

It is hoped that this study has contributed to the area of natural product discovery from cryptic BGC activation. Future work should focus on the identification and complete characterization of new antibacterial metabolites from the UVMs of *Streptomyces* strains. As development of novel antibiotics requires extensive pre-clinical evaluation before its entry into the prolonged and costly clinical trials, purified compounds should be thoroughly investigated for their antimicrobial spectrum of activity, mode of action study, and *in vivo* cell cytotoxicity in animal model (O'Neill and Chopra 2004; Silver, 2011). All these studies will provide a preliminary evaluation of the therapeutic potential of a novel antibacterial.

It is expected that UV mutagenesis approach would give the motivation of discovering new chemistry from the existing collection of streptomycetes or easily accessible source of streptomycetes; thus reduce the time to find novel chemical scaffold with desired antimicrobial activity. To make this as high-throughput approach, it will require automatic screening by robotics and miniaturization of the microbial metabolite extraction. The key labour-intensive steps include picking individual mutants, preparation and dispensing sterile culture media, inoculation of mutants into new media, assays from broth/agar media and reconfirmation of their bioactivity (if required).

The results presented in this thesis show that UVMs exhibited with different colony characteristics are likely to produce different secondary metabolites, thus screening of distinct changes in colony morphology post UV-irradiation appears to be a viable

strategy for selecting UVMs that likely produce different antibiotics. Although the experienced eye of a microbiologist can detect this altered morphology easily; it is again labour-intensive and a low throughput approach. Instead robotics with the capability of sensing specific changes, for instance, changes in the colony colour will speed up the screening process. Furthermore, automated screening for antimicrobial activity against a range of indicator microorganisms will give the opportunity to select UVMs exhibiting narrow spectrum or broad-spectrum bioactivity. It will give an extra advantage to screen with biosensors to detect the primary mode of action at the same time from the whole cell assay. Such biosensors can detect antimicrobial agent with a specific mode of action, for example, biosensor coupled with fluorescence system would allow visual detection of cell wall/protein synthesis inhibitor, and can categorized mutants according to their initial mode of action (Silver, 2011).

Extraction of metabolites and performing LC-MS can also be automated to match the throughput of the screening stage. MEDINA (Granada) already has semi-automated strategies for the bioassay-guided purification of natural products from microbial extract. Another alternative could be the ‘micrometabolomics platform’ developed by Barkal *et. al.* (2016) to adopt large-scale extraction and LC-MS metabolomics analysis in an automated integrated system. This microscale workflow allows for arrayed inoculation and on-chip metabolite extraction without the need for processing of broth culture or homogenization of agar. Another advantage of using this platform is the use of ~1000× less solvent, consequently cutting down on evaporation time and faster preparing of sample for LC-MS analysis.

Metabolite detection software used in this research has shown its efficiency and reproducibility to detect new metabolites from LC-MS data. Since it can detect traces of ions in complex mixtures, it can be employed in a miniaturized platform. Additionally, automated data acquisition from LC-MS in a batch processing manner and comparative metabolomics will accelerate the detection of new ions/metabolites in selected UVMs.

Whilst pharmaceutical industries are employing robotics and miniaturized platform for high-throughput screens (HTS) to identify pharmacologically active lead antibiotic compounds by testing hundreds of thousands of compounds in highly automated assays, these HTS approaches are beyond the reach of most academic researchers due to its high costs and labour requirements. Thus, collaborative and open discovery platform with knowledge sharing will accelerate the discovery of new antibiotics from natural products.

In addition, the study described in Chapter five has provided significance of exploring fungal natural products to discover novel and new analogues of known compounds with Gram-negative antibacterial activity. Although further work is required to establish the connectivity and stereochemistry of the molecule, primary *in vitro* susceptibility assay has shown promising results. However, the first thing to resolve will be the separation of the SAD analogues and purify them individually for *in vitro* antibacterial activity against MG1699. The pre-clinical evaluation should be performed to investigate if the compound is a membrane damaging agent by BacLight^{TM} assay (Nass, 2017), and macromolecular synthesis should be performed to reveal the mode of action of the compound (O'Neill and Chopra, 2004). Since SAD is a mycotoxin and teratogen, analogues of SAD should process through *in*

vitro human cell cytotoxicity assay (Nass, 2017) and *in vivo Galleria mellonella* (wax moth larvae) killing assay (Nass *et al*., 2017). Furthermore, a recent study has revealed the potential of SAD in eliminating *S. aureus* biofilm and synergistic bactericidal effects with vancomycin in *S. aureus* (Wang *et al*., 2017). Biofilm formation is particularly problematic for medical implants. Pathogens can irreversibly attach to the surface of these devices to form biofilm and cause persistent infections (Shrestha *et al.,* 2016). It would be useful to evaluate new analogue's antibiofilm activity as well.

Eight unusual heterodimeric tetrahydroxanthone derivatives, lentulins A−H reported from *Aspergillus lentulus* fungal with antibacterial activities (Li *et al*., 2016). The most active compound lentulin B had a hydroxyl group at position C-8 (8–OH) compare to the other lentulins; and it was speculated that 8-OH might be important for their antibacterial activities. Since the new SAD analogue has an –OH moiety in 8a position, further investigation should be conducted to determine the effect of hydroxyl ions on their antimicrobial activity.

The discovery of antibacterial from *P. dipodomyicola* points the urge for screening fungi to discover clinically relevant novel antibiotic candidate. It has been estimated that out of ~1 million different fungal species only ~100,000 have been described (Hawksworth and Rossman 1997). Hence, the vast group of fungi remains unexploited. Furthermore, like streptomycetes, the presence of silent BGCs offers fungi as an interesting source of new antibacterial compounds. And re-exploring fungal NPs could generate novel drug candidates to tackle this antibiotic resistance crisis, especially to fightback against Gram-negative clinical pathogens.

Appendices

Appendix I

Table 1 UV-induced mutation and survival studies in five *Streptomyces* species.

	S. griseoluteus					***S. kanamyceticus***			
UV µJ/cm^2	MF	Std ev	survival %	std dev	UV µJ/cm^2	MF	Std ev	survival %	std dev
0	1.24E-06	1.42E-07	100	0.00E+00	0	1.29E-07	8.19E-09	100	0.00E+00
10000	1.23E-05	2.52E-06	22.22333333	9.62E+00	10000	2.33E-05	1.04E-05	0.413	1.50E-01
30000	2.59E-05	1.85E-05	11.06	2.40E+00	30000	1.13E-03	1.25E-04	0.008716667	1.37E-03
60000	3.50E-04	0.00E+00	0.167	0.00E+00	60000	7.22E-03	2.55E-03	0.000315333	5.85E-05
100000	8.50E-04	1.32E-04	0.022133333	9.41E-03	100000	0.00E+00	0.00E+00	0.000206333	7.47E-05
200000	2.42E-03	1.42E-03	0.003126667	2.24E-03	200000	0.00E+00	0.00E+00	3.15333E-05	5.85E-06
300000	0.00E+00	0.00E+00	0.00265	1.42E-03	300000	0.00E+00	0.00E+00	2.43333E-05	1.07E-05
400000	0.00E+00	0.00E+00	0.000333333	1.67E-04	400000	0.00E+00	0.00E+00	0.000333333	1.67E-04

Table 1 UV-induced mutation and survival studies in five *Streptomyces* species. (**Continued)**

	S. antibioticus					***S. ficellus***			
UV µJ/cm^2	MF	Std ev	survival percentage	std dev	UV µJ/cm^2	MF	Std ev	survival percentage	std dev
0	1.35E-08	2.65448E-10	100	0.00E+00	0	7.71E-09	5.93241E-09	100	0.00E+00
10000	4.20E-08	2.09469E-09	32.21	1.56E+00	10000	1.92E-08	2.28968E-09	26	9.64E+00
30000	5.85E-08	2.25599E-08	12.95	2.71E+00	30000	2.59E-08	1.283E-08	12.77666667	2.55E+00
50000	1.29E-07	6.35321E-08	6.08	2.70E+00	60000	6.12E-08	3.36819E-08	6.666666667	2.89E+00
60000	6.02E-08	6.25E-10	11.27333333	1.33E-01	100000	0.00E+00	0	0.816666667	1.04E-01
100000	0.00E+00	0	20.95	0.00E+00	200000	0.00E+00	0	0.099	1.46E-01
200000	0.00E+00	0	4.32	0.00E+00	300000	0.00E+00	0	0.001556667	5.10E-04
300000	0.00E+00	0	0.45	0.00E+00	400000	0.00E+00	0	1.78E-04	6.93E-05

Table 1 UV-induced mutation and survival studies in five *Streptomyces* species. (**Continued)**

	S. ceolescens			
UV µJ/cm^2	MF	Std ev	survival percentage	std dev
0	4.83E-08	4.0104E-08	100	0.00E+00
10000	9.26E-08	4.98935E-08	40	3.00E+01
30000	3.50E-07	8.66025E-08	16.66666667	5.77E+00
60000	5.00E-07	0	1.666666667	5.77E-01
100000	0	0	1.166666667	2.89E-01
200000	0	0	0.016666667	5.77E-03
300000	0	0	0.00235	6.50E-04
400000	0	0	0.000266667	5.77E-05

Table 2 UV mutagenesis and survival studies in *Streptomyces kanamyceticus* with mycelial treatment.

UV $\mu J/cm^2$	MF	Std ev	Survival percentage	Std dev
0	6.35E-07	2.20552E-07	100	0
15000	1.88E-06	3.46987E-07	26.11666667	5.782061
20000	1.59E-06	1.67033E-07	36.71666667	13.18495
30000	2.15E-06	4.35086E-07	21.41666667	7.264477
40000	3.90E-06	1.39429E-06	13.71333333	3.68869
50000	1.00E-05	9.07544E-06	9.836666667	2.100508
60000	1.50E-05	1.10095E-05	8.783333333	2.650987
100000	1.54E-05	5.27686E-06	12.47333333	10.12976
200000	2.28E-05	1.65307E-05	15.81666667	12.3701
400000	2.80E-04	6.01041E-05	0.905	0.038184

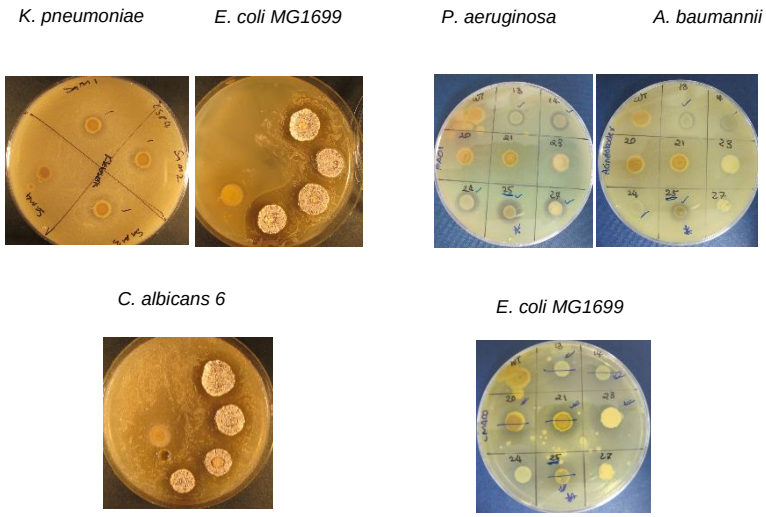


Figure 1 Antimicrobial activity of SaMs against range of indicator microorganisms**. (left: SaM3, right: SaM13,14,20,21,23,24,25 and 27)**

Appendix II

List of metabolites from *Streptomyces* species used in this study

Table 1 *S. antibioticus*

Metabolites	Biological Activity	MW
Actinomycin X (beta)	Anti-tumor, antibacterial	1271.41644
Clavam antibiotic	Anti-bacterial, anti-fungal and anti-tumor	329.352
Boromycin	Gram-positive, coccidiosstat	878.879
Chlorothicin	Macrolide, antibiotic, gram-positive	955.491
Cinerubin A	Gram-positive, virus, tumor	827.878
Cinerubin B	Gram-positive, virus, tumor	825.862
Cinerubin C	Gram-positive, virus, tumor	784.810
Esmeraldine A		518 / 652 (?)
Furanone	Against *Pseudomonas*	200.234, 186.207
Indanomycin	Gram-positive, growth promoter for ruminant	493.67742
Nosiheptide/ multhiomycin	Broad spectrum antibacterial, veterinary growth stimulator, gram-positive	1222.35702
Oleandomycin	Broad spectrum antibacterial, mainly gram-positive	687.85334
Rubromycin	Gram-positive,	536.4403
Azetomycin I, II	Antibacterial and anti-tumour	1241.405, 1227.379
Antimycin I, II, A3	Antifungal	549.2729 (M+H), 509.2 (M+H), 520.57204
Antibiotic Tu 1718	Antimicrobial properties	260.289
Sa AZ-Z710	Antifungal	351.20 (peaks at 355, 374 and 395)
ketomycin	Antibacterial, gram-positive mainly	154.1632
Oviedomycin	Anti-tumour	349.27056
Related to anisomycin	Antifungal	265.22

Table 2 *S. griseoluteus*

Metaolites	Biological activity	MW
Griseolutic acid	Antibiotic,antibacterial	284.26678
Griseolutin A (reddish yellow)	Antibiotic (gram+, gram -)	342.30286
Griseolutin B (light yellow)	Antibiotic,antibacterial	344.31874
U-77863/O-methyl-cinnamide	Anti tumor	161.20044
U-77864	Anti-metastatic	179.21572
Duramycin B	Lantibiotic , toxic to human	2000.727608
539A (Piperazinedione)	Antibiotic,antibacterial, anti tumor	349.25608

** the phenazine ring (C12H8N2, MW= 180.08).

Table 3 *S. ficellus*

Metabolites	Biological Activity	MW
Ficellomycin	Gram-positive, Gram-negative	**312.36806**
U-47, 929	Inhibitors of bacterial semi-conservative DNA replication	
Feldamycin	Gram-positive, Gram-negative Inhibitors of bacterial semi-conservative DNA replication	**407.42**
Nojirimycin	Glycosidase inhibitor	**179.17**

Table 4 Data shows the masses from *S. antibioticus* Mutant 9. The active fractions (each of peaks) and accurate mass

Fractions	Selected masses after considering adducts
Colourless	398.9278 592.1332
Light yellow	478.2250, 477.56 832.2435, 980.00 978.4253
Light pink	391.55 464.465, 855.00 613.3339
Dark pink	391.55, 374.52

** Highlighted masses were subjected to further mass-directed HPLC fractionation

Appendix III

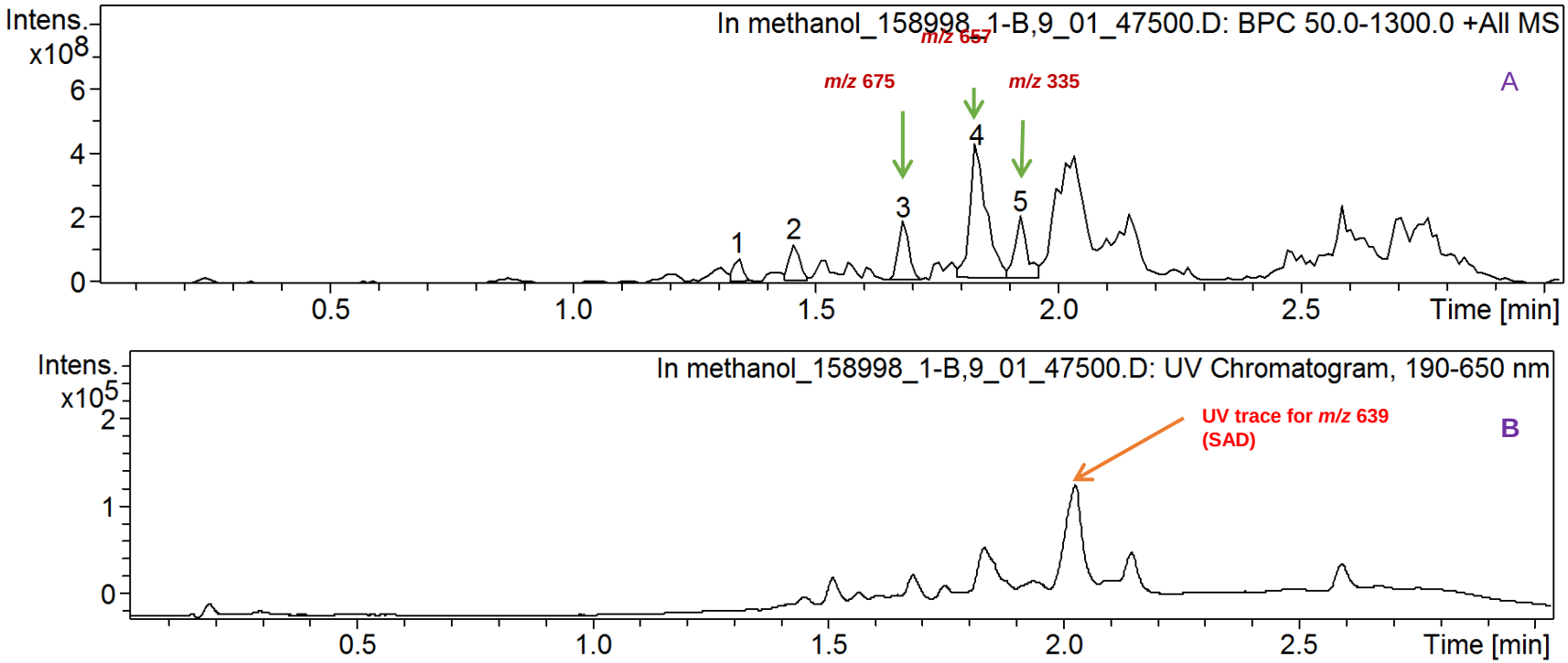


Figure 1 LC-MS analysis of methanol dissolved active) crude extract (against *E. coli* MG1699 from *P. dipodomyicola*. (A) all the peaks present in the crude;(B) UV trace for all the compounds, interestingly, UV trace for Secalonic acid D (SAD) was also present.

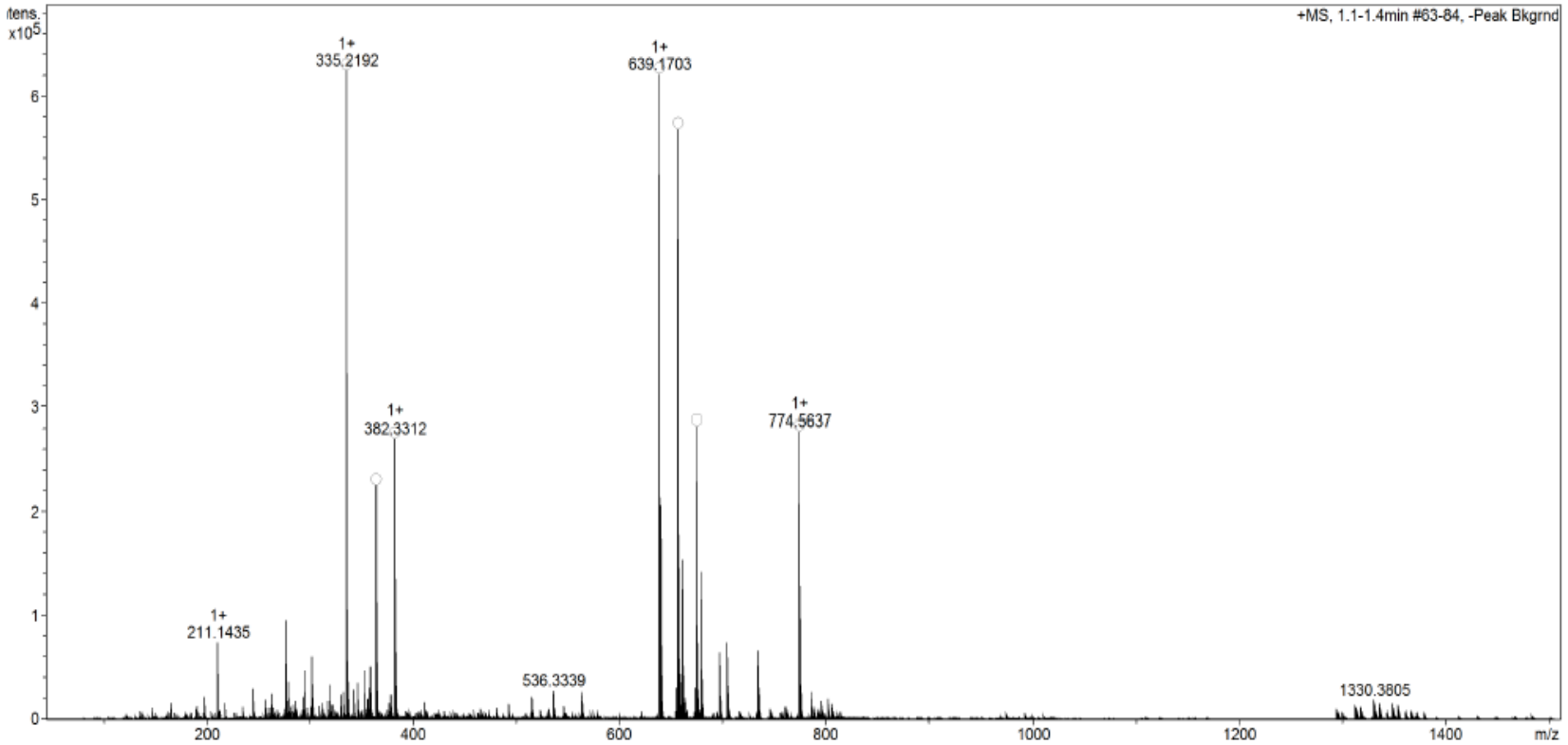


Figure 2 Accurate mass analysis of brown fraction dissolved in Methanol showing the presence of *m/z* 335, 639, 657, 675

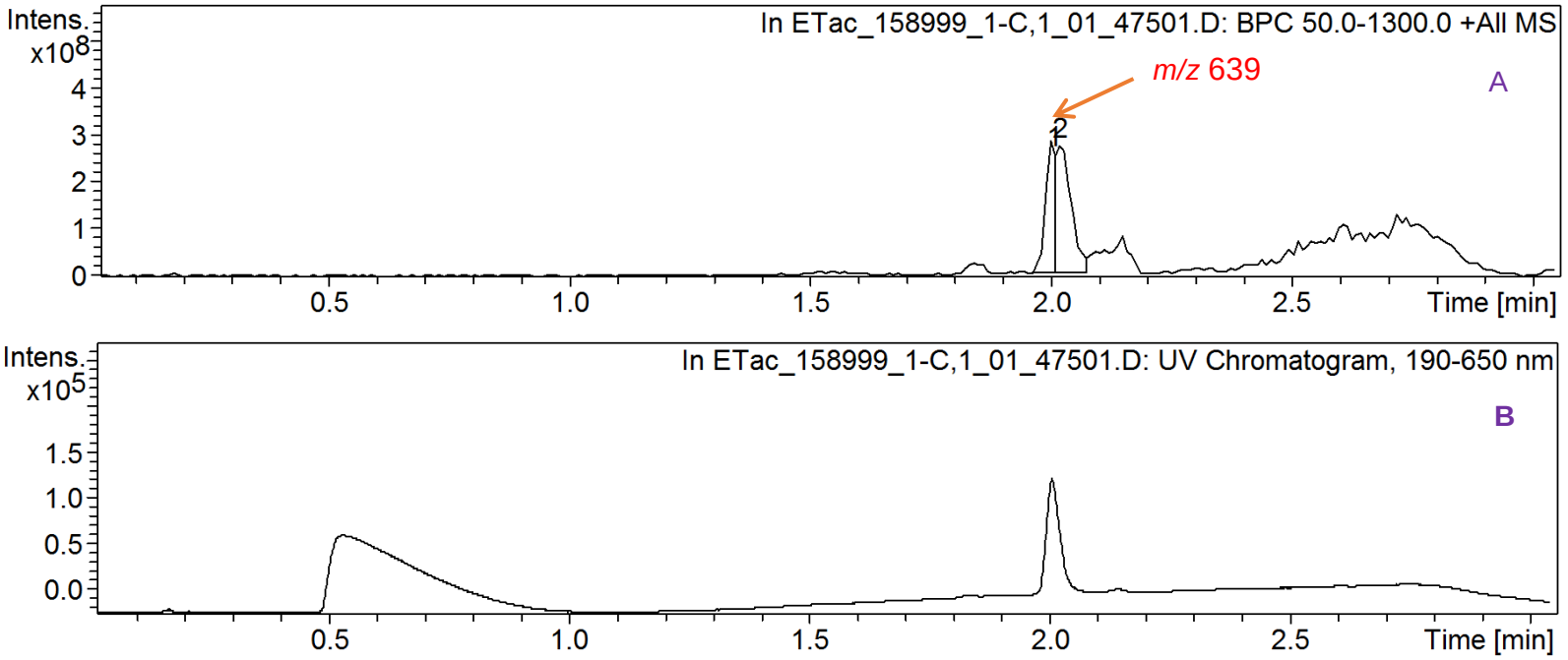


Figure 3 LC-MS analysis of ethyl acetate dissolved inactive (against *E. coli* MG1699) crude extract from *P. dipodomyicola*. (A) all the peaks present in the crude (B) UV trace for all the compounds.

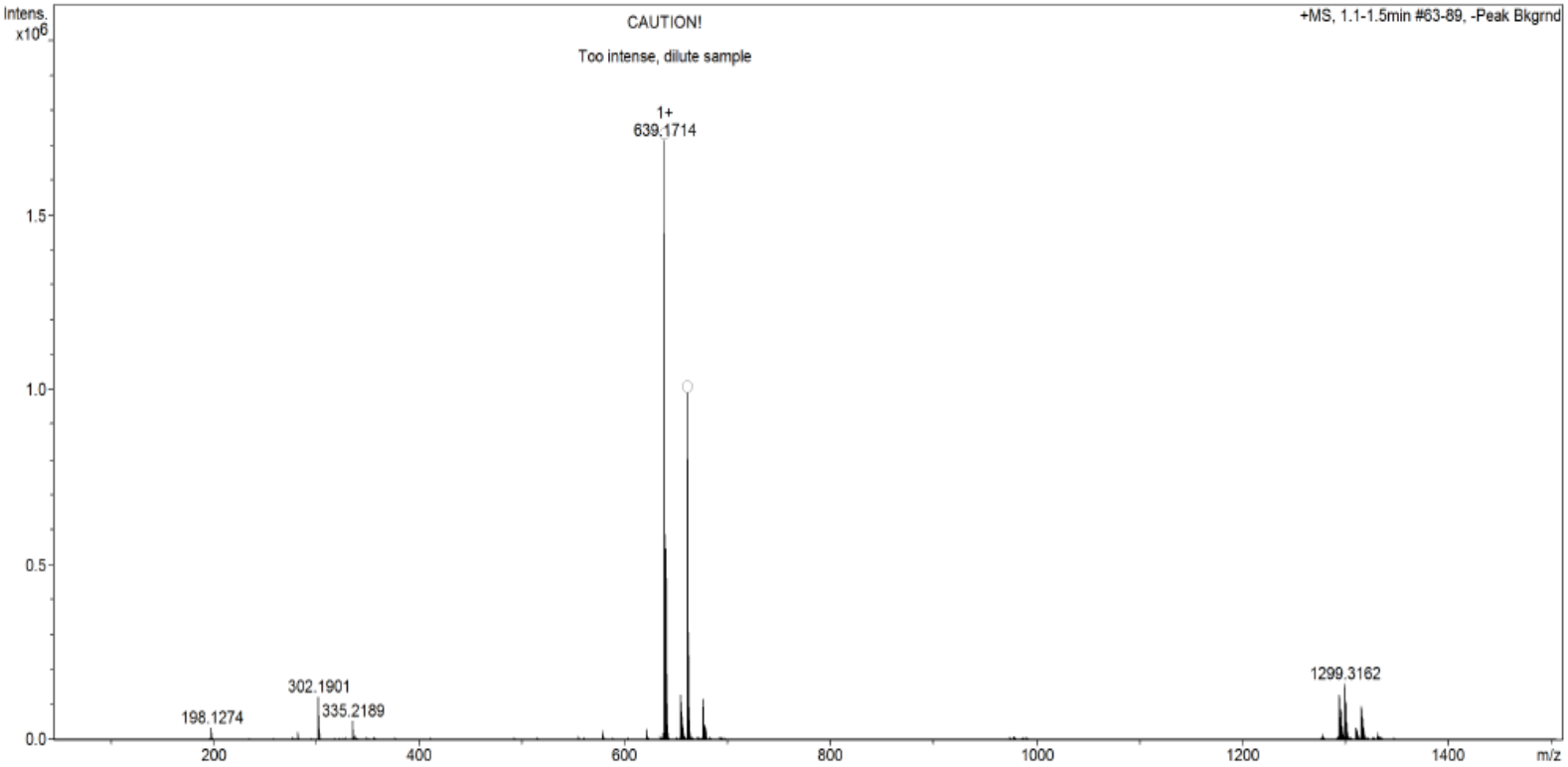


Figure 4 Accurate mass analysis of yellow fraction dissolved in ethyl acetate showing *m/z* 639 corresponding to SAD.

Table 1 List of metabolites from different strains of *Penicillium dipodomyicola*

Metabolites	MW	Colour/ appearance	UV nm for detection	Molecular formula
Peniphenone A	332.1621	Pale yellow crystals in EtOAc	231, 274	$C_{19}H_{24}O_5$
Peniphenone B	398.0996	Yellow crystals in acetone	285, 353	$C_{21}H_{18}O_8$
Peniphenone C	330.1098	White amorphous solid in MeOH	267, 231	$C_{18}H_{18}O_6$
Peniphenone D	292.0941	Colourless crystals in EtOAc	239, 292, 325	$C_{15}H_{16}O_6$
Cyclopiazonic acid	336.39	white to off-white ; Soluble in DMSO and ethanol	284	$C_{20}H_{20}N_2O_3$
Patulin	154.12	White; Soluble in ethyl acetate	276	$C_7H_6O_4$
Griseofulvin	352.766	-	291	$C_{17}H_{17}ClO_6$
Speradine B	423.1163	White amorphous powder in MeOH	-	$C_{20}H_{20}O_7N_2$
2,4-dihydroxy-3,5-dimethylacetophenone	180.20000			$C_{10}H_{12}O_3$
2,4-dihydroxy-5-methylacetophenone	210.22646			$C_{11}H_{14}O_4$

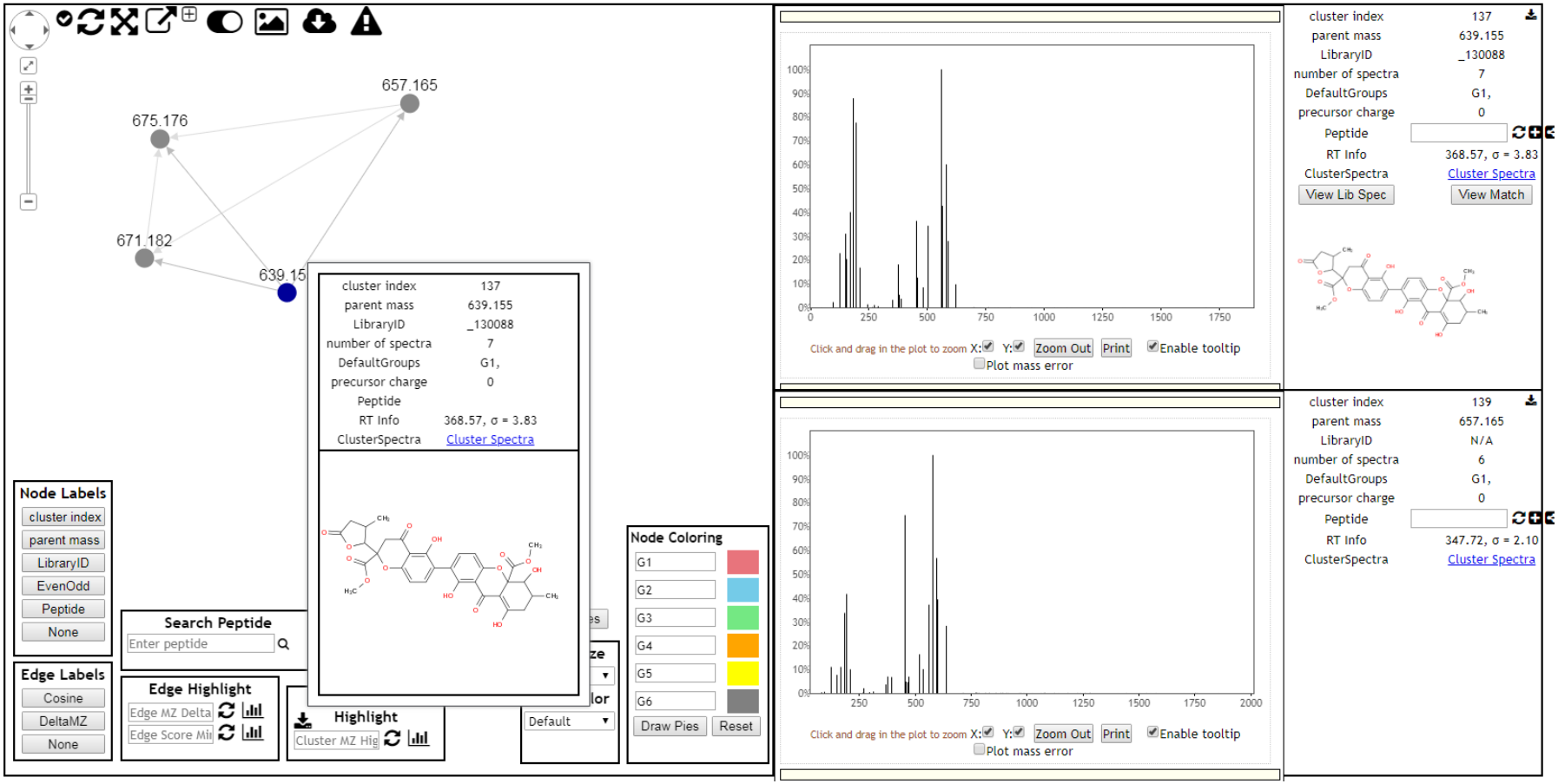


Figure 5 Molecular network of purified *m/z* 639 fungal compound (created by GNPS platform).

References

A-E

ADEDEJI, W.A., 2016. The Treasure Called Antibiotics. *Annals of Ibadan Postgraduate Medicine*, **14**(2), p.56.

ADNANI, N., CHEVRETTE, M.G., ADIBHATLA, S.N., ZHANG, F., YU, Q., BRAUN, D.R., NELSON, J., SIMPKINS, S.W., MCDONALD, B.R., MYERS, C.L. AND PIOTROWSKI, J.S. 2017. Coculture of Marine Invertebrate-Associated Bacteria and Interdisciplinary Technologies Enable Biosynthesis and Discovery of a New Antibiotic, Keyicin. *ACS Chemical Biology*, **12**(12), pp.3093-3102.

ALBERTI, F., FOSTER, G.D. AND BAILEY, A.M. 2017. Natural products from filamentous fungi and production by heterologous expression. *Applied Microbiology and Biotechnology*, **101**(2), pp.493-500.

ALBERTI, F., KHAIRUDIN, K., VENEGAS, E.R., DAVIES, J.A., HAYES, P.M., WILLIS, C.L., BAILEY, A.M. AND FOSTER, G.D., 2017. Heterologous expression reveals the biosynthesis of the antibiotic pleuromutilin and generates bioactive semi-synthetic derivatives. *Nature Communications*, **8**(1), p.1831.

ALLARD, P.M., PÉRESSE, T., BISSON, J., GINDRO, K., MARCOURT, L., PHAM, V.C., ROUSSI, F., LITAUDON, M. AND WOLFENDER, J.L. 2016. Integration of molecular networking and in-silico MS/MS fragmentation for natural products dereplication. *Analytical Chemistry*, **88**(6), pp.3317-3323.

AMINOV, R.I. 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in Microbiology*, **1,** p.134.

ANDERSEN, R., BUECHI, G., KOBBE, B. AND DEMAIN, A.L. 1977. Secalonic acids D and F are toxic metabolites of *Aspergillus aculeatus*. *The Journal of Organic Chemistry*, **42**(2), pp.352-353.

ARIAS, P., FERNÁNDEZ-MORENO, M.A. AND MALPARTIDA, F., 1999. Characterization of the pathway-specific positive transcriptional regulator for actinorhodin biosynthesis in *Streptomyces coelicolor* A3 (2) as a DNA-binding protein. *Journal of Bacteriology*, **181**(22), pp.6958-6968.

ARTSIMOVITCH, I., PATLAN, V., SEKINE, S.I., VASSYLYEVA, M.N., HOSAKA, T., OCHI, K., YOKOYAMA, S. AND VASSYLYEV, D.G. 2004. Structural basis for transcription regulation by alarmone ppGpp. *Cell*, **117**(3), pp.299-310.

ATLAS, R.M. 2010. Handbook of microbiological media. Boca Raton: CRC press.

ASOLKAR, R.N., KIRKLAND, T.N., JENSEN, P.R. AND FENICAL, W., 2010. Arenimycin, an antibiotic effective against rifampin-and methicillin-resistant Staphylococcus aureus from the marine actinomycete Salinispora arenicola. *The Journal of Antibiotics*, **63**(1), p.37.

AUERBACH, C. 2013. Mutation research: problems, results and perspectives. *Springer*.

AWAD, G., MATHIEU, F., COPPEL, Y. AND LEBRIHI, A. 2005. Characterization and regulation of new secondary metabolites from *Aspergillus ochraceus* M18 obtained by UV mutagenesis. *Canadian Journal of Microbiology*, **51(**1), pp.59-67.

BABA, T., T. ARA, M. HASEGAWA, Y. TAKAI, Y. OKUMURA, M. BABA, K. A. DATSENKO, M. TOMITA, B. L. WANNER and H. MORI. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology,* **2,** pp.1-11.

BADER, J., MAST-GERLACH, E., POPOVIĆ, M.K., BAJPAI, R. AND STAHL, U. 2010. Relevance of microbial coculture fermentations in biotechnology. *Journal of Applied Microbiology*, **109**(2), pp.371-387.

BALTZ, R.H., 1978. Genetic recombination in *Streptomyces fradiae* by protoplast fusion and cell regeneration. *Microbiology*, **107**(1), pp.93-102.

BALTZ, R.H. AND STONESIFER, J. 1985. Mutagenic and error-free DNA repair in *Streptomyces*. *Molecular and General Genetics*, **200**(3), pp.351-355.

BALTZ, R. H. 1986. Mutation in *Streptomyces*, p. 61-93. *In* S. W. Queener and L. E. Day (ed.), The bacteria, vol. 9. Antibiotic-producing *Streptomyces*. Academic Press, New York, NY.

BALTZ, R. H. 1999. Mutagenesis, p. 1819-1822. *In* M. C. Flickinger and S. W. Drew (ed.), Encyclopedia of bioprocess technology: fermentation, biocatalysis, and separation. Wiley, New York, NY.

BALTZ, R.H. 2006. Marcel Faber Roundtable: is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration? *Journal of Industrial Microbiology and Biotechnology*, **33**(7), pp.507-513.

BALTZ, R.H. 2007. Antimicrobials from actinomycetes: back to the future. *Microbe-American Society for Microbiology*, ***2***(3), p.125.

BALTZ, R.H. 2014. Spontaneous and induced mutations to rifampicin, streptomycin and spectinomycin resistances in actinomycetes: mutagenic mechanisms and applications for strain improvement. *The Journal of Antibiotics*, ***67***(9), pp.619-624.

BANSKOTA, A.H., MCALPINE, J.B., SØRENSEN, D., IBRAHIM, A., AOUIDATE, M., PIRAEE, M., ALARCO, A.M., FARNET, C.M. AND ZAZOPOULOS, E. 2006. Genomic analyses lead to novel secondary metabolites. *The Journal of Antibiotics*, **59**(9), p.533.

BARKAL, L.J., THEBERGE, A.B., GUO, C.J., SPRAKER, J., RAPPERT, L., BERTHIER, J., BRAKKE, K.A., WANG, C.C., BEEBE, D.J., KELLER, N.P. AND BERTHIER, E., 2016. Microbial metabolomics in open microscale platforms. *Nature Communications*, **7**, p.10610.

BARONA-GÓMEZ, F., WONG, U., GIANNAKOPULOS, A.E., DERRICK, P.J. AND CHALLIS, G.L. 2004. Identification of a Cluster of Genes that Directs Desferrioxamine Biosynthesis in *Streptomyces coelicolor* M145. *Journal of the American Chemical Society*, **126**(50), pp.16282-16283.

BARTLETT, D.W., CLOUGH, J.M., GODWIN, J.R., HALL, A.A., HAMER, M. AND PARR-DOBRZANSKI, B. 2002. The strobilurin fungicides. *Pest Management Science*, **58**(7), pp.649-662.

BENTLEY, S.D., CHATER, K.F., CERDENO-TARRAGA, A.M., CHALLIS, G.L., THOMSON, N.R., JAMES, K.D., HARRIS, D.E., QUAIL, M.A., KIESER, H., HARPER, D. AND BATEMAN, A. 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3 (2). *Nature*, **417**(6885), p.141.

BENNETT JW, KLICH M. 2003. Mycotoxins. *Clinical Microbiology Reviews*, **16**(3),497-516.

BEUTLER, J.A., ALVARADO, A.B., SCHAUFELBERGER, D.E., ANDREWS, P. AND MCCLOUD, T.G. 1990. Dereplication of phorbol bioactives: Lyngbya majuscula and Croton cuneatus. *Journal of Natural Products*, **53**(4), pp.867-874.

BIBB, M., 1996. The regulation of antibiotic production in *Streptomyces coelicolor* A3 (2). *Microbiology*, **142**(6), pp.1335-1344.

BIBB, M.J. 2005. Regulation of secondary metabolism in streptomycetes. *Current Opinion in Microbiology*, **8**(2), pp.208-215.

BIEDENBACH, D.J., RHOMBERG, P.R., MENDES, R.E. AND JONES, R.N. 2010. Spectrum of activity, mutation rates, synergistic interactions, and the effects of pH and serum proteins for fusidic acid (CEM-102). *Diagnostic Microbiology and Infectious disease*, **66**(3), pp.301-307.

BIRCH, A., HÄUSLER, A. AND HÜTTER, R. 1990. Genome rearrangement and genetic instability in *Streptomyces* spp. *Journal of Bacteriology*, **172**(8), p.4138.

BITZER, J., KÖPCKE, B., STADLER, M., HELLWIG, V., JU, Y.M., SEIP, S. AND HENKEL, T. 2007. Accelerated dereplication of natural products, supported by reference libraries. *International Journal for Chemistry*, **61**(6), pp.332-338.

BLIN, K., MEDEMA, M.H., KOTTMANN, R., LEE, S.Y. AND WEBER, T., 2016. The antiSMASH database, a comprehensive database of microbial secondary metabolite biosynthetic gene clusters. *Nucleic Acids Research*, p.gkw960.

BLUMAUEROVA, M., POKORNÝ, V., ŠŤASTNÁ, J., HOŠŤÁLEK, Z. AND VANĚK, Z. 1978. Developmental mutants of *Streptomyces coeruleorubidus*, a producer of anthracyclines: Isolation and preliminary characterization. *Folia Microbiologica*, **23**(3), pp.177-182.

BODE, H.B., WALKER, M. AND ZEECK, A. 2000. Structure and Biosynthesis of Mutolide, a Novel Macrolide from a UV Mutant of the Fungus F-24′ 707. *European Journal of Organic Chemistry,* **8**, pp.1451-1456.

BOSE, U., HEWAVITHARANA, A.K., NG, Y.K., SHAW, P.N., FUERST, J.A. AND HODSON, M.P., 2015. LC-MS-Based metabolomics study of marine bacterial secondary metabolite and antibiotic production in *Salinispora arenicola*. *Marine Drugs*, **13**(1), pp.249-266.

BRÄSE, S., ENCINAS, A., KECK, J. AND NISING, C.F. 2009. Chemistry and biology of mycotoxins and related fungal metabolites. *Chemical Reviews,* **109**(9), pp.3903-3990.

BRADE, K. D., RYBAK, J. M., & RYBAK, M. J. 2016. Oritavancin: A New Lipoglycopeptide Antibiotic in the Treatment of Gram-Positive Infections. *Infectious Diseases and Therapy*, **5**(1), pp.1–15.

BRADFORD, P.A., 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews*, 14(4), pp.933-951.

BROWN, P. AND DAWSON, M.J. 2015. A perspective on the next generation of antibacterial agents derived by manipulation of natural products. *Progress in Medicinal Chemistry*, **54**, pp.135-184.

BROWN, E. D. and G. D. WRIGHT. 2016. Antibacterial drug discovery in the resistance era. *Nature,* **529**(7586), pp.336-343.

BUTLER, M.S. 2004. The role of natural product chemistry in drug discovery. *Journal of Natural Products*, **67**(12), pp.2141-2153.

BUTLER, M.S., M.A. BLASKOVICH, AND M.A. COOPER, *Antibiotics in the clinical pipeline in 2013.* The *Journal of Antibiotics* (*Tokyo*), 2013. **66**(10), p. 571-91.

BYCROFT, B.W. ED. 1987. Dictionary of antibiotics & related substances. CRC Press.

CAPONE, A., M. GIANNELLA, D. FORTINI, A. GIORDANO, M. MELEDANDRI, M. BALLARDINI, M. VENDITTI, E. BORDI, D. CAPOZZI, M. P. BALICE, A. TARASI, G. PARISI, A. LAPPA, A. CARATTOLI, N. PETROSILLO and S.-G. N. ON BEHALF OF THE. 2013. High rate of colistin resistance among patients with carbapenem-resistant *Klebsiella pneumoniae* infection accounts for an excess of mortality. *Clinical Microbiology and Infection,* **19**(1), pp.E23-E30.

CARR, G., POULSEN, M., KLASSEN, J.L., HOU, Y., WYCHE, T.P., BUGNI, T.S., CURRIE, C.R. AND CLARDY, J. 2012. Microtermolides A and B from termite-associated *Streptomyces* sp. and structural revision of vinylamycin. *Organic Letters*, **14**(11), pp.2822-2825.

CARDOSO-MARTÍNEZ, F., BECERRIL-ESPINOSA, A., BARRILA-ORTÍZ, C., TORRES-BELTRÁN, M., OCAMPO-ALVAREZ, H., IÑIGUEZ-MARTÍNEZ, A.M. AND SORIA-MERCADO, I.E., 2015. Antibacterial and cytotoxic bioactivity of marine Actinobacteria from Loreto Bay National Park, Mexico. *Hidrobiológica*, **25**(2), p.223.

CDC, Antibiotic resistance threats in the United States, 2013. http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf.[Accessed 20 May 2014]

CHAKRABARTY, S.L. AND NANDI, P. 1971. A new actinomycin-like antibiotic produced by a mutant strain of *Streptomyces indicus*. *Experientia,* **27**(5), pp.595-596.

CHAKRABURTTY, R. AND BIBB, M., 1997. The ppGpp synthetase gene (*rel*A) of Streptomyces coelicolor A3 (2) plays a conditional role in antibiotic production and morphological differentiation. *Journal of Bacteriology*, **179**(18), pp.5854-5861.

CHALLIS, G.L., 2008. Mining microbial genomes for new natural products and biosynthetic pathways. *Microbiology,* **154**(6), pp.1555-1569.

CHANDRASEKHAR, D. AND VAN HOUTEN, B. 2000. In vivo formation and repair of cyclobutane pyrimidine dimers and 6-4 photoproducts measured at the gene and nucleotide level in *Escherichia coli.* Mutation Research/Fundamental and *Molecular Mechanisms of Mutagenesis*, **450**(1), pp.19-40.

CHATER, K.F. AND CHANDRA, G. 2008. The use of the rare UUA codon to define “expression space” for genes involved in secondary metabolism, development and environmental adaptation in *Streptomyces*. *The Journal of Microbiology*, **46**(1), pp.1-11.

CHENG, Y.R., HUANG, J., QIANG, H., LIN, W.L. AND DEMAIN, A.L. 2001. Mutagenesis of the rapamycin producer *Streptomyces hygroscopicus* FC904. *The Journal of Antibiotics*, **54**(11), pp.967-972.

CHIANG, Y.M., CHANG, S.L., OAKLEY, B.R. AND WANG, C.C. 2011. Recent advances in awakening silent biosynthetic gene clusters and linking orphan clusters to natural products in microorganisms. *Current Opinion in Chemical Biology*, **15**(1), pp.137-143.

CHOPRA, I. 2013. The 2012 Garrod lecture: discovery of antibacterial drugs in the 21st century. *Journal of Antimicrobial agents and Chemotherapy,* **68**(3), pp.496-505.

CHOPRA I., HESSE L., O'NEILL A. 2002. Discovery and development of new anti-bacterial drugs in Pharmacochemistry Library, vol 32, *Trends in Drug Research III*, ed. van der Goot H., editor. (Amsterdam: Elsevier;), pp.213–225.

CHUTRAKUL, C., BOONRUANGPRAPA, T., SUVANNAKAD, R., ISAKA, M., SIRITHUNYA, P., TOOJINDA, T. AND KIRTIKARA, K. 2009. Ascherxanthone B from Aschersonia luteola, a new antifungal compound active against rice blast pathogen *Magnaporthe grisea*. *Journal of Applied Microbiology*, **107**(5), pp.1624-1631.

CIMERMANCIC, P., MEDEMA, M.H., CLAESEN, J., KURITA, K., BROWN, L.C.W., MAVROMMATIS, K., PATI, A., GODFREY, P.A., KOEHRSEN, M., CLARDY, J. AND BIRREN, B.W., 2014. Insights into secondary metabolism from a global analysis of prokaryotic biosynthetic gene clusters. *Cell*, **158**(2), pp.412-421.

CLARDY, J., M. A. FISCHBACH and C. T. WALSH. 2006. New antibiotics from bacterial natural products. *Nature Biotechnology,* **24**(12), pp.1541-1550.

CLARKE, C.H. AND HOPWOOD, D.A. 1976. Ultraviolet mutagenesis in *Streptomyces coelicolor*: induction of reversions in a polyauxotrophic strain. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **41**(2), pp.201-207.

CLSI, 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Ninth Edition. CLSI document M07-A8. Clinical Laboratory Standards Institute, Wayne.

COCCHIA, M., KOUPRINA, N., KIM, S.J., LARIONOV, V., SCHLESSINGER, D. AND NAGARAJA, R. 2000. Recovery and potential utility of YACs as circular YACs/BACs. *Nucleic Acids Research*, **28**(17), pp. e81-e81.

COHEN, S. S. 1977. A strategy for the chemotherapy of infectious disease. *Science,* **197**(4302), pp.431-2.

COVINGTON, B. C., MCLEAN, J. A., & BACHMANN, B. O. 2017. Comparative mass spectrometry-based metabolomics strategies for the investigation of microbial secondary metabolites. *Natural Product Reports*, **34**(1),pp.6–24. http://doi.org/10.1039/c6np00048g

COX, G., SIERON, A., KING, A.M., DE PASCALE, G., PAWLOWSKI, A.C., KOTEVA, K. AND WRIGHT, G.D., 2017. A common platform for antibiotic dereplication and adjuvant discovery. *Cell Chemical Biology*, 24(1), pp.98-109.

COYNE, V.E., USDIN, K. AND KIRBY, R. 1984. The effect of inhibitors of DNA repair on the genetic instability of *Streptomyces cattleya*. *Microbiology*, **130**(4), pp.887-892.

CRANEY, A., AHMED, S. AND NODWELL, J. 2013. Towards a new science of secondary metabolism. *The Journal of Antibiotics*, **66**(7), p.387.

DA SILVA, P.H., DE SOUZA, M.P., BIANCO, E.A., DA SILVA, S.R., SOARES, L.N., COSTA, E.V., DA SILVA, F.M., BARISON, A., FORIM, M.R., CASS, Q.B. AND DE SOUZA, A.D. 2018. Antifungal Polyketides and Other Compounds from Amazonian Endophytic Talaromyces Fungi. *Journal of Brazilian. Chemical Society*, **29(**3), pp.622-630.

DAVIES, J. AND D. DAVIES. 2010. Origins and evolution of antibiotic resistance*. Microbiology and Molecular Biology Reviews*,**74**(3),pp.417-33.

DE CASTRO, A. P., FERNANDES, G. DA R., & FRANCO, O. L. 2014. Insights into novel antimicrobial compounds and antibiotic resistance genes from soil metagenomes. *Frontiers in Microbiology*, **5**, pp.489.

DE LIMA PROCÓPIO, R.E., DA SILVA, I.R., MARTINS, M.K., DE AZEVEDO, J.L. AND DE ARAÚJO, J.M., 2012. Antibiotics produced by *Streptomyces. The Brazilian Journal of Infectious Diseases*, **16**(5), pp.466-471.

DEN HENGST, C.D., TRAN, N.T., BIBB, M.J., CHANDRA, G., LESKIW, B.K. AND BUTTNER, M.J. 2010. Genes essential for morphological development and antibiotic production in Streptomyces coelicolor are targets of BldD during vegetative growth. *Molecular Microbiology*, **78(**2), pp.361-379.

DERESINSKI, S., 2005. Methicillin-resistant *Staphylococcus aureus*: an evolutionary, epidemiologic, and therapeutic odyssey. *Clinical infectious diseases*, **40**(4), pp.562-573.

DESHMUKH, S.K., VEREKAR, S.A. AND BHAVE, S.V. 2015. Endophytic fungi: a reservoir of antibacterials. *Frontiers in Microbiology*, ***5***, p.715.

DE VRIES, R.P., RILEY, R., WIEBENGA, A., AGUILAR-OSORIO, G., AMILLIS, S., UCHIMA, C.A., ANDERLUH, G., ASADOLLAHI, M., ASKIN, M., BARRY, K. AND BATTAGLIA, E. 2017. Comparative genomics reveals high biological

diversity and specific adaptations in the industrially and medically important fungal genus Aspergillus. *Genome Biology*, **18**(1), p.28.

DEVON RULE AND NAOWARAT CHEEPTHAM. 2013. The effects of UV light on the antimicrobial activities of cave actinomycetes. *International Journal of SPE,* **42**(2), p.147-153.

DHARMARATNE, H.R.W., SAKAGAMI, Y., PIYASENA, K.G.P. AND THEVANESAM, V. 2013. Antibacterial activity of xanthones from Garcinia mangostana (L.) and their structure–activity relationship studies. *Natural Product Research*, **27**(10), pp.938-941.

DONKOR, E. 2013. Sequencing of Bacterial Genomes: Principles and Insights into Pathogenesis and Development of Antibiotics*. Genes*, **4**(4): p.556-572.

DOROGHAZI, J.R. AND METCALF, W.W., 2013. Comparative genomics of actinomycetes with a focus on natural product biosynthetic genes. *BMC Genomics*, **14**(1), p.611.

DOWHAN, W. 1997. Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annual Review of Biochemistry*, **66**(1), pp.199-232.

ELANDER, R.P. 2003. Industrial production of β-lactam antibiotics. *Applied Microbiology and Biotechnology*, **61**(5-6), pp.385-392.

EL-ELIMAT, T., FIGUEROA, M., RAJA, H.A., GRAF, T.N., SWANSON, S.M., FALKINHAM, J.O., WANI, M.C., PEARCE, C.J. AND OBERLIES, N.H. 2015. Biosynthetically distinct cytotoxic polyketides from *Setophoma terrestris*. *European Journal of Organic Chemistry*, **1**, pp.109-121.

ELLIOT, M., DAMJI, F., PASSANTINO, R., CHATER, K. AND LESKIW, B., 1998. The bldD gene of *Streptomyces coelicolor* A3 (2): a regulatory gene involved in morphogenesis and antibiotic production. *Journal of Bacteriology*, **180**(6), pp.1549-1555.

ESKILSSON, C.S. AND BJÖRKLUND, E. 2000. Analytical-scale microwave-assisted extraction. *Journal of Chromatography A*, **902**(1), pp.227-250.

F-J

FANG, S.-M., WU, C.-J., LI, C.-W., and CUI, C.-B. 2014. A Practical Strategy to Discover New Antitumor Compounds by Activating Silent Metabolite Production in Fungi by Diethyl Sulphate Mutagenesis. *Marine Drugs*, **12**(4), 1788–1814.

FENG, Z., J.H. KIM, AND S.F. BRADY. 2010. Fluostatins produced by the heterologous expression of a TAR reassembled environmental DNA derived type II PKS gene cluster. *Journal of American Chemical Society*, **132**(34), p. 11902-3.

FENICAL, W. AND JENSEN, P.R., 2006. Developing a new resource for drug discovery: marine actinomycete bacteria. *Nature Chemical Biology*, **2**(12), p.666.

FIEHN, O., 2002. Metabolomics—the link between genotypes and phenotypes. In *Functional Genomics* (pp. 155-171). Springer Netherlands.

FISCHBACH, M. A. and C. T. WALSH. 2009. Antibiotics for emerging pathogens. *Science,* **325**(5944), pp.1089-1093.

FIX, D. AND BOCKRATH, R., 1981. Thermal resistance to photoreactivation of specific mutations potentiated in E. coli B/r ung by ultraviolet light. *Molecular and General Genetics*, **182**(1), pp.7-11.

FLEMING, A. G. 1929. Responsibilities and opportunities of the private practitioner in preventive medicine. *Canadian Medical Association Journal,* **20**, pp.11-13.

FRISVAD, J.C. AND SAMSON, R.A., 2004. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Studies in Mycology*, **49**(1), pp.1-174.

FRISVAD, J.C., FILTENBORG, O. AND WICKLOW, D.T., 1987. Terverticillate penicillia isolated from underground seed caches and cheek pouches of banner-tailed kangaroo rats (*Dipodomys spectabilis*). *Canadian Journal of Botany*, **65**(4), pp.765-773.

GABRIELA ZUREK, ANDREAS GERMANUS, MARYANN SHEN, CARSTEN BAESSMANN, ARND INGENDOH BRUKER DALTONIK GMBH. 2002. Novel Strategies for Metabolite Identification using HPLC-Ion Trap Mass Spectrometry and the MetaboliteTools software. Bruker Daltonik GmbH) http://bruker.poznan.pl/images/stories/Daltonics/noty/lcms39.pdf

GAO, H., LIU, M., ZHUO, Y., ZHOU, X., LIU, J., CHEN, D., ZHANG, W., GOU, Z., SHANG, P. AND ZHANG, L., 2010. Assessing the potential of an induced-mutation strategy for avermectin overproducers. *Applied and Environmental Microbiology,* **76**(13), pp.4583-4586.

GARG, N., KAPONO, C.A., LIM, Y.W., KOYAMA, N., VERMEIJ, M.J., CONRAD, D., ROHWER, F. AND DORRESTEIN, P.C. 2015. Mass spectral similarity for untargeted metabolomics data analysis of complex mixtures. *International Journal of Mass Spectrometry*, **377**, pp.719-727.

GENILLOUD, O. 2017. Actinomycetes: still a source of novel antibiotics. *Natural Product Reports*, **34**(10), pp.1203-1232.

GOMEZ-ESCRIBANO, J.P. AND BIBB, M.J., 2011. Engineering Streptomyces coelicolor for heterologous expression of secondary metabolite gene clusters. *Microbial Biotechnology*, **4**(2), pp.207-215.

GOTTELT, M., KOL, S., GOMEZ-ESCRIBANO, J.P., BIBB, M. AND TAKANO, E., 2010. Deletion of a regulatory gene within the *cpk* gene cluster reveals novel antibacterial activity in *Streptomyces coelicolor* A3 (2). *Microbiology*, **156**(8), pp.2343-2353.

GOUBA, N., RAOULT, D. AND DRANCOURT, M. 2013. Plant and fungal diversity in gut microbiota as revealed by molecular and culture investigations. *PLoS One*, **8**(3), p. e59474.

GRANATA, G. AND PETROSILLO, N., 2017. Resistance to colistin in Klebsiella pneumoniae: a 4.0 strain? *Infectious Disease Reports*, **9**(2). p.7104.

GREINERT, R., VOLKMER, B., HENNING, S., BREITBART, E. W., GREULICH, K. O., CARDOSO, M. C., & RAPP, A. 2012. UVA-induced DNA double-strand breaks result from the repair of clustered oxidative DNA damages. *Nucleic Acids Research*, **40**(20), pp. 10263–10273.

GULLO, V.P., MCALPINE, J., LAM, K.S., BAKER, D. AND PETERSEN, F., 2006. Drug discovery from natural products. *Journal of Industrial Microbiology and Biotechnology*, 33(7), pp.523-531.

GUO, F., XIANG, S., LI, L., WANG, B., RAJASÄRKKÄ, J., GRÖNDAHL-YLI-HANNUKSELA, K., AI, G., METSÄ-KETELÄ, M. AND YANG, K., 2015. Targeted activation of silent natural product biosynthesis pathways by reporter-guided mutant selection. *Metabolic Engineering*, **28**, pp.134-142.HAMMERUM, A.M., TOLEMAN, M.A., HANSEN, F., KRISTENSEN, B., LESTER, C.H., WALSH, T.R. AND FUURSTED, K., 2010. Global spread of New Delhi metallo-β-lactamase 1. *The Lancet Infectious Diseases*, **10**(12), pp.829-830.

HAMMAMI, R., ZOUHIR, A., LE LAY, C., HAMIDA, J.B. AND FLISS, I., 2010. BACTIBASE second release: a database and tool platform for bacteriocin characterization. *BMC Microbiology*, **10**(1), p.22.

HANSFORD, K.A., BLASKOVICH, M.A. AND COOPER, M.A. 2016. Chemical philanthropy: a path forward for antibiotic discovery? *Future Medicinal Chemistry*, **8**(9), pp. 925–929.

HAROLD, R.J. AND D.A. HOPWOOD, 1970. Ultraviolet-sensitive mutants of Streptomyces coelicolor I. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **10**(5), p. 427-448.

HARVEY, C.J., TANG, M., SCHLECHT, U., HORECKA, J., FISCHER, C.R., LIN, H.C., LI, J., NAUGHTON, B., CHERRY, J., MIRANDA, M. AND LI, Y.F., 2018. HEx: a heterologous expression platform for the discovery of fungal natural products. *BioRxiv*, p.247940.

HAWKSWORTH, D.L. AND ROSSMAN, A.Y. 1997. Where are all the undescribed fungi? *Phytopathology*, **87**(9), pp.888-891.

HEIDEMANN S, MONOD M, GRÄSER Y. 2010. Signature polymorphisms in the internal transcribed spacer region relevant for the differentiation of zoophilic and anthropophilic strains of *Trichophyton interdigitale* and other species of *T. mentagrophytes sensu lato*. *Brazilian Journal of Dermatology*, **162**, pp. 282-295.

HEYNICK, F. 2009. The original 'magic bullet' is 100 years old - extra*. Brazilian Journal of Psychiatry.* **195**(5), p. 456.

HOLT, G. AND SAUNDERS, G., 1985. Genetic modification of industrial microorganisms, in: 'Comprehensive Biotechnology', C.L. Cooney and A. E. Humphrey, ed., Pergamon Press.

HOLDEN, M. T., E. J. FEIL, J. A. LINDSAY, S. J. PEACOCK, N. P. DAY, M. C. ENRIGHT, T. J. FOSTER, C. E. MOORE, L. HURST, R. ATKIN, A. BARRON, N. BASON, S. D. BENTLEY, C. CHILLINGWORTH, T. CHILLINGWORTH, C. CHURCHER, L. CLARK, C. CORTON, A. CRONIN, J. DOGGETT, L. DOWD, T. FELTWELL, Z. HANCE, B. HARRIS, H. HAUSER, S. HOLROYD, K. JAGELS, K. D. JAMES, N. LENNARD, A. LINE, R. MAYES, S. MOULE, K. MUNGALL, D. ORMOND, M. A. QUAIL, E. RABBINOWITSCH, K. RUTHERFORD, M. SANDERS, S. SHARP, M. SIMMONDS, K. STEVENS, S. WHITEHEAD, B. G. BARRELL, B. G. SPRATT and J. PARKHILL. 2004. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proceedings of the National Academy of Sciences of the United States of America*, **101**(26), pp.9786-91.

HOLLOWAY, B.W. 1955. Genetic Recombination in *Pseudomonas aeruginosa. Journal of Microbiology*, **13**(3), pp. 572-581.

HOSAKA, T., OHNISHI-KAMEYAMA, M., MURAMATSU, H., MURAKAMI, K., TSURUMI, Y., KODANI, S., YOSHIDA, M., FUJIE, A. AND OCHI, K. 2009. Antibacterial discovery in actinomycetes strains with mutations in RNA polymerase or ribosomal protein S12. *Nature Biotechnology*, **27**(5), p.462.

HOU, Y., BRAUN, D.R., MICHEL, C.R., KLASSEN, J.L., ADNANI, N., WYCHE, T.P. AND BUGNI, T.S. 2012. Microbial strain prioritization using metabolomics tools for the discovery of natural products. *Analytical Chemistry*, **84**(10), pp.4277-4283.

HOVER, B.M., KIM, S.H., KATZ, M., CHARLOP-POWERS, Z., OWEN, J.G., TERNEI, M.A., MANIKO, J., ESTRELA, A.B., MOLINA, H., PARK, S. AND PERLIN, D.S., 2018. Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens. *Nature Microbiology*, **3**(4), p.415.

HUANG, H., ZHENG, G., JIANG, W., HU, H. AND LU, Y. 2015. One-step high-efficiency CRISPR/Cas9-mediated genome editing in Streptomyces. *Acta biochimica et biophysica Sinica*, **47**(4), pp.231-243.

HUANG, R., DING, Z.G., LONG, Y.F., ZHAO, J.Y., LI, M.G., CUI, X.L. AND WEN, M.L. 2013. A new isoflavone derivative from *Streptomyces* sp. YIM GS3536. *Chemistry of Natural Compounds*, **48**(6), pp.966-969.

.

HUANG, T. AND LIN, S., 2017. Microbial natural products: A promising source for drug discovery. *Journal of Applied Microbiology and Biochemistrey*, **2**, pp.1-3.

IAN, E., MALKO, D.B., SEKUROVA, O.N., BREDHOLT, H., RÜCKERT, C., BORISOVA, M.E., ALBERSMEIER, A., KALINOWSKI, J., GELFAND, M.S. AND ZOTCHEV, S.B., 2014. Genomics of sponge-associated *Streptomyces* spp. closely related to *Streptomyces albus* J1074: insights into marine adaptation and secondary metabolite biosynthesis potential. *PloS One*, **9**(5), p.e96719.

IKEHATA, H. AND ONO, T., 2011. The mechanisms of UV mutagenesis. *Journal of Radiation Research*, **52**(2), pp.115-125.

JACOBY, G.A., 2009. AmpC β-lactamases. *Clinical Microbiology Reviews*, **22**(1), pp.161-182.

JIA, K., Y. ZHANG, AND Y. LI. 2010. Systematic engineering of microorganisms to improve alcohol tolerance. *Engineering in Life Science*, **10**(5), p. 422-429.

JOSHI, G.K., J. JUGRAN, AND J.P. BHATT, 2014. Metagenomics: The Exploration of Unculturable Microbial World, in Advances in Biotechnology, I. Ravi, M. Baunthiyal, and J. Saxena, Editors. 2014, Springer India. pp. 105-115.

JU, K.S., GAO, J., DOROGHAZI, J.R., WANG, K.K.A., THIBODEAUX, C.J., LI, S., METZGER, E., FUDALA, J., SU, J., ZHANG, J.K. AND LEE, J., 2015. Discovery of phosphonic acid natural products by mining the genomes of 10,000 actinomycetes. *Proceedings of the National Academy of Sciences*, **112**(39), pp.12175-12180.

K-O

KAEBERLEIN, T., K. LEWIS, AND S.S. EPSTEIN., 2002. Isolating "Uncultivable" Microorganisms in Pure Culture in a Simulated Natural Environment. *Science*, **296**(5570), pp.1127-1129.

KELNER, A. 1949. Studies on the genetics of antibiotic formation: the induction of antibiotic-forming mutants in Actinomycetes*. Journal of Bacteriology*, **57**(1),p.73.

KELNER, A. 1951. Action spectra for photoreactivation of ultraviolet-irradiated *Escherichia coli* and *Streptomyces griseus*. *The Journal of General Physiology*, **34**(6), pp.835–852.

KHALIQ, S., AKHTAR, K., GHAURI, M.A., IQBAL, R., KHALID, A.M. AND MUDDASSAR, M., 2009. Change in colony morphology and kinetics of tylosin production after UV and gamma irradiation mutagenesis of *Streptomyces fradiae* NRRL-2702. *Microbiological research*, **164**(4), pp.469-477.

KHAMTHONG N., RUKACHAISIRIKUL V., PHONGPAICHIT S., PREEDANON S., SAKAYAROJ J., 2014. An antibacterial cytochalasin derivative from the marine-derived fungus *Diaporthaceae* sp. PSU-SP2/4. *Phytochemical Letters*,**10**, pp.5–9.

KIESER, T., BIBB, M.J., BUTTNER, M.J., CHATER, K.F., AND HOPWOOD, D.A. 2000. *Practical Streptomyces Genetics*. John Innes Foundation, Norwich, UK.

KIMMIG, J., *[GERHARD DOMAGK, 1895-1964.* Contribution to the chemotherapy of bacterial infections*].* Internist (Berl), 1969. **10**(3): pp. 116-20.

KLANOVA, K., BLUMAUEROVA, M. AND VANĚK, Z., 1977. Spontaneous and induced variability in *Streptomyces nogalater* producing nogalamycin. *Folia Microbiologica*, **22**(4), pp.286-294.

KODYM, A. AND AFZA, R., 2003. Physical and chemical mutagenesis. *In Plant functional genomics* (pp. 189-203). Humana Press.

KOL, S., MERLO, M.E., SCHELTEMA, R.A., DE VRIES, M., VONK, R.J., KIKKERT, N.A., DIJKHUIZEN, L., BREITLING, R. AND TAKANO, E., 2010. Metabolomic characterization of the salt stress response in *Streptomyces coelicolor*. *Applied and Environmental Microbiology*, **76**(8), pp.2574-2581.

KOMATSU, M., UCHIYAMA, T., ŌMURA, S., CANE, D.E. AND IKEDA, H., 2010. Genome-minimized *Streptomyces* host for the heterologous expression of

secondary metabolism. *Proceedings of the National Academy of Sciences*, **107**(6), pp.2646-2651.

KONDRAT, R.W., MCCLUSKY, G.A. AND COOKS, R.G., 1978. Multiple reaction monitoring in mass spectrometry/mass spectrometry for direct analysis of complex mixtures. *Analytical chemistry*, **50**(14), pp.2017-2021.

KORBEKANDI, H., DARKHAL, P., HOJATI, Z., ABEDI, D., HAMEDI, J. AND POURHOSEIN, M., 2010. Overproduction of clavulanic acid by UV mutagenesis of *Streptomyces clavuligerus.* Iranian *Journal of Pharmaceutical Research*, **9**(2), p.177.

KOYAMA, N., NAGAHIRO, T., YAMAGUCHI, Y., MASUMA, R., TOMODA, H. AND ŌMURA, S., 2005. Stemphones, novel potentiators of imipenem activity against methicillin-resistant *Staphylococcus aureus*, produced by *Aspergillus* sp. FKI-2136. *The Journal of Antibiotics*, **58**(11), pp.695-703.

KÜCK, U., BLOEMENDAL, S., & TEICHERT, I., 2014. Putting Fungi to Work: Harvesting a Cornucopia of Drugs, Toxins, and Antibiotics. *PLoS Pathogens*, **10**(3), e1003950.

KUMAR, S. AND RZHETSKY, A., 1996. Evolutionary relationships of eukaryotic kingdoms. *Journal of Molecular Evolution*, **42**(2), pp.183-193.

KUMAR, N., GORANTLA, J.N., MOHANDAS, C., NAMBISAN, B. AND LANKALAPALLI, R.S., 2013. Isolation and antifungal properties of cyclo (d-Tyr-l-Leu) diketopiperazine isolated from Bacillus sp. associated with rhabditid entomopathogenic nematode. *Natural Product Research*, **27**(23), pp.2168-2172.

KUROBANE, I., IWAHASHI, S. AND FUKUDA, A., 1987. Cytostatic activity of naturally isolated isomers of secalonic acids and their chemically rearranged dimers. *Drugs Under Experimental and Clinical Research,* **13**(6), pp.339-344.

LABEDA, D.P., GOODFELLOW, M., BROWN, R., WARD, A.C., LANOOT, B., VANNCANNEYT, M., SWINGS, J., KIM, S.B., LIU, Z., CHUN, J. AND TAMURA, T., 2012. Phylogenetic study of the species within the family Streptomycetaceae. *Antonie Van Leeuwenhoek*, **101**(1), pp.73-104.

LABEDA, D.P., 2016. Taxonomic evaluation of putative *Streptomyces scabiei* strains held in the ARS Culture Collection (NRRL) using multi-locus sequence analysis. *Antonie van Leeuwenhoek*, **109**(3), pp.349-356.

LAURETI, L., SONG, L., HUANG, S., CORRE, C., LEBLOND, P., CHALLIS, G.L. AND AIGLE, B., 2011. Identification of a bioactive 51-membered macrolide complex by activation of a silent polyketide synthase in *Streptomyces*

ambofaciens. *Proceedings of the National Academy of Sciences*, **108**(15), pp.6258-6263.

LEBLOND, P. AND DECARIS, B., 1994. New insights into the genetic instability of *Streptomyces*. *FEMS Microbiology Letters,* **123**(3), pp.225-232.

LECK, A., 1999. Preparation of lactophenol cotton blue slide mounts. *Journal of Community Eye Health*. **12**, p. 24.

LECLERC, J.E., BORDEN, A. AND LAWRENCE, C.W., 1991. The thymine-thymine pyrimidine-pyrimidone (6-4) ultraviolet light photoproduct is highly mutagenic and specifically induces 3'thymine-to-cytosine transitions in *Escherichia coli*. *Proceedings of the National Academy of Sciences*, **88**(21), pp.9685-9689.

LEE, J.-H., BAE, S.-H., & CHOI, B.-S., 2000. The Dewar photoproduct of thymidylyl(3′→5′)- thymidine (Dewar product) exhibits mutagenic behavior in accordance with the "A rule." *Proceedings of the National Academy of Sciences of the United States of America*, **97**(9), pp. 4591–4596.

LEE, S.H. AND RHO, Y.T., 1999. Improvement of tylosin fermentation by mutation and medium optimization. *Letters in Applied Microbiology*, **28**(2), pp.142-144.

LEE, J.-H., BAE, S.-H., AND CHOI, B.-S., 2000. The Dewar photoproduct of thymidylyl(3′→5′)- thymidine (Dewar product) exhibits mutagenic behavior in accordance with the "A rule." *Proceedings of the National Academy of Sciences of the United States of America*, **97**(9), pp. 4591–4596.

LEE, S.H. AND RHO, Y.T., 1999. Improvement of tylosin fermentation by mutation and medium optimization. *Letters in Applied Microbiology*, **28**(2), pp.142-144.

LESKIW, B.K., LAWLOR, E.J., FERNANDEZ-ABALOS, J.M. AND CHATER, K.F., 1991. TTA codons in some genes prevent their expression in a class of developmental, antibiotic-negative, *Streptomyces* mutants. *Proceedings of the National Academy of Sciences*, **88**(6), pp.2461-2465.

LEUVEN iGEM. 2013. Colony PCR for *Streptomyces*. *Team KU Leuven Protocol online.*http://2013.igem.org/Team:KU_Leuven/Protocols#Colony%20PCR%20for%20Streptomyces. [Accessed September 2016]

LEWIS, K., 2013. Platforms for antibiotic discovery. *Nature Reviews Drug Discovery*, **12**(5), p. 371-87.

LEWIS, K., 2012. Antibiotics: Recover the lost art of drug discovery. *Nature*, **485**(7399), pp. 439-440.

LI, J., LIU, Z., TAN, C., GUO, X., WANG, L., SANCAR, A. AND ZHONG, D., 2010. Dynamics and mechanism of repair of ultraviolet-induced (6-4) photoproduct by photolyase. *Nature*, **466**(7308), pp.887-890.

LI, H., JIANG, J., LIU, Z., LIN, S., XIA, G., XIA, X., DING, B., HE, L., LU, Y. AND SHE, Z., 2014. Peniphenones A–D from the mangrove fungus *Penicillium dipodomyicola* HN4-3A as inhibitors of *Mycobacterium tuberculosis* Phosphatase MptpB. *Journal of Natural Products*, **77**(4), pp.800-806.

LI, J.W.H. AND VEDERAS, J.C., 2009. Drug discovery and natural products: end of an era or an endless frontier? *Science*, **325**(5937), pp.161-165.

LI, C.W., WU, C.J., CUI, C.B., XU, L.L., CAO, F. AND ZHU, H.J., 2016. Penicimutamides A–C: rare carbamate-containing alkaloids from a mutant of the marine-derived *Penicillium purpurogenum* G59. *RSC Advances*, **6**(77), pp.73383-73387.

LI, T.X., YANG, M.H., WANG, Y., WANG, X.B., LUO, J., LUO, J.G. AND KONG, L.Y., 2016. Unusual dimeric tetrahydroxanthone derivatives from Aspergillus lentulus and the determination of their axial chiralities. *Scientific Reports*, **6**, p.38958.

LI Y., SUN K.L., WANG Y., FU P., LIU P.P., WANG C., ZHU W.M., 2013. A cytotoxic pyrrolidinoindoline diketopiperazine dimer from the algal fungus *Eurotium herbariorum* HT-2. *China Chemical Letters*, **24**, pp.1049-1052.

LIAO, G., ZHOU, J., WANG, H., MAO, Z., XIAO, W., WANG, H., SHE, Z. AND ZHU, Y., 2010. The cell toxicity effect of secalonic acid D on GH3 cells and the related mechanisms. *Oncology Reports*, **23**(2), pp.387-395.

LIN, K.E.V.I.N. AND WANG, A.L.I.C.E., 2001. UV mutagenesis in Escherichia coli K-12: Cell survival and mutation frequency of the chromosomal genes lacZ, rpoB, ompF, and ampA. *Journal of Experimental Microbiology and Immunology*, **1**, pp.32-46.

LING, L.L., SCHNEIDER, T., PEOPLES, A.J., SPOERING, A.L., ENGELS, I., CONLON, B.P., MUELLER, A., SCHÄBERLE, T.F., HUGHES, D.E., EPSTEIN, S. AND JONES, M., 2015. A new antibiotic kills pathogens without detectable resistance. *Nature*, 517(7535), p.455.

LIU, J.T.; LU, X.L.; LIU, X.Y.; GAO, Y.; HU, B.; JIAO, B.H.; ZHENG, H. **2013.** Bioactive natural products from the antarctic and arctic organisms. *Mini Review in Medicinal Chemistry*, **13**, pp. 617–626.

LIU, N., WANG, H., LIU, M., GU, Q., ZHENG, W. AND HUANG, Y., 2009. Streptomyces alni sp. nov., a daidzein-producing endophyte isolated from a root of Alnus nepalensis D. Don. *International Journal of Systematic and Evolutionary Microbiology*, **59**(2), pp.254-258.

LOBANOVSKA, M., & PILLA, G., 2017. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? *The Yale Journal of Biology and Medicine*, **90**(1), pp. 135–145.

LUO, Y., 2014. Natural products discovery and characterization via synthetic biology (Doctoral dissertation, University of Illinois at Urbana-Champaign).

LUO, Y., HUANG, H., LIANG, J., WANG, M., LU, L., SHAO, Z., COBB, R.E. AND ZHAO, H., 2013. Activation and characterization of a cryptic polycyclic tetramate macrolactam biosynthetic gene cluster. *Nature Communications*, **4**, p.2894.

MA, H.G., LIU, Q., ZHU, G.L., LIU, H.S. AND ZHU, W.M., 2016. Marine natural products sourced from marine-derived *Penicillium* fungi. *Journal of Asian Natural Products Research*, **18**(1), pp.92-115.

MACARRON, R., BANKS, M.N., BOJANIC, D., BURNS, D.J., CIROVIC, D.A., GARYANTES, T., GREEN, D.V., HERTZBERG, R.P., JANZEN, W.P., PASLAY, J.W. AND SCHOPFER, U., 2011. Impact of high-throughput screening in biomedical research. *Nature Reviews Drug Discovery*, **10**(3), p.188.

MANIVASAGAN, P., VENKATESAN, J., SIVAKUMAR, K. AND KIM, S.K., 2014. Pharmaceutically active secondary metabolites of marine actinobacteria. *Microbiological Research,* **169**(4), pp.262-278.

MARCET-HOUBEN, M. AND GABALDÓN, T., 2010. Acquisition of prokaryotic genes by fungal genomes. *Trends in Genetics*, **26**(1), pp.5-8.

MARINER, K. R., OOI, N., ROEBUCK, D., O'NEILL, A. J., & CHOPRA, I., 2011. Further Characterization of *Bacillus subtilis* Antibiotic Biosensors and Their Use for Antibacterial Mode-of-Action Studies . *Antimicrobial Agents and Chemotherapy*, **55**(4), 1784–1786.

MARTEL, C. M., J. E. PARKER, O. BADER, M. WEIG, U. GROSS, A. G. WARRILOW, N. ROLLEY, D. E. KELLY and S. L. KELLY. 2010. Identification and characterization of four azole-resistant erg3 mutants of *Candida albicans*. *Antimicrobial Agents and Chemotherapy,* **54**(11), pp.4527-33.

MARMANN, A., ALY, A.H., LIN, W., WANG, B. AND PROKSCH, P., 2014. Co-cultivation—A powerful emerging tool for enhancing the chemical diversity of microorganisms. *Marine Drugs*, **12**(2), pp.1043-1065.

MASCHER, T., HELMANN, J.D. AND UNDEN, G., 2006. Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiology and Molecular Biology Reviews*, **70**(4), pp.910-938.

MASTERS, K.S. AND BRÄSE, S., 2012. Xanthones from fungi, lichens, and bacteria: the natural products and their synthesis. *Chemical Reviews,* **112**(7), pp.3717-3776.

MATSELIUKH, B.P., POLISHCHUK, L.V., LUTCHENKO, V.V., BAMBURA, O.I. AND KOPIYKO, O.P., *2005.* Synthesis of daidzein and genistein by streptomycetes and their effect on production of antibiotics. *Mikrobiolohichnyi zhurnal (Kiev, Ukraine: 1993),* **67**(2), pp.12-21*.*

MAYER, A. M. S., K. B. GLASER, C. CUEVAS, R. S. JACOBS, W. KEM, R. D. LITTLE, J. M. MCINTOSH, D. J. NEWMAN, B. C. POTTS and D. E. SHUSTER. 2010. The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends in Pharmacological Sciences,* **31**(6), pp.255-265.

MCALPINE, J.B., BACHMANN, B.O., PIRAEE, M., TREMBLAY, S., ALARCO, A.M., ZAZOPOULOS, E. AND FARNET, C.M., 2005. Microbial genomics as a guide to drug discovery and structural elucidation: ECO-02301, a novel antifungal agent, as an example. *Journal of Natural Products*, **68(**4), pp.493-496.

MCCABE, P., 1990. Production of Pimaricin by *Streptomyces natalensis* in submerged culture (Doctoral dissertation, Dublin City University).

MCCORMICK, J.R. AND FLÄRDH, K., 2011. Signals and regulators that govern *Streptomyces* development. *FEMS Microbiology Reviews*, **36**(1), pp.206-231.

MEDEMA, M.H., R. BREITLING, AND E. TAKANO, 2011. *Synthetic biology in Streptomyces bacteria. Methods in Enzymology*, **497**: p. 485-502.

MEDINA. Natural Product Research. High-throughput Screening. [on line] Available from:http://www.medinadiscovery.com/services-view/high-throughput-screening/ [Accessed September 2017]

MINCER, T.J., JENSEN, P.R., KAUFFMAN, C.A. AND FENICAL, W., 2002. Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Applied and Environmental Microbiology*, **68**(10), pp.5005-5011.

MITRA, S., SIVAKUMAR, P., OUGHTON, J. AND OSSUETTA, I., 2011. National surveillance study of extended spectrum β lactamase (ESBL) producing organism infection in neonatal units of england and wales. *Archives of Disease in Childhood*, **96**(Suppl 1), pp. A47-A47.

MISHRA, K.P., GANJU, L., SAIRAM, M., BANERJEE, P.K. AND SAWHNEY, R.C., 2008. A review of high throughput technology for the screening of natural products. *Biomedicine & Pharmacotherapy*, **62**(2), pp.94-98.

MISLIVEC, P.B. AND TUITE, J., 1970. Species of *Penicillium* occurring in freshly-harvested and in stored dent corn kernels. *Mycologia*, pp.67-74.

MOHANTA, T.K. AND BAE, H., 2015. The diversity of fungal genome. *Biological Procedures Online*, **17**(1), p.8.

MOORE, D. AND FRAZER, L.N., 2002. Essential fungal genetics. Springer Science & Business Media.

MOORE, J.M., ET AL., 2012. Use and discovery of chemical elicitors that stimulate biosynthetic gene clusters in *Streptomyces* bacteria*. Methods in Enzymology*, **517**, pp. 367-85.

MOREE, W.J., PHELAN, V.V., WU, C.H., BANDEIRA, N., CORNETT, D.S., DUGGAN, B.M. AND DORRESTEIN, P.C., 2012. Interkingdom metabolic transformations captured by microbial imaging mass spectrometry. *Proceedings of the National Academy of Sciences*, **109**(34), pp.13811-13816.

MORTELMANS, K. 1989. Effect of photoreactivating light on survival of ultraviolet-light-irradiated *Streptomyces lividans* 66*.* J*ournal of Industrial Microbiology*, **4**(1), pp. 7-12.

MOSS, S., BOVERMANN, G., DENAY, R., FRANCE, J., GUENAT, C., OBERER, L., PONELLE, M. AND SCHRÖDER, H., 2007. Efficient structure elucidation of natural products in the pharmaceutical industry. *CHIMIA International Journal for Chemistry*, **61**(6), pp.346-349.

NASS, N.M., Farooque, S., Hind, C., Wand, M.E., Randall, C.P., Sutton, J.M., Seipke, R.F., Rayner, C.M. and O'Neill, A.J., 2017. Revisiting unexploited antibiotics in search of new antibacterial drug candidates: the case of γ-actinorhodin. *Scientific Reports*, **7**(1), p.17419.

NASS, N.M., 2017. Revisiting unexploited natural products in search of new antibacterial drug candidates: the case of the benzoisochromanequinone and dithiolopyrrolone classes (Doctoral dissertation, University of Leeds).

NAH, H.-J., PYEON, H.-R., KANG, S.-H., CHOI, S.-S., & KIM, E.-S. 2017. Cloning and Heterologous Expression of a Large-sized Natural Product Biosynthetic Gene Cluster in *Streptomyces* Species. *Frontiers in Microbiology*, **8**,p. 394.

NETT, M., IKEDA, H., & MOORE, B. S. 2009. Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Natural Product Reports*, **26**(11), 1362–1384.

NETZ, N. AND OPATZ, T., 2015. Marine indole alkaloids. *Marine Drugs*, **13**(8), pp.4814-4914.

NICHOLS, D., 2007. Cultivation gives context to the microbial ecologist. FEMS *Microbiology and Ecology*, **60**(3), pp.351-357.

NIELSEN, K.F., MÅNSSON, M., RANK, C., FRISVAD, J.C. AND LARSEN, T.O., 2011. Dereplication of microbial natural products by LC-DAD-TOFMS. *Journal of Natural Products*, **74**(11), pp.2338-2348.

NIESELT, K., BATTKE, F., HERBIG, A., BRUHEIM, P., WENTZEL, A., JAKOBSEN, Ø.M., SLETTA, H., ALAM, M.T., MERLO, M.E., MOORE, J. AND OMARA, W.A., 2010. The dynamic architecture of the metabolic switch in *Streptomyces coelicolor*. *BMC Genomics*, **11**(1), p.10.

NGUYEN, K.T., TENOR, J., STETTLER, H., NGUYEN, L.T., NGUYEN, L.D. AND THOMPSON, C.J., 2003. Colonial differentiation in *Streptomyces coelicolor* depends on translation of a specific codon within the adpA gene. *Journal of Bacteriology*, **185**(24), pp.7291-7296.

NOSKOV, V., KOUPRINA, N., LEEM, S.H., KORIABINE, M., BARRETT, J.C. AND LARIONOV, V., 2002. A genetic system for direct selection of gene-positive clones during recombinational cloning in yeast. *Nucleic Acids Research*, **30**(2), pp.e8-e8.

NOSKOV, V.N., KOUPRINA, N., LEEM, S.H., OUSPENSKI, I., BARRETT, J.C. AND LARIONOV, V., 2003. A general cloning system to selectively isolate any eukaryotic or prokaryotic genomic region in yeast. *BMC Genomics*, **4**(1), p.16.

NOVAK, R. AND SHLAES, D.M., 2010. The pleuromutilin antibiotics: a new class for human use. Current opinion in investigational drugs (London, England: 2000), **11**(2), pp.182-191.

OCHI, K., OKAMOTO, S., TOZAWA, Y., INAOKA, T., HOSAKA, T., XU, J. AND KUROSAWA, K., 2004. Ribosome engineering and secondary metabolite production. *Advances in applied microbiology*, **56**(1), pp.155-184.

OCHI, K., 2007. From microbial differentiation to ribosome engineering. *Bioscience, Biotechnology, and Biochemistry*, **71**(6), pp.1373-1386.

OCHI, K. AND HOSAKA, T., 2013. New strategies for drug discovery: activation of silent or weakly expressed microbial gene clusters. *Applied Microbiology and Biotechnology*, **97**(1), pp.87-98.

OHNISHI, Y., KAMEYAMA, S., ONAKA, H. AND HORINOUCHI, S., 1999. The A-factor regulatory cascade leading to streptomycin biosynthesis in Streptomyces griseus: identification of a target gene of the A-factor receptor. *Molecular Microbiology*, **34**(1), pp.102-111.

OMURA, S., IKEDA, H., ISHIKAWA, J., HANAMOTO, A., TAKAHASHI, C., SHINOSE, M., TAKAHASHI, Y., HORIKAWA, H., NAKAZAWA, H.,

OSONOE, T. AND KIKUCHI, H., 2001. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. *Proceedings of the National Academy of Sciences of the United States of America*, **98**(21), pp.12215-12220.

O'NEILL, A. J., J. H. COVE AND I. CHOPRA. 2001. Mutation frequencies for resistance to fusidic acid and rifampicin in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy,* **47**(5), pp.647-650.

O'NEILL, A. J. AND I. CHOPRA. 2004. Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. *Expert Opinion on Investigational Drugs,* **13**(8), pp.1045-1063.

O'NEILL, A. J. 2010. *Staphylococcus aureus* SH1000 and 8325-4: comparative genome sequences of key laboratory strains in staphylococcal research. *Letters in Applied Microbiology,* **51**(3), pp.358-61.

O'NEILL, J., 2016. Tackling drug-resistant infections globally: final report and recommendations. The review on antimicrobial resistance. London: AMR, 2016.

P-T

PARADKAR, A. 2013. Clavulanic acid production *by Streptomyces clavuligerus:* biogenesis, regulation and strain improvement. *The Journal of Antibiotics(Tokyo)*, **66**: p. 411-20.

PAREKH, S., VINCI, V. A. & STROBEL, R. J. 2000. Improvement of microbial strains and fermentation processes. *Applied Microbiology and Biotechnology,* **54**, p. 287-301.

PATERSON, D.L. AND BONOMO, R.A., 2005. Extended-spectrum β-lactamases: a clinical update. *Clinical Microbiology Reviews*, **18**(4), pp.657-686.

PAULUS, C., REBETS, Y., TOKOVENKO, B., NADMID, S., TEREKHOVA, L.P., MYRONOVSKYI, M., ZOTCHEV, S.B., RÜCKERT, C., BRAIG, S., ZAHLER, S. AND KALINOWSKI, J., 2017. New natural products identified by combined genomics-metabolomics profiling of marine *Streptomyces* sp. MP131-18. *Scientific Reports*, **7**, p.42382.

PAYNE, D.J., GWYNN, M.N., HOLMES, D.J. AND POMPLIANO, D.L., 2007. Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature Reviews Drug Discovery*, **6**(1), p.29.

PAWLIK, K., KOTOWSKA, M., CHATER, K.F., KUCZEK, K. AND TAKANO, E., 2007. A cryptic type I polyketide synthase (*cpk*) gene cluster in Streptomyces coelicolor A3 (2). *Archives of Microbiology*, **187**(2), pp.87-99

TYRKKÖ, E. AND OJANPERÄ, I., 2009. *In silico* methods for predicting metabolism and mass fragmentation applied to quetiapine in liquid chromatography/time-of-flight mass spectrometry urine drug screening. *Rapid Communications in Mass Spectrometry*, **23**(4), pp.506-514.

PETER CIMERMANCIC, MARNIX H. MEDEMA, JAN CLAESEN, KENJI KURITA, LAURA C. WIELAND BROWN, KONSTANTINOS MAVROMMATIS, AMRITA PATI, PAUL A. GODFREY, MICHAEL KOEHRSEN, JON CLARDY, BRUCE W. BIRREN, ERIKO TAKANO, ANDREJ SALI, ROGER G. LININGTON, MICHAEL A. FISCHBACH, 2014. Insights into Secondary Metabolism from a Global Analysis of Prokaryotic Biosynthetic Gene Clusters, *Cell*, **158**(2), pp.412-421.

PITOUT, J.D., NORDMANN, P., LAUPLAND, K.B. AND POIREL, L., 2005. Emergence of Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs) in the community. *Journal of Antimicrobial Chemotherapy*, **56**(1), pp.52-59.

QUINN, R.A., NOTHIAS, L.F., VINING, O., MEEHAN, M., ESQUENAZI, E. AND DORRESTEIN, P.C., 2017. Molecular networking as a drug discovery, drug metabolism, and precision medicine strategy. *Trends in Pharmacological Sciences*, **38**(2), pp.143-154.

QU, X., LEI, C. AND LIU, W., 2011. Transcriptome mining of active biosynthetic pathways and their associated products in *Streptomyces flaveolus. Angewandte Chemie International Edition*, **50**(41), pp.9651-9654.

RAHFELD, J., SCHIERBORN, M., HARTRODT, B., NEUBERT, K. AND HEINS, J., 1991. Are diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) inhibitors or substrates of dipeptidyl peptidase IV? *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, **1076**(2), pp.314-316.

RASTOGI, R.P., KUMAR, A., TYAGI, M.B. AND SINHA, R.P., 2010. Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. *Journal of Nucleic Acids*, 2010.

RATEB, M.E. AND EBEL, R., 2011. Secondary metabolites of fungi from marine habitats. *Natural Product Reports*, **28**(2), pp.290-344.

RAWAT, D. AND NAIR, D., 2010. Extended-spectrum β-lactamases in Gram Negative Bacteria. *Journal of Global Infectious Diseases*, **2**(3), p.263.

REN, H., WANG, B. AND ZHAO, H., 2017. Breaking the silence: new strategies for discovering novel natural products. *Current Opinion in Biotechnology*, **48**, pp.21-27.

RICHARD A STEIN. Colistin:revival in times of hardship. *The International Journal of Clinical Practice*, **70**,pp. 703-705.

RICHARDSON, M.D. AND WARNOCK, D.W., 2012. *Fungal infection: diagnosis and management*. John Wiley & Sons.

RIEDLINGER, J., A. REICKE, H. ZAHNER, B. KRISMER, A. T. BULL, L. A. MALDONADO, A. C. WARD, M. GOODFELLOW, B. BISTER, D. BISCHOFF, R. D. SUSSMUTH and H. P. FIEDLER., 2004. Abyssomicins, inhibitors of the para-aminobenzoic acid pathway produced by the marine *Verrucosispora* strain AB-18-032. *Journal of Antibiotics (Tokyo),* **57**(4), pp.271-9.

RIGALI, S., TITGEMEYER, F., BARENDS, S., MULDER, S., THOMAE, A.W., HOPWOOD, D.A. AND VAN WEZEL, G.P., 2008. Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by Streptomyces. *EMBO Reports*, **9**(7), pp.670-675.

RIGALI, S., ANDERSSEN, S., NAÔMÉ, A. AND VAN WEZEL, G.P., 2018. Cracking the Regulatory Code of Biosynthetic Gene Clusters as a Strategy for Natural Product Discovery. *Biochemical pharmacology*.

ROJAS-CHERTÓ, M., KASPER, P.T., WILLIGHAGEN, E.L., VREEKEN, R.J., HANKEMEIER, T. AND REIJMERS, T.H., 2011. Elemental composition determination based on MS n. *Bioinformatics*, **27**(17), pp.2376-2383.

RUTLEDGE, P.J. AND CHALLIS, G.L., 2015. Discovery of microbial natural products by activation of silent biosynthetic gene clusters. *Nature Reviews Microbiology*, 1**3**(8), p.509.

RYDING, N.J., ANDERSON, T.B. AND CHAMPNESS, W.C., 2002. Regulation of the Streptomyces coelicolor calcium-dependent antibiotic by *abs*A, encoding a cluster-linked two-component system. *Journal of Bacteriology*, **184**(3), pp.794-805.

SALERNO, P., PERSSON, J., BUCCA, G., LAING, E., AUSMEES, N., SMITH, C.P. AND FLÄRDH, K., 2013. Identification of new developmentally regulated genes involved in *Streptomyces coelicolor* sporulation. *BMC Microbiology*, 13(1), p.281.

SAMBROOK, J., and D. W. RUSSELL. 2006. Agarose Gel Electrophoresis. *Cold Spring Harbor Protocol*s. 2006: pdb. prot4020.

SAMSON, R.A., SEIFERT, K.A., KUIJPERS, A.F., HOUBRAKEN, J.A.M.P. AND FRISVAD, J.C., 2004. Phylogenetic analysis of *Penicillium* subgenus

Penicillium using partial β-tubulin sequences. *Studies in Mycology*, **49**(1), pp.175-200.

SARKER, S.D. AND NAHAR, L., 2012. Hyphenated techniques and their applications in natural products analysis. In *Natural Products Isolation* (pp. 301-340). Humana Press.

SASHIDHARA, K.V. AND ROSAIAH, J.N., 2007. Various dereplication strategies using LC-MS for rapid natural product lead identification and drug discovery. *Natural Product Communications*, **2**(2), pp.193-202.

SAUNDERS, G. AND HOLT, G. 1982. Far Ultraviolet Light Sensitive Derivatives of *Streptomyces clavuligerus*. *Microbiology*, **128**(2), pp.381-385.

SAUNDERS, G., ALLSOP, A. AND HOLT, G., 1982. Modern developments in mutagenesis. *Journal of Chemical Technology and Biotechnology*, **32**(1), pp.354-364.

SAWANA, A., ADEOLU, M. AND GUPTA, R.S., 2014. Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species. *Frontiers in genetics*, **5**, p.429.

SCHÄBERLE, T.F., ORLAND, A. AND KÖNIG, G.M., 2014. Enhanced production of undecylprodigiosin in Streptomyces coelicolor by co-cultivation with the corallopyronin A-producing myxobacterium, Corallococcus coralloides. *Biotechnology Letters*, **36**(3), pp.641-648.

SCHMIDT, E.W., NELSON, J.T., RASKO, D.A., SUDEK, S., EISEN, J.A., HAYGOOD, M.G. AND RAVEL, J., 2005. Patellamide A and C biosynthesis by a microcin-like pathway in Prochloron didemni, the cyanobacterial symbiont of *Lissoclinum patella*. *Proceedings of the National Academy of Sciences of the United States of America*, **102**(20), pp.7315-7320.

SCHROECKH, V., SCHERLACH, K., NÜTZMANN, H.W., SHELEST, E., SCHMIDT-HECK, W., SCHUEMANN, J., MARTIN, K., HERTWECK, C. AND BRAKHAGE, A.A., 2009. Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proceedings of the National Academy of Sciences*, **106**(34), pp.14558-14563.

SIDDIQUE, S., SYED, Q., ADNAN, A. AND QURESHI, F.A., 2014. Production and screening of high yield Avermectin B1b mutant of Streptomyces avermitilis 41445 Through Mutagenesis. *Jundishapur Journal of Microbiology*, **7**(2).

SIDEBOTTOM, A.M., JOHNSON, A.R., KARTY, J.A., TRADER, D.J. AND CARLSON, E.E., 2013. Integrated metabolomics approach facilitates

discovery of an unpredicted natural product suite from Streptomyces coelicolor M145. *ACS Chemical Biology*, **8**(9), pp.2009-2016.

SILVER, L. L. 2011. Challenges of antibacterial discovery. *Clinical Microbiology Reviews,* **24**(1), pp.71-109.

SINGH, B. K. and C. A. MACDONALD. 2010. Drug discovery from uncultivable microorganisms. *Drug Discovery Today,* **15**(17-18), pp.792-9.

SINGH, S., PRASAD, P., SUBRAMANI, R. AND AALBERSBERG, W., 2014. Production and purification of a bioactive substance against multi-drug resistant human pathogens from the marine-sponge-derived Salinispora sp. *Asian Pacific Journal of Tropical Biomedicine*, **4**(10), pp.825-831.

SHAO, Z., LUO, Y., & ZHAO, H.,2011. Rapid Characterization and Engineering of Natural Product Biosynthetic Pathways via DNA Assembler. *Molecular BioSystems*, ***7***(4), pp.1056–1059.

SHAO, Z., RAO, G., LI, C., ABIL, Z., LUO, Y. AND ZHAO, H., 2013. Refactoring the silent spectinabilin gene cluster using a plug-and-play scaffold. *ACS Synthetic Biology*, **2**(11), pp.662-669.

SHEN, B., 2015. A new golden age of natural products drug discovery. *Cell*, **163**(6), pp.1297-1300.

SHEPHERD, M.D., KHAREL, M. K., BOSSERMAN, M. A., AND ROHR, J. 2010. Laboratory maintenance of *Streptomyces* species. *Current Protocols in Microbiology,* Chapter 10, Unit 10E.11, Willy, New York.

SHERIDAN, H., KRENN, L., JIANG, R., SUTHERLAND, I., IGNATOVA, S., MARMANN, A., LIANG, X. AND SENDKER, J., 2012. The potential of metabolic fingerprinting as a tool for the modernisation of TCM preparations. *Journal of Ethnopharmacology*, **140**(3), pp.482-491.

SHIBAI, A., TAKAHASHI, Y., ISHIZAWA, Y., MOTOOKA, D., NAKAMURA, S., YING, B.W. AND TSURU, S., 2017. Mutation accumulation under UV radiation in Escherichia coli. *Scientific Reports*, **7**(1), p.14531.

SHIM, S.H., BALTRUSAITIS, J., GLOER, J.B. AND WICKLOW, D.T., 2011. Phomalevones A− C: dimeric and pseudodimeric polyketides from a fungicolous Hawaiian isolate of *Phoma* sp.(Cucurbitariaceae). *Journal of Natural Products*, **74**(3), pp.395-401.

SHIN, Y.G. AND VAN BREEMEN, R.B., 2001. Analysis and screening of combinatorial libraries using mass spectrometry. *Biopharmaceutics & Drug Disposition*, **22**(7-8), pp.353-372.

STONESIFER, J.I.L.L. AND BALTZ, R.H., 1985. Mutagenic DNA repair in Streptomyces. *Proceedings of the National Academy of Sciences*, **82**(4), pp.1180-1183.

SHRESTHA, L., KAYAMA, S., SASAKI, M., KATO, F., HISATSUNE, J., TSURUDA, K., KOIZUMI, K., TATSUKAWA, N., YU, L., TAKEDA, K. AND SUGAI, M., 2016. Inhibitory effects of antibiofilm compound 1 against *Staphylococcus aureus* biofilms. *Microbiology and Immunology*, **60**(3), pp.148-159.

SKROPETA, D. AND WEI, L., 2014. Recent advances in deep-sea natural products. *Natural Product Reports*, **31**(8), pp.999-1025.

SONG, L., JENNER, M., MASSCHELEIN, J., JONES, C., BULL, M.J., HARRIS, S.R., HARTKOORN, R.C., VOCAT, A., ROMERO-CANELON, I., COUPLAND, P. AND WEBSTER, G., 2017. Discovery and biosynthesis of gladiolin: a *Burkholderia gladioli* antibiotic with promising activity against *Mycobacterium tuberculosis*. *Journal of the American Chemical Society*, **139**(23), pp.7974-7981.

SONG, Y., LIU, G., LI, J., HUANG, H., ZHANG, X., ZHANG, H. AND JU, J., 2015. Cytotoxic and antibacterial angucycline-and prodigiosin-analogues from the deep-sea derived *Streptomyces* sp. SCSIO 11594. *Marine Drugs*, **13**(3), pp.1304-1316.

STEPHANOPOULOS, G., ALPER, H. AND MOXLEY, J., 2004. Exploiting biological complexity for strain improvement through systems biology. *Nature Biotechnology*, **22**(10), p.1261.

STEWART, M., CAPON, R.J., WHITE, J.M., LACEY, E., TENNANT, S., GILL, J.H. AND SHADDOCK, M.P., 2004. Rugulotrosins A and B: Two new antibacterial metabolites from an Australian isolate of a *Penicillium* sp. *Journal of Natural Products*, **67**(4), pp.728-730.

STEWART, E.J., 2012. Growing unculturable bacteria. *Journal of Bacteriology*, **194**(16), pp.4151-4160.

STEYN, P. ED., 2012. The biosynthesis of mycotoxins: a study in secondary metabolism. Elsevier.

STOLL, A., RENZ, J. AND BRACK, A., 1952. On yellow dyes in ergot. 11. Communication on antibacterial substances. Hel*vetica Chimica Acta* , **35**(6), pp.2022-2034.

STREGE, M.A., 1999. High-performance liquid chromatographic–electrospray ionization mass spectrometric analyses for the integration of natural products with modern high-throughput screening. *Journal of Chromatography B: Biomedical Sciences and Applications,* **725**(1), pp.67-78.

SUBBAN, K., SUBRAMANI, R. AND JOHNPAUL, M., 2013. A novel antibacterial and antifungal phenolic compound from the endophytic fungus Pestalotiopsis mangiferae. *Natural product research*, **27**(16), pp.1445-1449.

SUSIĆ, E., 2004. Mechanisms of resistance in Enterobacteriaceae towards beta-lactamase antibiotics. *Acta medica Croatica: casopis Hravatske akademije medicinskih znanosti*, **58**(4), pp.307-312.

TAKANO, E., GRAMAJO, H.C., STRAUCH, E., ANDRES, N., WHITE, J. AND BIBB, M.J., 1992. Transcriptional regulation of the redD transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3 (2). *Molecular Microbiology*, **6**(19), pp.2797-2804.

TAKANO, E., TAO, M., LONG, F., BIBB, M.J., WANG, L., LI, W., BUTTNER, M.J., BIBB, M.J., DENG, Z.X. AND CHATER, K.F., 2003. A rare leucine codon in adpA is implicated in the morphological defect of bldA mutants of *Streptomyces coelicolor*. *Molecular Microbiology*, **50**(2), pp.475-486.

TAYLOR, P. W. 2013. Alternative natural sources for a new generation of antibacterial agents. *International Journal of Antimicrobial Agents,* **42**(3), pp.195-201.

TRACANNA, V., DE JONG, A., MEDEMA, M.H. AND KUIPERS, O.P., 2017. Mining prokaryotes for antimicrobial compounds: from diversity to function. *FEMS Microbiology Reviews*, **41**(3), pp.417-429.

TREMBLAY, N., HILL, P., CONWAY, K.R. AND BODDY, C.N., 2016. The use of clustermine360 for the analysis of polyketide and nonribosomal peptide biosynthetic pathways. *Nonribosomal Peptide and Polyketide Biosynthesis: Methods and Protocols*, pp.233-252.

T-TEST CALCULATOR, QuickCalcs, GRAPHPAD (ON LINE), 2018. [Accessed 09 May 2018] Availbale from: https://www.graphpad.com/quickcalcs/ttest1.cfm

U-Z

URBAN, A., ECKERMANN, S., FAST, B., METZGER, S., GEHLING, M., ZIEGELBAUER, K., RÜBSAMEN-WAIGMANN, H. AND FREIBERG, C., 2007. Novel whole-cell antibiotic biosensors for compound discovery. *Applied and Environmental Microbiology*, **73**(20), pp.6436-6443.

VALGAS, C., SOUZA, S.M.D., SMÂNIA, E.F. AND SMÂNIA JR, A., 2007. Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology*, **38**(2), pp.369-380

VAN MEER, G., VOELKER, D.R. AND FEIGENSON, G.W., 2008. Membrane lipids: where they are and how they behave. *Nature Reviews Molecular Cell Biology*, **9**(2), p.112.

VENTOLA, C. L., 2015.) The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *Pharmacy and Therapeutics*, **40**(4), 277–283.

VICKERS, A. A., A. J. O'NEILL and I. CHOPRA. 2007. Emergence and maintenance of resistance to fluoroquinolones and coumarins in *Staphylococcus aureus*: predictions from in vitro studies. *Journal of Antimicrobial Chemotherapy,* **60**(2), pp.269-273.

WALLEN, L.L., LYONS, A.J. and PRIDHAM, T.G., 1980. Antimicrobial activity of patulin derivatives: a preliminary report. *The Journal of Antibiotics*, ***33***(7), pp.767-769.

WANG, J., NONG, X.H., ZHANG, X.Y., XU, X.Y., AMIN, M. AND QI, S.H., 2017. Screening of Anti-Biofilm Compounds from Marine-Derived Fungi and the Effects of Secalonic Acid D on *Staphylococcus aureus* Biofilm. *Journal Of Microbiology and Biotechnology*, **27**(6), pp.1078-1089.

WANG, L., KUANG, L., PAN, X., LIU, J., WANG, Q., DU, B., LI, D., LUO, J., LIU, M., HOU, A. AND QIAN, M., 2010. Isoalvaxanthone inhibits colon cancer cell proliferation, migration and invasion through inactivating Rac1 and AP-1. *International Journal of Cancer*, **127**(5), pp.1220-1229.

WANG, L.N., DI, W.J., ZHANG, Z.M., ZHAO, L.L., ZHANG, T., DENG, Y.R. AND YU, L.Y., 2016. Small-molecule inhibitors of the tuberculosis target, phenylalanyl-t RNA synthetase from *Penicillium griseofulvum* CPCC-400528. *Cogent Chemistry*, ***2***(1), p.1181536.

WANG, M., J. J. CARVER, V. V. PHELAN, L. M. SANCHEZ, N. GARG, Y. PENG, D. D. NGUYEN, J. WATROUS, C. A. KAPONO, T. LUZZATTO-KNAAN, C. PORTO, A. BOUSLIMANI, A. V. MELNIK, M. J. MEEHAN, W.-T. LIU, M. CRUSEMANN, P. D. BOUDREAU, E. ESQUENAZI, M. SANDOVAL-CALDERON, R. D. KERSTEN, L. A. PACE, R. A. QUINN, K. R. DUNCAN, C.-C. HSU, D. J. FLOROS, R. G. GAVILAN, K. KLEIGREWE, T. NORTHEN, R. J. DUTTON, D. PARROT, E. E. CARLSON, B. AIGLE, C. F. MICHELSEN, L. JELSBAK, C. SOHLENKAMP, P. PEVZNER, A. EDLUND, J. MCLEAN, J. PIEL, B. T. MURPHY, L. GERWICK, C.-C. LIAW, Y.-L. YANG, H.-U. HUMPF, M. MAANSSON, R. A. KEYZERS, A. C. SIMS, A. R. JOHNSON, A. M. SIDEBOTTOM, B. E. SEDIO, A. KLITGAARD, C. B. LARSON, C. A. BOYA P, D. TORRES-MENDOZA, D. J. GONZALEZ, D. B. SILVA, L. M. MARQUES, D. P. DEMARQUE, E. POCIUTE, E. C. O'NEILL, E. BRIAND, E. J. N. HELFRICH, E. A. GRANATOSKY, E. GLUKHOV, F. RYFFEL, H. HOUSON, H. MOHIMANI, J. J. KHARBUSH, Y. ZENG, J. A. VORHOLT, K. L. KURITA, P. CHARUSANTI, K. L. MCPHAIL, K. F. NIELSEN, L. VUONG, M. ELFEKI, M. F. TRAXLER, N. ENGENE, N. KOYAMA, O. B. VINING, R.

BARIC, R. R. SILVA, S. J. MASCUCH, S. TOMASI, S. JENKINS, V. MACHERLA, T. HOFFMAN, V. AGARWAL, P. G. WILLIAMS, J. DAI, R. NEUPANE, J. GURR, A. M. C. RODRIGUEZ, A. LAMSA, C. ZHANG, K. DORRESTEIN, B. M. DUGGAN, J. ALMALITI, P.-M. ALLARD, P. PHAPALE*, et al.* 2016. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nature Biotechnology,* **34**(8), pp.828-837.

WARIDEL, P., WOLFENDER, J.L., NDJOKO, K., HOBBY, K.R., MAJOR, H.J. AND HOSTETTMANN, K., 2001. Evaluation of quadrupole time-of-flight tandem mass spectrometry and ion-trap multiple-stage mass spectrometry for the differentiation of C-glycosidic flavonoid isomers. *Journal of Chromatography A*, **926**(1), pp.29-41.

WEBER, T., K. BLIN, S. DUDDELA, D. KRUG, H. U. KIM, R. BRUCCOLERI, S. Y. LEE, M. A. FISCHBACH, R. MULLER, W. WOHLLEBEN, R. BREITLING, E. TAKANO and M. H. MEDEMA. 2015. antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Research,* **43**(W1), pp.W237-43.

WEBER, T., 2014. *In silico* tools for the analysis of antibiotic biosynthetic pathways. *International Journal of Medical Microbiology*, **304**(3-4), pp.230-235.

WEBER, T. AND KIM, H.U., 2016. The secondary metabolite bioinformatics portal: computational tools to facilitate synthetic biology of secondary metabolite production. *Synthetic and Systems Biotechnology*, **1**(2), pp.69-79.

WEZEMAN, T. AND MASTERS, K.S., 2015. Xanthones are Privileged Scaffolds in Medicinal Chemistry–but are they Over-privileged?. In *Privileged Scaffolds in Medicinal Chemistry* (pp. 312-347).

WEZEMAN, T., BRÄSE, S. AND MASTERS, K.S., 2015. Xanthone dimers: a compound family which is both common and privileged. *Natural Product Reports*, **32**(1), pp.6-28.

WIDYARINI, S., SPINKS, N., HUSBAND, A.J. AND REEVE, V.E., 2001. Isoflavonoid compounds from red clover (Trifolium pratense) protect from inflammation and immune suppression induced by UV radiation. *Photochemistry and Photobiology*, **74**(3), pp.465-470.

WISECAVER, J.H., SLOT, J.C. AND ROKAS, A., 2014. The evolution of fungal metabolic pathways. *PLoS Genetics*, **10**(12), p. e1004816.

WHO. 2017. WHO publishes list of bacteria for which new antibiotics are urgently needed. *Saudi Medical Journal,* **38**(4), pp.444-445.

WU, C.J., LI, C.W., GAO, H., HUANG, X.J. AND CUI, C.B., 2017. Penicimutamides D–E: two new prenylated indole alkaloids from a mutant of the marine-derived Penicillium purpurogenum G59. RSC Advances, **7**(40), pp.24718-24722.

WRIGHT, G.D., 2010. Q&A: Antibiotic resistance: where does it come from and what can we do about it?. *BMC Biology*, **8**(1), p.123.

WRIGHT, P.M., SEIPLE, I.B. AND MYERS, A.G., 2014. The evolving role of chemical synthesis in antibacterial drug discovery. *Angewandte Chemie International Edition*, **53**(34), pp.8840-8869.

WRIGHT, G.D., 2017. Opportunities for natural products in 21 st century antibiotic discovery. *Natural Product Reports*, 34(7), pp.694-701.

WYCHE, T.P., PIOTROWSKI, J.S., HOU, Y., BRAUN, D., DESHPANDE, R., MCILWAIN, S., ONG, I.M., MYERS, C.L., GUZEI, I.A., WESTLER, W.M. AND ANDES, D.R., 2014. Forazoline A: Marine-Derived Polyketide with Antifungal In Vivo Efficacy. Angewandte Chemie International Edition, **53**(43), pp.11583-11586.

Xia, M.W., Cui, C.B., Li, C.W. and Wu, C.J., 2014. Three new and eleven known unusual C25 steroids: Activated production of silent metabolites in a marine-derived fungus by chemical mutagenesis strategy using diethyl sulphate. *Marine Drugs*, **12**(3), pp.1545-1568.

Xu, J., Zhang, J., Zhuo, J., Li, Y., Tian, Y. and Tan, H., 2017. Activation and mechanism of a cryptic oviedomycin gene cluster via the disruption of a global regulatory gene, adpA, in Streptomyces ansochromogenes. *Journal of Biological Chemistry*, **292**(48), pp.19708-19720.

Xu, Z., Wang, Y., Chater, K.F., Ou, H.Y., Xu, H.H., Deng, Z. and Tao, M., 2017. Large-scale transposition mutagenesis of Streptomyces coelicolor identifies hundreds of genes influencing antibiotic biosynthesis. Applied and *Environmental Microbiology*, **83**(6), pp.e02889-16.

YAMANAKA, K., REYNOLDS, K.A., KERSTEN, R.D., RYAN, K.S., GONZALEZ, D.J., NIZET, V., DORRESTEIN, P.C. AND MOORE, B.S., 2014. Direct cloning and refactoring of a silent lipopeptide biosynthetic gene cluster yields the antibiotic taromycin A. *Proceedings of the National Academy of Sciences*, **111**(5), pp.1957-1962.

YANG, J.Y., SANCHEZ, L.M., RATH, C.M., LIU, X., BOUDREAU, P.D., BRUNS, N., GLUKHOV, E., WODTKE, A., DE FELICIO, R., FENNER, A. AND WONG, W.R., 2013. Molecular networking as a dereplication strategy. *Journal Of Natural Products*, **76**(9), pp.1686-1699.

YANG, G., SANDJO, L., YUN, K., LEUTOU, A.S., KIM, G.D., CHOI, H.D., KANG, J.S., HONG, J. AND SON, B.W., 2011. Flavusides A and B, antibacterial cerebrosides from the marine-derived fungus *Aspergillus flavus*. *Chemical and Pharmaceutical Bulletin*, **59**(9), pp.1174-1177.

YANG, Y.L., XU, Y., STRAIGHT, P. AND DORRESTEIN, P.C., 2009. Translating metabolic exchange with imaging mass spectrometry. *Nature Chemical Biology*, **5**(12), pp. nchembio-252.

YU, C. Y., G. Y. ANG, P. S. CHIN, Y. F. NGEOW, W. F. YIN and K. G. CHAN. 2016. Emergence of mcr-1-mediated colistin resistance in *Escherichia coli* in Malaysia. *International Journal of Antimicrobial Agents,* **47**(6), pp.504-505.

YULIANA, N.D., KHATIB, A., VERPOORTE, R., AND CHOI, Y.H., 2011. Comprehensive extraction method integrated with NMR metabolomics: a new bioactivity screening method for plants, adenosine A1 receptor binding compounds in Orthosiphon stamineus benth. *Analytical Chemistry,* **83**(17):6902–6906

ZABURANNYI, N., RABYK, M., OSTASH, B., FEDORENKO, V. AND LUZHETSKYY, A., 2014. Insights into naturally minimised *Streptomyces albus* J1074 genome. *BMC Genomics*, **15**(1), p.97.

ZENG, K. J., Y. H. DOI, S. PATIL, X. HUANG AND G. B. TIAN. 2016. Emergence of the plasmid-mediated mcr-1 gene in colistin-resistant *Enterobacter aerogenes* and *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy,* **60**(6), pp.3862-3863.

ZERIKLY, M. AND CHALLIS, G.L., 2009. Strategies for the discovery of new natural products by genome mining. *ChemBioChem*, **10**(4), pp.625-633.

ZIEMERT, N., A. LECHNER, M. WIETZ, N. MILLAN-AGUINAGA, K. L. CHAVARRIA AND P. R. JENSEN. 2014. Diversity and evolution of secondary metabolism in the marine actinomycete genus *Salinispora*. *Proceedings of the National Academy of Sciences of the United States of America,* **111**(12), pp.E1130-E1139.

ZIN, N.M., BABA, M.S., ZAINAL-ABIDIN, A.H., LATIP, J., MAZLAN, N.W. AND EDRADA-EBEL, R., 2017. Gancidin W, a potential low-toxicity antimalarial agent isolated from an endophytic *Streptomyces* SUK10. *Drug Design, Development and Therapy*, **11**, p.351.

ZHANG, A., SUN, H., WANG, P., HAN, Y. AND WANG, X., 2012. Modern analytical techniques in metabolomics analysis. *Analyst*, **137**(2), pp.293-300.

ZHANG, J.Y., TAO, L.Y., LIANG, Y.J., YAN, Y.Y., DAI, C.L., XIA, X.K., SHE, Z.G., LIN, Y.C. AND FU, L.W., 2009. Secalonic acid D induced leukemia cell

apoptosis and cell cycle arrest of G1 with involvement of GSK-3β/β-catenin/c-Myc pathway*. Cell Cycle*, **8**(15), pp.2444-2450.

ZHANG, M.M., WONG, F.T., WANG, Y., LUO, S., LIM, Y.H., HENG, E., YEO, W.L., COBB, R.E., ENGHIAD, B., ANG, E.L. AND ZHAO, H., 2017. CRISPR–Cas9 strategy for activation of silent Streptomyces biosynthetic gene clusters. *Nature Chemical Biology*, **13**(6), p.607.

ZHANG, W., KROHN, K., FLÖRKE, U., PESCITELLI, G., DI BARI, L., ANTUS, S., KURTÁN, T., RHEINHEIMER, J., DRAEGER, S. AND SCHULZ, B., 2008. New Mono-and Dimeric Members of the Secalonic Acid Family: Blennolides A–G Isolated from the Fungus *Blennoria sp. Chemistry-A European Journal,* **14**(16), pp.4913-4923.

ZHAO, X. Q. 2011. Genome-based studies of marine microorganisms to maximize the diversity of natural products discovery for medical treatments. *Evidence-Based Complementary and Alternative Medicine*, 2011.

ZHONG, X., TIAN, Y., NIU, G. AND TAN, H., 2013. Assembly and features of secondary metabolite biosynthetic gene clusters in *Streptomyces ansochromogenes*. *Science China Life Sciences*, **56**(7), pp.609-618.

ZUREK, G., GERMANUS, A., SHEN, M., BAESSMANN, C. AND INGENDOH, A. 2003. Novel strategies for metabolite identification using HPLC-ion trap mass spectrometry. *Lc Gc North America*. **21**, pp. 13-14.