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METABOLIC PATHWAY ENGINEERING OF ACTINOMYCETES FOR NOVEL ANTIBIOTICS DISCOVERY

Bikash Baral

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For everyone contemplating to heal
the Mother Earth!

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ABSTRACT

Microbes harbouring a profound wealth of chemical space have instigated tremendous attention for developing crucial therapeutic drugs. The genes encoding the enzymes responsible for synthesizing these specialized metabolites are often organized in so-called biosynthetic gene clusters (BGCs). While these clusters are potentially capable of producing novel drug candidate, most of them remain dormant in natural environmental conditions (or settings). Microorganisms seldom synthesize substantial quantities of the desired molecules in natural settings. Harnessing the dormant compound production requires careful optimization of the host cellular machinery, which can be accomplished by thorough engineering of silent biosynthetic pathways. My target, the genus *Streptomyces* is endowed with tremendous abilities to secrete a diverse array of metabolites. Besides, *in-silico* analysis of their genomic sequences reveals enormous potential to generate novel metabolites not biosynthesized in natural environmental settings. Realizing such bountiful resources, I attempt to unveil *Streptomyces*' true potential to generate novel metabolites by using various approaches.

In the present dissertation, various novel approaches have allowed me to unveil novel specialized metabolites encoded by otherwise silent biosynthetic clusters. For this, (i) I developed single cell mutant selection (SCMS) platform, where mutants harboring a silent promoter are probed with a double reporter system using classical mutagenesis techniques. Mutants were sorted using FACS based on expression of reporter genes and mutants generated a novel metabolite with a distinct chemical scaffold, referred to as mutaxanthene. (ii) Next approach involved binary physical interaction studies between dead yeast and *Streptomyces* where the contact induced production of prodigiosin. My studies identified a master-regulator, namely *mbkZ,* for its regulatory roles in prodigiosin production in different hosts (*S. coelicolor* and *Streptomyces* sp. MBK6). (iii) Third approach exploited CRISPR/Cas9 system to unveil the functional role of *sdmA* within the showdomycin biosynthetic pathway. (iv) Final approach revealed the characterization of new bacterial lineage (*Streptomonospora* sp. PA3) isolated from the high-salt environment, which helped me to isolate and identify a novel polyketide persiamycin A. Using these different approaches allowed me to unveil the secret knowledge sealed within biosynthetic pathways of the studied organisms.

In a nutshell, adopting these techniques has helped me discover and characterize novel metabolites. I believe these strategies may aid in the fight against antimicrobial resistance and speed up the drug discovery process. Furthermore, this dissertation has not only implications for future engineering of *Streptomyces* to increase metabolites production, but it also illustrates a SCMS state-of-art approach for generating novel therapeutic leads.

TURUN YLIOPISTO
Teknillinen tiedekunta
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BIKASH BARAL: Aktinomykeettien Aineenvaihduntareittien Muokkaus Uusien Antibioottien Löytämiseksi
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TIIVISTELMÄ

Mikrobien tuottamat monimuotoiset luonnonyhdisteet ovat herättäneet suurta kiinnostusta lääkekehityksessä. Näiden erikoistuneiden metaboliittien tuotannosta vastaavia entsyymejä koodaavat geenit ovat yleensä järjestyneet niin sanottuiksi biosynteettisiksi geeniryppäiksi (BGC, *engl.* biosynthetic gene cluster). Nämä geeniryppäät saattavat tuottaa vielä tuntemattomia metaboliitteja, mutta yleensä ne ovat hiljaisia luonnollisessa ympäristössä. Yleensä mikro-organismit eivät tuota haluttua yhdistettä merkittäviä määriä luonnollisissa olosuhteissa. Näiden hiljaisten yhdisteiden hyödyntäminen lääkeaineiden kehityksessä vaatii solukoneiston huolellista optimointia, mikä voidaan saavuttaa muokkaamalla hiljaisia biosynteesireittejä. Kohdeorganismimme *Streptomyces* -bakteerit kykenevät tuottamaan lukuisia erilaisia sekundäärimetaboliitteja, jotka ovat kemialliselta rakenteeltaan erittäin vaihtelevia. Tämän lisäksi genomisekvenssien analyysi on paljastanut lukuisia lupaavia hiljaisia geeniklustereita, jotka saattaisivat aktivoituina tuottaa aikaisemmin tuntemattomia yhdisteitä. Tämän työn tarkoitus on käyttää useita erilaisia tekniikoita tämän hiljaisen biosynteettisen potentiaalin valjastamiseen.

Väitöskirjatyössä käytin useita uusia menetelmiä hiljaisten geeniryppäiden koodaamien uusien yhdisteiden tuottamiseksi. Tätä tarkoitusta varten (i) kehitin yksisolujen mutanttivalinta (SCMS, *engl.* single cell mutant selection) alusta – menetelmän, missä hiljaisen geeniryppään aktiivisuutta seurataan tuplareportterisysteemillä. Seuloin menetelmällä mutanttikirjastoja reportterigeenien ilmenemisen perusteella FACS –laitteistoilla ja tuotin erityisen kemiallisen rakenteen omaavia mutaxanthene -yhdisteitä. (ii) Seuraavaksi tutkin hiivojen ja streptomykeettien fyysisen vuorovaikutuksen vaikutusta prodigiosiini –yhdisteen tuottoon. Tutkimukseni paljasti säätelygeeni *mbkZ*:n roolin prodigiosiinien tuotossa kahdessa eri isäntäkannassa (*S. coelicolor* ja *Streptomyces* sp. MBK6). iii) Kolmannessa menetelmässä käytin CRISPR/Cas9-menetelmää selvittääkseni *sdmA* geenin roolin showdomysiinin biosynteesireitillä. iv) Viimeisenä menetelmänä eristin uuden halofiilisen bakteerikannan (*Streptomonospora* sp. PA3) korkean suolapitoisuuden kasvuympäristöstä meren pohjasta. Kannasta eristettiin uusi persiamysiini A polyketidi. Näiden erilaisten lähestymistapojen avulla pystyin paljastamaan tutkittujen organismien biosynteettisten reittien sisälle suljetut salaisuudet.

Lyhyesti, olemme pystyneet löytämään ja karakterisoimaan uusia metaboliitteja käyttämiemme tekniikoiden avulla. Uskomme, että käyttämämme strategiat auttavat kamppailuissa antibioottiresistenssiä vastaan ja nopeuttamaan uusien lääkkeiden löytämistä. Tämän lisäksi tutkielman tulokset sekä auttavat sekundaarimetabolliittien tuotannon tehostamista tulevaisuudessa streptomykeettejä muokkaamalla, että havainnollistaa SCMS – menetelmän käyttökelpoisuuden uusien terapeuttisten yhdisteiden tuotossa.

Table of Contents

Abbreviations

AA	Amino acid
ABC	ATP-binding cassettes
ACP	Acyl carrier protein
AMR	Antimicrobial resistance
antiSMASH	Antibiotics & Secondary Metabolite Analysis Shell
AT	Acyltransferase
BGC	Biosynthetic gene cluster
CAM	Chloramphenicol
Cas9	CRISPR associated protein-9
CDS	Coding Sequence
CRISPR	Clustered regularly interspaced short palindromic repeats
CSRs	Cluster-situated regulators
DiPaC	Direct pathway cloning
EMS	Ethylmethanesulphonate
FACS	Fluorescence-activated cell sorting
GFP	Green fluorescent protein
GlcNAc	*N*-acetylglucosamine
HTS	High-throughput screening
HPLC	High-performance liquid chromatography
Hyg	Hygromycin
kb	Kilo base-pairs
LA	Luria-bertani agar
LB	Lysogeny broth
LCHR	Linear-circular homologous recombination
LC-MS	Liquid chromatography-mass spectrometry
LLHR	Linear-linear homologous recombination
MEGA	Molecular evolutionary genetics analysis
MeOD	Methanol-d_4
MFS	Multi-facilitator transport proteins
MIBiG	Minimum Information about a Biosynthetic Gene Cluster
minPKS	Minimal polyketide synthase

MS	Mass spectrometry
neo	Kanamycin
NGS	Next-generation sequencing
NPs	Natural products
NRPS	Nonribosomal peptide synthetase
NTG	*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine
OD	Optical density
OSMAC	One strain many compounds (concept)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKS	Polyketide synthase
ppGpp	Guanosine tetraphosphate
PPtases	Phosphopantetheinyl transferase
RAST	Rapid annotations using subsystems technology
RGMS	Reporters-guided mutant selection
RNAP	RNA polymerase
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase-Polymerase chain reaction
SARP	*Streptomyces* antibiotic regulatory protein
SBGC	Silent biosynthetic gene cluster
SCMS	Single-cell mutant selection
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SM	Secondary metabolite
TAR	Transformation-associated recombination
UV/Vis	Ultraviolet-visible light

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Akhgari A*, **Baral B***, Koroleva A, Siitonen V, Fewer D, Melancon C, Rahkila J, Metsä-Ketelä M. Single cell mutant selection for metabolic engineering of actinomycetes (Submitted).

II **Baral B***, Siitonen V*, Laughlin, M, Yamada, K, Ilomäki M, Metsä-Ketelä, M, Niemi J. Differential regulation of undecylprodigiosin biosynthesis in the yeast-scavenging *Streptomyces* strain MBK6. *FEMS Microbiol. Letters,* 2021; fnab044.

III Palmu K, Rosenqvist P, Thapa K, Ilina Y, Siitonen V, **Baral B**, Mäkinen J, Belogurov G, Virta P, Niemi J, Metsä-Ketelä M. Discovery of the showdomycin gene cluster from *Streptomyces showdoensis* ATCC 15227 yields insight into the biosynthetic logic of *C*-nucleoside antibiotics. *ACS Chem Biol*. 2017; 12: 1472-1477.

IV Matroodi S, Siitonen V, **Baral B**, Yamada K, Akhgari A, Metsä-Ketelä M. Genotyping-guided discovery of persiamycin A from halophilic *Streptomonospora* sp. PA3. *Front. Microbiol*. 2020; 11: 1237.

*Equal contributions.

1 Introduction

1.1 Microbial therapeutics

Microbial natural products (NPs) or their derivatives possess exotic chemical structures derived from various biochemical sources [1]. These NPs are low-molecular-weight molecules (<3,000) and are often referred to as specialized metabolites rather than secondary metabolites (SMs) because of their remarkable biological and ecological functions [2], such as signaling molecules, antimicrobial agents, and bioregulators [3,4]. Microorganisms are prolific and efficient chemists, which generate NPs with attractive chemical scaffolds and diverse bioactivities [5,6]. Biosynthetic genes encoding the enzymes are responsible for producing NPs and are organized into biosynthetic gene clusters (BGC; a group of genes involved in NP synthesis). These BGCs can be transcriptionally silent (not expressed) or are cryptic (product of the BGC is unknown) depending on the environmental stimulus [7–9]. Because the metabolites produced by these silent biosynthetic clusters are functionally inaccessible, organisms harbouring them are believed to have a huge potential to generate novel molecules. These untapped producer strains could generate diverse small molecules with high bioactivity, ushering in a new era in microbial therapeutics [10].

NPs have huge biological/evolutionary functions and mediate ecological interactions (such as competition and predation), resource mutualism, cytoplasmic signaling [11], etc. However, NPs are mostly used as chemical weapons (antagonistic properties) against competing microbes. Their unique role and structure (skeletons) have made them a clear target for the development of antibiotic therapeutics [12].

The mortality rate caused by a rapid upsurge in antimicrobial resistance (AMR) is expected to surpass 10 million deaths per year by 2050 [13,14]. Moreover, our antibiotic arsenal is becoming more outdated due to the AMR displayed by the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) group of pathogens [15]. The pathogens resistance to existing antibiotics exemplifies a critical need for new antibiotics in modern medicine [12]. Fortunately, new advanced technologies employed to screen thousands of novel strains from extreme

environmental niches have enticed possibilities in tracing a potent strain with the capability to generate molecules with new chemical scaffolds [16–18].

Beyond the ability to produce critical antibiotics, it has become apparent that the chemical diversity afforded by *Streptomyces* can also be used in other therapeutics, including anti-cancer drugs. Doxorubicin and daunorubicin from *Streptomyces peucetius* are the most prominent and clinically significant antitumor agents that are in use to treat various cancers [19,20]. Other antitumor drugs discovered from *Streptomyces* include aclarubicin, epirubicin, pirarubicin, streptozotocin, and zorubicin [21,22]. Such tremendous antitumor metabolites obtained from these organisms imply that *Streptomyces* has been and will remain one of the crucial sources of developing anticancer drugs and is likely to be exploited extensively in the future.

1.2 History of antibiotics

Microbial domains are enriched with synthesizing structurally unique and biologically active NPs that have evolved over millions of years [23–25]. Penicillin's fortuitous discovery from *Penicillium notatum* in 1928 [26] and streptomycin from *Streptomyces griseus* in 1944 [27] marks the 'Golden Age' of antibiotic development. The use of soil microorganisms as a source for novel antibiotics has now remained the mainstay of drug discovery, in which a soil-dwelling bacterium, namely *Streptomyces*, has been chemically exploited in search of new chemical scaffolds.

As the pathogens' resistivity to antibiotics is on the rise, new antimicrobials are needed to combat the resistance. The antibiotics discovery void in therapeutics plus the rising superbugs has compelled the pharmaceutical industry to look for alternatives, leading to the development of synthetic antimicrobials. Sulfa drugs, quinolones, nitrofurans, and oxazolidinone are among the few antibiotics that are not NPs. Sulfa drugs, such as prontosils are synthetic drugs with a long history, having been created in 1929, long before penicillin was introduced to the market [28]. Thus, scientists may have sought for synthetic antibacterial chemicals before discovering the NPs.

Due to the difficulties in discovering novel antimicrobials, repurposing existing bioactive compounds might be a viable alternative. Drug repurposing (drug repositioning) approach detects new uses for authorized drugs that are not related to their earliest medical use. This strategy has a low risk of failure, a shorter drug development timeline, and requires less investment [29]. Several drugs have been repurposed by now, such as sildenafil citrate (Viagra), developed initially to cure hypertension, is repurposed to cure erectile dysfunction [30,31]. Furthermore, a combination therapy of different active ingredients may aid in the discovery of a

more effective molecule. For example, a combination therapy of clavulanic acid and amoxicillin can inhibit various serine β-lactamases, which would otherwise render most penicillin inactive [32].

Several bioactive antibiotics have been discovered and are now being evaluated for clinical trials for human consumption utilizing a variety of contemporary techniques. Because there are so many procedures to follow when releasing novel antibiotics, it takes a long time for a newly discovered drug lead to be developed into an effective drug ready to be distributed globally. For instance, a narrow spectrum pleuromutilin (inhibitor of protein synthesis) discovered in 1950 from *Clitopilus passeckerianus* was first commercialized in 2020 [33] (Fig. 1).

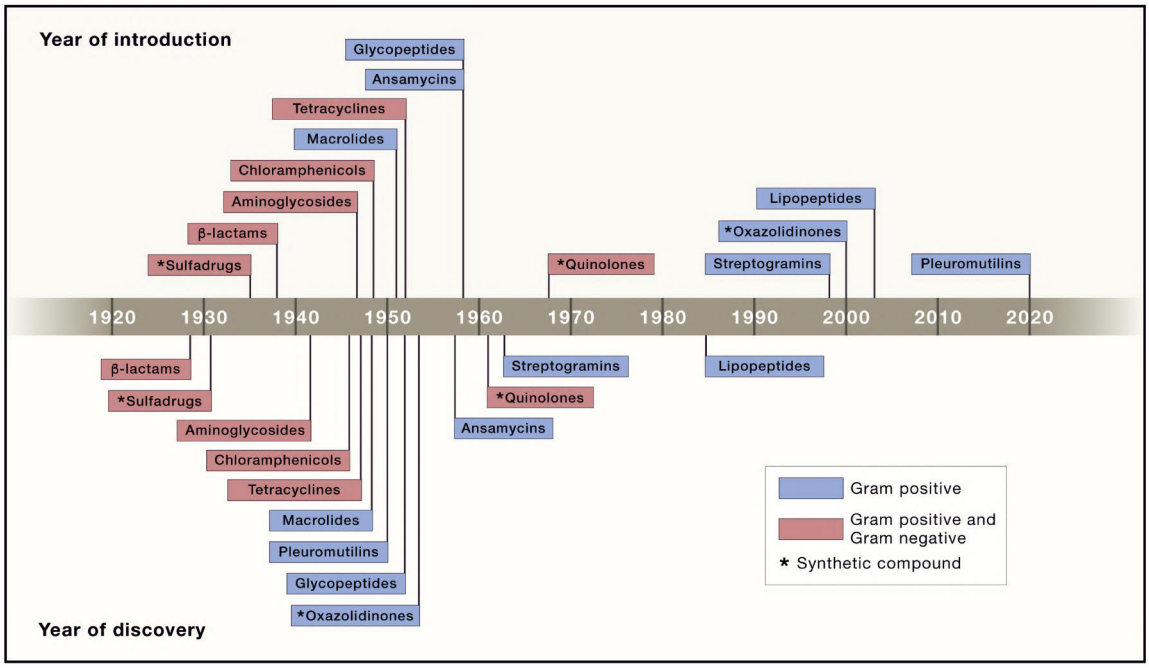

Figure 1. Antibiotics discovery timeline. The upper panel shows antibiotics' year of introduction in clinical practice, while the lower panel shows the year they were discovered. Antibiotics in red indicate broad-spectrum antibiotics. Synthetic medicines are indicated with an asterisk (*). Adapted from [33].

1.3 Natural products synthesizing microbes

Numerous microbes (bacteria, fungi, myxobacteria, and archaea) are housed in soil environments and have potential to generate diverse NPs in varying conditions [34–36]. Several thousands of 'hit molecules' showing an incredible array of unique chemical structures [37], complexity [38,39], and physiological functions [40] have been identified from these organisms. However, identifying the actual conditions

that could trigger metabolites production by a microbe is not trivial. Thus, microorganisms inhabiting exotic regions could be an attractive alternative for producing novel bioactive compounds. Microbes belonging to or isolated from marine sponge-related species [41–44], eukaryotes [45–47], animals/insects residing species (symbionts and/pathogenic organisms) [17,48,49], human microbiomes [50,51], hypogean [52,53], plants [54–56], and species from extremely harsh climatic settings, such as higher Himalayas [57,58] are possible candidates for novel NPs discovery. These areas/sources, which have received little attention, are established virgin regions with an increased likelihood of harbouring a plethora of abundant species with the most remarkable therapeutic leads [59].

The total number of compounds characterized from microbes exceeds 50,000, with bioactive NPs ranging between 22,000 and 23,000 [60]. However, only a tiny fraction (<1%) of them are in direct use in our daily life [61]. Most of these compounds belong to *Streptomyces* origin [62], followed by fungal, bacterial, and rare actinomycetes. *Circa* 45% (>10,000) of known bioactive compounds are produced by filamentous Actinomycetales that are of *Streptomyces* (7,600) and rare actinomycetes (2,500) origin. Microscopic fungi generate >8,600 (38%) of known microbial products [61]. Similarly, prokaryotes such as *Bacillus* and *Pseudomonas* sp. produce *ca.* 3,800 bioactive compounds (17% of total microbial metabolites). Until now, more than 2,140,000 NPs have been discovered from all species (including plants, microbes, etc.), and characterized depending on their functional and structural analysis as well as biosynthetic potentials [63].

Among several potent species for NPs production, *Streptomyces* continue to remain an excellent genus for the generation of NPs. The production of NPs from *Streptomyces* have received approval as antibiotics (erythromycin, tetracycline), as anticancer agents (bleomycin, doxorubicin) [64], immunosuppressants (ascomycin, rapamycin) [65], as antifungal compounds (amphotericin B, nystatin) [66], in diabetes treatment (acarbose), and as anthelmintic drugs (avermectin) [67]. Moreover, small molecules from *Streptomyces* have profound effects in eukaryotic systems. For instance, some small molecules induce eukaryotic cellular differentiation, inhibit apoptosis events [68], and compounds such as staurosporine act as kinase inhibitors with antitumor activity [69,70] (Fig. 2).

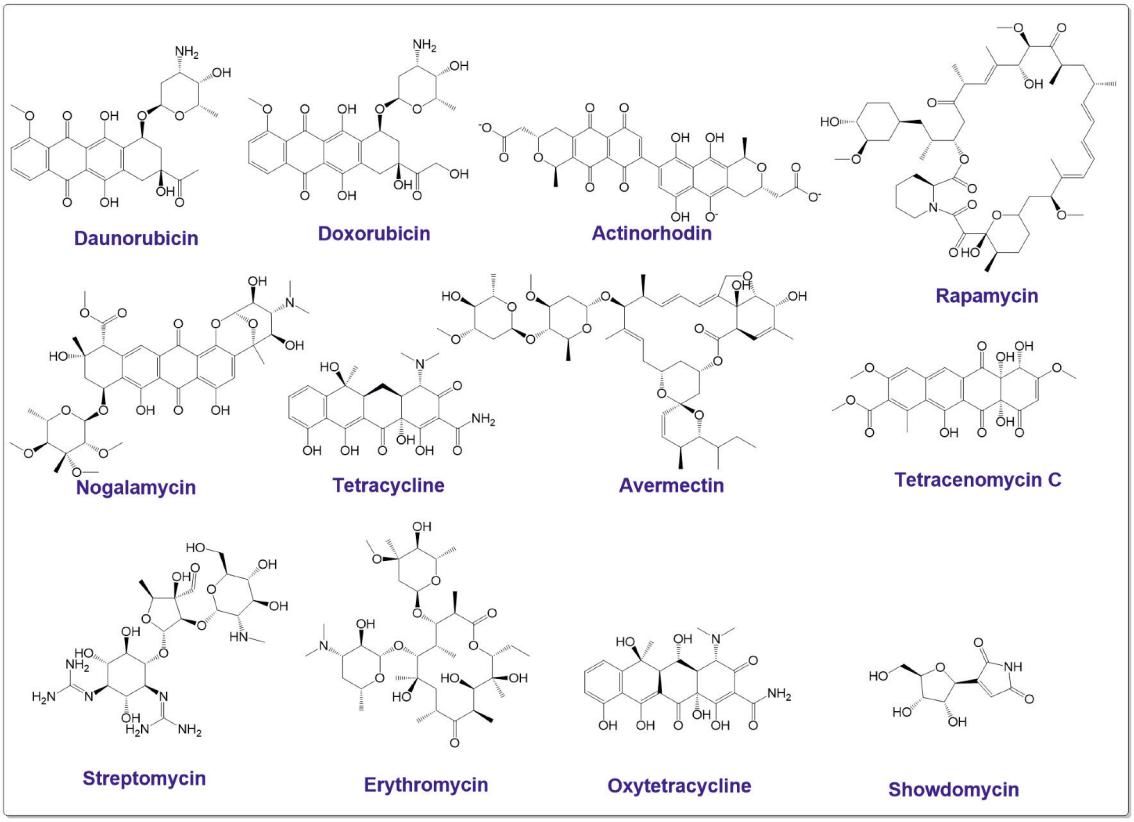

Figure 2. Examples of natural products from *Streptomyces* origin.

1.4 Antibiotics developmental pipeline

Modern drug production programs have been severely hampered by the fact that majority of new hits turn out to be previously known strains and compounds instead of new ones [71]. Estimates suggest that *ca.* 99% of hits in these randomly generated screenings are false positives [72,73]. Microbial-derived 'hit molecules' are in various stages of development [74], and the discovery of new synthetic analogs could revitalize the dried antibiotic discovery pipeline [26,75]. Since NPs are produced in limited amount and appear as mixtures in extracts, their isolation is tedious and demands lengthy preparation time [76]. Despite significant investment in the search for novel small molecules of microbial origin, high attrition rates have caused pharma companies to shift their efforts to developing libraries of synthetic compounds [75,77–79]. However, most new antibiotics are still NPs, and pharma investments for synthetic antibiotics did not lead to the development of any new drugs. Currently, utilizing various strategies, 41 new antibiotics with high efficacy are in various phases of clinical development [80].

Since NPs have a complex structure with several functional groups, their modification is difficult [81,82]. Fortunately, as new techniques are being applied, there has been a revival in the discovery of "magic bullets (the antibiotic molecule)" [83]. An alternative for keeping the dried antibiotic pipeline running could be the hunt for microbes from extreme habitats and catalogued for their biosynthetic abilities [61,84–89]. Thus, a willingness to utilize cutting-edge technologies could aid in discovering new, broad-spectrum antibiotics and revolutionizing the NPs screening process [90].

1.5 *Streptomyces* as a model organism and its biology

Streptomyces- the prolific antibiotic factories- are saprophytic, soil-dwellers who spend most of their lives as semi-dormant spores, allowing them to flourish in nutrient-depleted conditions [91]. Nonetheless, this phylum has evolved to various environments, including soils, freshwater, deep seas and in symbiosis with other organism such as ants and bees [92–94]. A minority of *Streptomyces* have been described as pathogenic, such as *S. scabies* and *S. ipomoeae* pathogenic to potato tubers [95,96] and *S. hygroscopicus* TP-A0451 endophyte in *Pteridium aquilinum* plants [97]. Alkaline soils and organic matter are known as the best habitats for these organisms and constitutes a large part of the microbial community [98].

An interesting aspect of *Streptomyces* is their abundance in soil with their optimal role in soil ecosystem functioning. One gram of soil harbours 10^6 - 10^9 cells of Actinomycetes, of which over 95% belong to streptomycetes genera. Temperature, pH, and soil moisture are among the factors that affect the development of actinomycetes [61]. Among Actinomycetes species, *Streptomyces* are mostly mesophilic (25-30°C), preferring low humidity; however, thermophilic isolates (50-60°C; *S. thermoviolaceus* strain SD8)[99] and strains with the capability to sustain acidic conditions (*S. yeochonensis* CN732) have also been identified [100,101]. Different exoenzymes secreted by *Streptomyces* in soil help break polymers to release carbon [102]. In addition, their extraordinary tendency to generate potent compounds (antibiotics, anticancer agents, immunosuppressants) makes these organisms incredibly useful [103].

An organism's morphology and detailed life cycle provide a broad range of information regarding its functional role in an ecosystem. *Streptomyces* are Gram-positive filamentous bacteria with fungus-like mycelial differentiation [104]. Reproduction in *Streptomyces* occurs by specialized aerial hyphae that matures into chains of uninucleoid spores [105]. The hyphae, which form after spore germination, tether to the substratum, allowing nutrient uptake and exploration to adjoining places. This eventually gives rise to a dense vegetative mycelium, which rapidly

expands into branched hyphal tips and undergoes cross-sectional division to form individual compartments [104–107]. Each sub-compartment harbours multiple copies of a chromosome, making streptomycetes a multicellular organism [108]. However, in adverse conditions, *Streptomyces* develop aerial hyphae and generate spores [109]. These spores are dormant and can rapidly disperse in the atmosphere ensuring the species' longevity. Volatile compounds, such as geosmin and 2-methylisoborneols released by *Streptomyces* act as chemical signals that attract arthropod and other insects and assists in spore dispersal [110]. With the onset of favourable environmental conditions, the dormant spores swell and become polarized (required for germination), culminating in the formation of a germ tube, which further grows into a hypha [111,112] (Fig. 3).

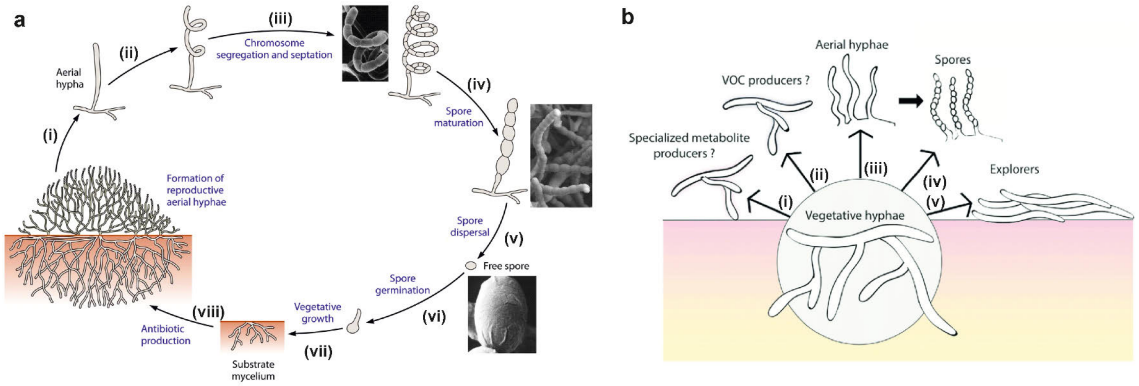

Figure 3. General biology of *Streptomyces*. **a**) Life cycle of *Streptomyces*, **i** and **ii**) Dense mycelium grows out of the substratum into aerial hyphae, **iii**) Cellular contents are sequestered at the cross-section, followed by the hyphal division into small compartments, **iv**) Newly formed small compartments maturate into spores, **v-viii**) Dormant spores get dispersed into the air and germinate with the advent of favourable conditions to give rise to mycelium. **b**) Fate of vegetative hyphae, **i**) hyphae elongates further to become specialized metabolite producers, **ii**) hyphae generates volatile compounds as a means of signaling, **iii-iv**) hyphae spread aerially in nutrient-deprived conditions to give rise to dormant spores, **v**) hyphae explores for the source of nutrients. Adapted from [112,113], respectively.

1.6 Regulation of antibiotic production in *Streptomyces*

Members of the *Streptomyces* genus multiply rapidly during their exponential growth phase, followed by the production of SMs during their idiophase. Varying environmental and physiological factors influence the production of antibiotic synthesis at various levels [114,115]. A diffusible hormone-like signaling molecule can stimulate antibiotics production by interacting with a specific receptor and controls gene expression at multiple levels [114,116,117]. For instance, A-factor

signaling molecule and its receptor ArpA regulate A-factor cascade, which results in the biosynthesis of streptomycin and grixazone in *S. griseus* [118,119]. Similarly, other signaling small-molecules, such as avenolide (*S. avermitilis*, *S. virginiae*, and *S. lavendulae* FRI-5) [120], γ-butyrolactones, SCB2 (2-[1'-hydroxyoctyl]-3-hydroxymethylbutanolide) [121,122], methylenomycin furan [121], butenolide (*S. rochei*; [121]), and godasporin [123] trigger metabolites production in *Streptomyces*.

1.7 Biosynthetic gene clusters, superclusters, and chromosome

Microbial genomes can harbour several BGCs that potentially encode NPs [124]. *S. coelicolor*, for example, has over 20 (with six known metabolites) [125], while *S. avermitilis* has up to 30 BGCs that encode NPs [126]. Other microbial species have a similar arrangement of NP encoding genes. These BGCs are self-contained cassettes that harbour genes encoding enzymes that are responsible for the generation of SM skeletons and for the subsequent tailoring of these skeletons into diverse NPs. The size and numbers of BGCs determine the nature of molecules they generate. For instance, pristinamycin cluster of size 210 kb is the largest supercluster known [127]. This cluster is unusual in the sense that its organization does not obey the general concept of gene clustering. Instead, a cryptic type II PKS gene cluster (90 kb; likely encoding a glycosylated aromatic polyketide) is found interwoven within this giant cluster [127]. Other superclusters, such as simocyclinone cluster in *S. antibioticus* can exist in cells [128], where two different clusters remain intermixed and form moieties of a more complex metabolite [129].

Genes with distinct functional roles are found within a typical BGC. For example, the prodigiosin (*red* cluster; a red-pigmented oligopyrrole antibiotic) encoding cluster in *S. coelicolor* comprises 23 candidate genes organized into four transcriptional units. This *red* cluster possesses biosynthetic genes (blue and red, Fig. 4), transport functions, cluster-specific regulators (orange, Fig. 4), and resistance functions [130] (Fig. 4).

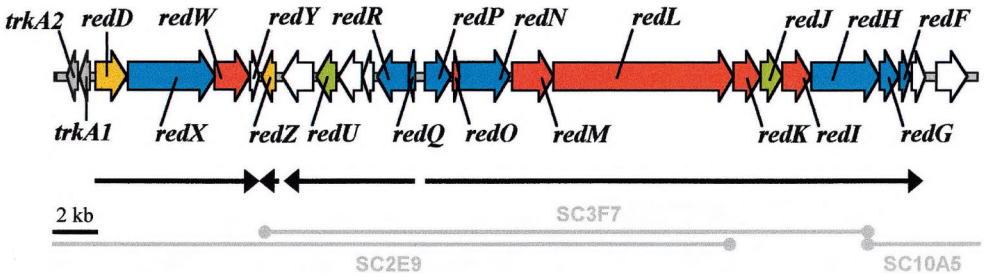

Figure 4. Prodiginine biosynthetic gene cluster organisation in *S. coelicolor* A3(2). (Orange: regulatory genes; blue: 2-undecylpyrrole biosynthetic genes; red: 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde biosynthetic genes; green: Putative housekeeping genes; white: unknown functions). Black arrows indicate four mRNA transcripts that are likely to be produced by the cluster. Adapted from [130].

Distinct gene arrangements may also be observed in different species that generate the same metabolite. Certain Gram-negative and Gram-positive bacteria fall within this category. For instance, prodigiosin cluster (*pig*) in Gram-negative (*Serratia marcescens* ATCC 274) is 21.6 kb in size and contains 14-15 genes that are unidirectionally oriented and transcribed [131]. However, in *S. coelicolor*, a different arrangement of genes in the same cluster (*red*) of 31.6 kb in size (containing 23 genes) possibly reflects greater complexity of prodigiosin regulation in this organism [70,125,132,133] (Fig. 5).

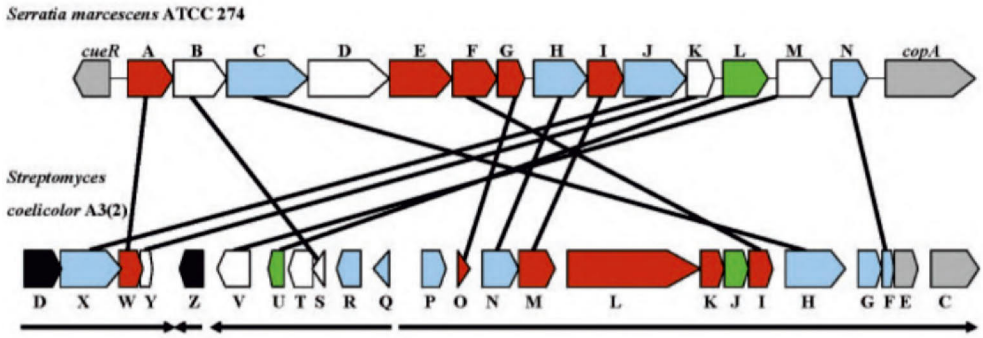

Figure 5. Comparative study of *S. marcescens* prodigiosin cluster (*pig* cluster) and *S. coelicolor* A3(2) undecylprodigiosin biosynthetic cluster (*red* cluster). The homologous genes' relative locations in the two clusters are indicated by the lines connecting them. In the *red* cluster, black denotes regulatory genes, red denotes genes engaged in bipyrrole moiety production, white denotes genes with unknown roles, and grey denotes genes not involved in BGC. Adapted from [132].

In general, antibiotics are generated by a complex and physically clustered biosynthetic genes in a chromosome with a common set of core biosynthetic genes [134]. *Streptomyces* possess a single linear chromosome, whose BGCs are arranged

together in distal parts of the chromosome. This specific arrangement facilitates easy and rapid transfer of the entire cluster to other species horizontally. However, in rare cases, these biosynthetic genes are also localized in plasmid DNA [135,136], which include plasmid-encoded α-hly in *E. coli* [137], genes for pantothenate biosynthesis in *Rhizobium etli* and *R. leguminosarum* [138].

1.8 Activation of BGCs

Accessing the elusive and concealed information regarding the NPs generation from microorganisms is typically challenging. However, data obtained from high throughput genome sequencing clarifies that organisms possess abilities to secrete several different metabolites, even though they generate only a few of metabolites in specific environments [26]. Finding an actual trigger that induces the expression of silent clusters is far from trivial. Hence, harnessing cryptic metabolites and resolving the multifaceted challenge of detecting and identifying SMs synthesized by organisms remains a significant bottleneck in new NP discovery. Despite these considerable downsides, technological advancements used for successfully expressing these otherwise silent/cryptic gene clusters could benefit the pharma industries [84].

In light of this, several techniques have been deployed to unlock silent/cryptic gene clusters and to gain access to their NPs. They are used either alone or in combination (detail reviewed in [84]). I have classified them into three major categories: viz., BGC-specific, pleiotropic, and targeted genome-wide methods. BGC specific approaches include modifications within biosynthetic clusters, which can be achieved by promoter engineering, cluster capture, exogenous expression, gene refactoring, and switching regulators. On the other hand, pleiotropic strategies include histone modifications (in eukaryotes), modifications in the translational machinery, and identifying signaling molecules. The third targeted genome-wide approach includes elicitation by chemicals and selection of mutants based on the expression of reporter genes. However, many of these tactics have their own set of benefits and drawbacks (Fig. 6).

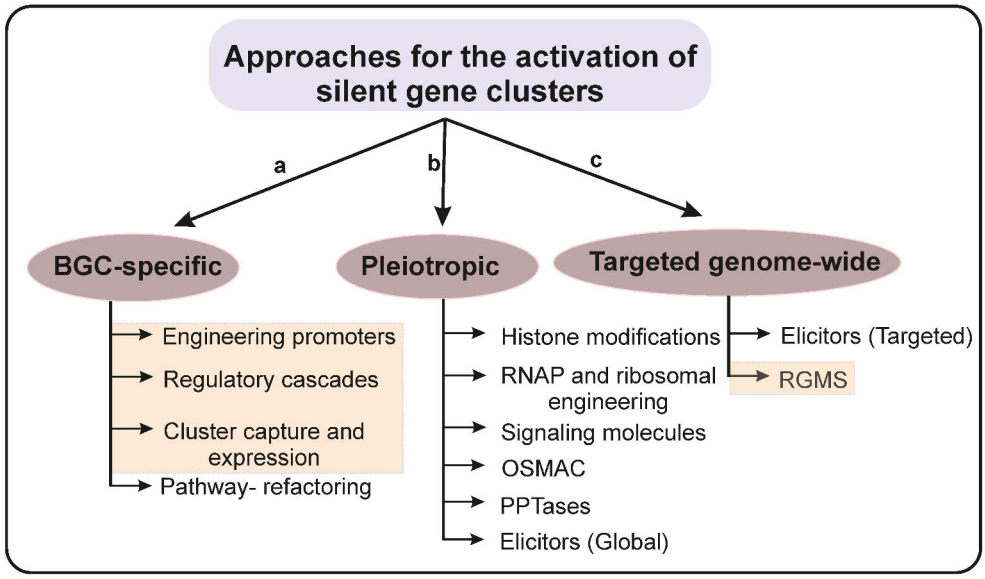

Figure 6. Several methods used for successfully activating silent/cryptic gene clusters. **a**) biosynthetic gene cluster specific. **b**) pleiotropic, and **c**) targeted genome-wide approach (Baral, unpublished data). Approaches highlighted were performed in my experiments and are included in present dissertation.

1.8.1 BGC-specific approaches

1.8.1.1 Engineering of promoters

Promoters are DNA sequences that are recognized by RNA polymerase and sigma factors and thus regulate the expression of metabolic pathways statically or dynamically [139]. The promoters' affinity for RNA polymerase plays a crucial role in gene expression [140,141]. Advantages of promoters engineering include their precision for a cluster of interest and the ability to be employed on all clusters. However, promoter engineering require advanced molecular biological tools and are labor-intensive [142]. Different promoters, viz., silent, weak, constitutive, and inducible, direct the complicated process of turning on and off the gene transcription [143]. Constitutively expressing promoters provide a higher level of transcripts [144], while silent promoters do not yield any [145].

For the target BGC expression, knowing the exact sequence and location of promoters is crucial. While eukaryotic promoters can be challenging to recognize, prokaryotic promoters are easily distinguishable due to their distinct characteristics. For instance, strong promoters of *E. coli* and *Bacillus subtilis* possess canonical consensus sequences (-35 box, TTGACA) and -10 box (TATAAT) [141]. The σ-factor makes RNA polymerase to bind to the promoters and thus play a crucial role

in transcription [146]. Different σ-factors, which are expressed in different conditions, can bind to different promoter sequences, and therefore regulate the expression of different genes. In prokaryotes, specific binding of RNA polymerase to promoters can occur without the presence of any ancillary transcription factors, while multicellular eukaryotes require complex control [147].

Since biosynthetic genes in prokaryotes are often arranged in separate operons, promoter activation in these operons must be performed in a specific proportion [148]. Activation of silent BGCs can be achieved by substituting constitutive promoters for silent promoters [149]. Furthermore, increasing specific metabolic flux of pathway can be improved by increasing the expression of key enzyme of the pathway. The expression can also be enhanced by using strong and appropriate constitutive promoters. This necessitates the development and application of static and dynamic promoters that control transcription processes and may be used to build increasingly complex metabolic networks in industrial microbes [150]. Since they have a higher binding affinity with RNA polymerase, highly utilized promoters *ermE**p, SP44, and other strong promoters are constitutively expressed and may efficiently control the gene expression. *Streptomyces* constitutive promoters include *ermE**p (a mutation in the promoter of *S. erythraeus*' erythromycin resistance gene; [151]), *gapdh* (promoter of glyceraldehyde-3-phosphate dehydrogenase in *S. griseus*; [152]), *kasO*p (promoter of SARP family regulator in *S. coelicolor* A3; [153]), *rpsL* (promoter of 30S ribosomal protein S12 in *S. griseus*; [152]), and *SF14P* (*S. ghanaensis* phage I19 promoter; [154]). Some inducible promoters that are applied in *Streptomyces* include *nitA (*ε-caprolactam-induced promoter; [155]), *P21-cmt* (cumate-induced promoter; [156]), *PA3-rolO* (resorcinol-induced promoter; [156]), *tcp830* (tetracycline-induced promoter; [157]), *tipA* (thiostrepton-induced promoter; [158], and *xylA* (xylose-induced promoter; [159]).

1.8.1.2 Regulatory cascades for cluster activation

Both pleiotropic and cluster-situated regulators affect BGC expression. Many global regulatory protein families tightly regulate SM expression and regulate complex regulatory pathways, which transmit signals to pathway-specific switches, primarily antibiotic regulatory proteins (SARP) family regulators, in all species [38,160,161]. Most current pathway regulators have been characterized by thorough investigations of individual genes residing within the pathway.

Global regulatory genes (GRGs) are scattered throughout the genome and regulate transcriptional regulatory networks and morphological development in *Streptomyces* [117]. Global regulators possess distinct roles and features, such as the genes they regulate, their effects on any co-regulators, σ-factors, and regulation of transcriptional factors [162]. GRGs can easily alter production profiles of SMs, but

they have a broad impact. Some examples of well-characterized regulators in *Streptomyces* are *adpA* regulator (activates streptomycin, grixazone, nikkomycin, and natamycin production, while represses ovideomycin biosynthesis) [163], *crp* (cyclic AMP receptor protein) which regulates biosynthesis of actinorhodin, undecylprodigiosin and calcium-dependent antibiotic biosynthesis in *S. coelicolor* [164], *atrA* regulator (activates the production of actinorhodin [165], daptomycin [166], lidamycin [167] and pristinamycin [168], while represses avermectin biosynthesis [169]).

Nevertheless, specific regulatory genes, known as cluster-situated regulators (CSRs), are master switches of antibiotic biosynthesis and are localized within the cluster. They have a direct influence on the transcription of biosynthetic genes. The engineering of CSRs has many advantages, including their specificity and the ability to alter the expression of an entire cluster with just one gene modulation. To my understanding, the *Streptomyces* genome harbours a tremendous number of CSRs belonging to different protein families. Some representatives of CSRs belonging to *Streptomyces* antibiotic regulatory protein (SARP) family are *actII*-ORF4 (enhancer of actinorhodin biosynthesis; [170]), *redD* (undecylprodigiosin biosynthesis activator; [171]), *cdaR* (calcium-dependent antibiotic biosynthesis activator; [172]), and *polR*, and *polY* (activator of polyoxin biosynthesis; [173,174]). The CSRs belonging to TetR family are *chlF1* (chlorothricin biosynthesis activator; [175]), *gouR* (gougerotin biosynthesis activator; [176]), and *ScbR* (repressor of coelimycin P1 biosynthesis; [177]). Large ATP-binding regulators of LuxR (LAL) group include *pikD* (pikromycin biosynthesis activator in *S. venezuelae*; [178]), *rapH* (rapamycin biosynthesis activator in *S. hygroscopicus*; [179]), *slnR* (activator of salinomycin biosynthesis in *S. albus*; [180]). Besides, some notable CSRs in *Streptomyces* include *nysRIV* (PAS-LuxR family, an activator of nystatin biosynthesis in *S. noursei*; [66]), *penR/pntR* (MarR family, pentalenolactone biosynthesis an activator; [181]), and *lmbU* (a novel family, an inducer of lincomycin biosynthesis; [182]).

1.8.1.3 Expression of biosynthetic clusters in non-native hosts

Expression of an exogenous gene cluster in a highly flexible and genetically amenable host devoid of related metabolites eases the functional characterization of a putative gene cluster. Several promising strains (e.g., *S. albus*, *S. avermitilis*, *S. chattanoogensis*, and *S. coelicolor*) have been engineered as expression hosts to achieve improved titers of target compounds. Endogenous specific BGCs are deleted and site-specific conjugation sites (e.g., φC31 *attB* loci) are introduced to engineer these host strains [183]. Examples include deleting actinorhodin biosynthetic genes (*act*), inactivating undecylprodigiosin (*red* cluster), and deleting

the *cpk* cluster [183]. *S. albus* Del14, a derivative of *S. albus* J1074 does not produce any compounds, because 15 endogenous gene clusters have been knocked out [184]. However, engineering host for expressing foreign BGCs is far from being trivial. The criteria for efficient engineering requires having functional promoters, gene-controlling components, specific-transcription factors, BGCs' number, and location in the genome. Knocking out/down aforementioned BGCs facilitate the process of creating a heterologous surrogate host that can be widely used for further experimental analysis [185–188].

More recently, direct cluster capture technology has acquired increased attention for metabolite production in surrogate hosts. This technique takes leverage of well-studied genetic systems to avoid the necessity for cultivating and modifying native producers [189]. Several strategies for capturing BGCs include transformation-associated recombination (TAR), and direct pathway cloning [190]. TAR system utilizes a 'plug-and-play' approach, whereby a locus of interest is captured, followed by desired genetic manipulations and expression in a suitable optimized host. For instance, a plug-and-play scaffold was employed for refactoring silent spectinabilin cluster [110]. The TAR cloning system benefits from using natural *in-vivo* homologous recombination of *E. coli* and budding yeast (*Sacch. cerevisiae*) to capture large genomic segments for synthesizing novel drug candidates. An excellent example of a TAR cloning system is a characterization of nonribosomal peptide synthetase gene cluster (67 kb) from *Saccharomonospora* sp. CNQ-490. Heterologously expressing this cluster in *S. coelicolor* generated taromycin A [191].

Homologous recombination-mediated direct cluster capture and expression have been recently utilized for cloning and expression of metabolic pathways. This uses linear-linear homologous recombination (LLHR) and (ii) linear-circular homologous recombination (LCHR). Examples of clusters cloned with homologous recombination include oxytetracycline, salinomycin, and spectinabilin clusters [188]. The Direct Pathway Cloning approach (DiPAC) has identified anabaenopeptins, fontizine A, and hapalosin [192]. Other methods, such as Red/ET recombination technique [184], LEXAS [188,193,194], restriction digestion, integrase-mediated site-specific recombination, DNA assembler [195,196], ExRec [197] are being employed for cloning and expression of biosynthetic clusters.

1.8.1.4 Pathway-refactoring

Reconstitution of SM biosynthetic genes arranged in clusters can reveal their roles in metabolites biosynthesis. However, most biosynthetic clusters' manipulation is far from trivial because of a complicated, redundant, and interconnected host regulation mechanism [198,199]. Currently used advanced metabolic engineering

tools and plummeting costs of DNA synthesis have eased the remodelling of BGCs. Refactoring provides a complete re-design and assembly of BGC from synthetic DNA in three different stages (design, assembly, and recombineering/cloning). The initial stage includes designing synthetic gene clusters from biological libraries to acquire desired promoter, gene, and terminator sequences within a frame. Upon pathway architectural design, pathway assembly is done using advanced cloning strategies such as recombineering or TAR cloning in budding yeast (*Sacc. cerevisiae*) or *E. coli*. The gene clusters responsible for spectinabilin [199] and bottromycin [200] biosynthesis have been refactored.

1.8.2 Pleiotropic approaches

1.8.2.1 RNA polymerase (RNAP) and ribosomal engineering

Ribosomal engineering can help to acquire mutants with increased NP productivity, which in *Streptomyces* has been widely used for two purposes: (i) discovery of natural products and (ii) productivity enhancement [84]. Previously, traditional strain improvement techniques, including chemical mutagenesis or physical mutagens, were used for engineering RNA polymerase (RNAP) [201]. Mutations in translational machinery (ribosomal subunits) or RNA polymerase has led to the overproduction of SMs [84,202–204]. For example, rifampicin resistance in bacteria was caused by a mutation in the β-subunit of RNAP [205]. Similarly, mutations in the ribosomal 30S subunit instigated bacteria to resist streptomycin effects [206] (Fig. 7).

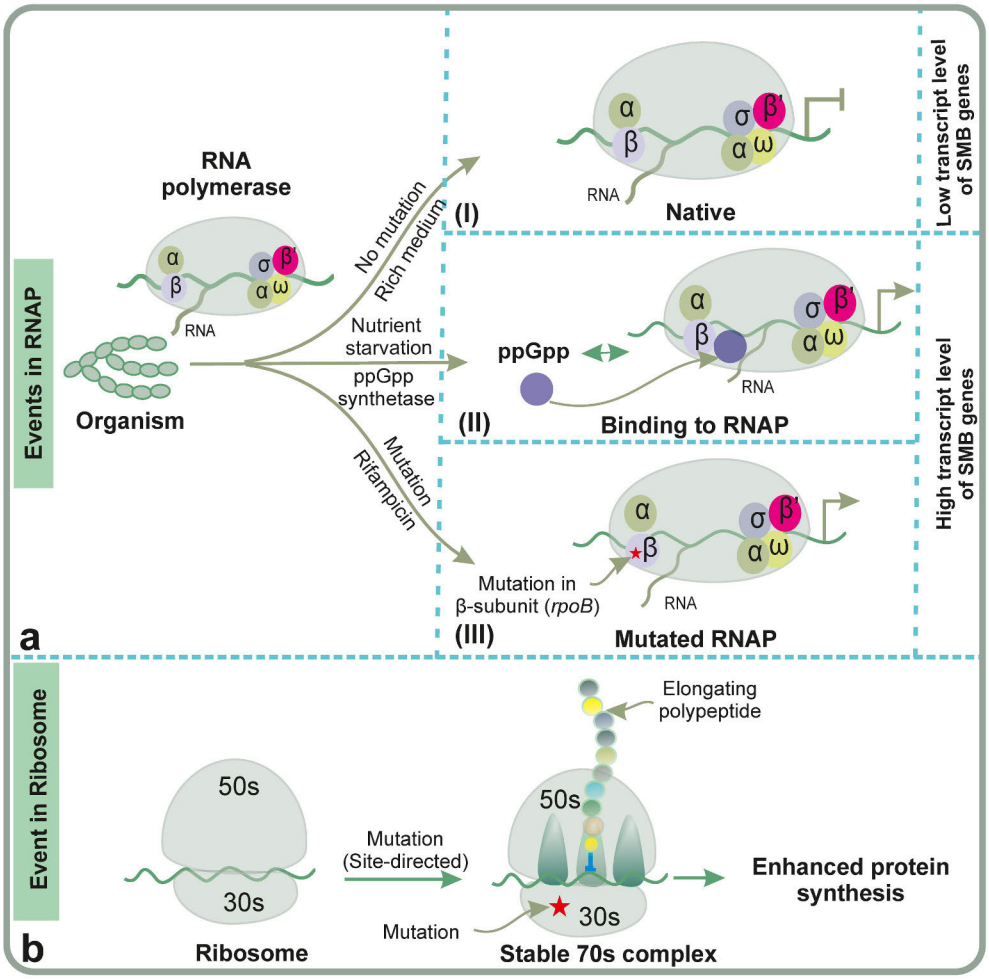

Figure 7. **Activation of silent gene clusters through pleiotropic approaches. a**) RNAP events when an organism is grown in different environments. **b**) A mutation in ribosomal 30S sub-unit upregulates protein synthesis in the stationary phase and stabilizes the 70S ribosomal complex. Adapted from [84].

Different events in RNAP and ribosomes led to activation of silent biosynthetic clusters or overexpression of cryptic biosynthetic clusters. Microbes exposed to nutritional downshift (amino-acid deficiency) generate ppGpp (guanosine tetraphosphate), which binds to RNAP and causes higher levels of BGC transcription. In addition, antibiotics like rifampicin cause a mutation in polymerase's β-subunit, which raises transcriptional levels of BGC. Similarly, in ribosomes, a mutation in the ribosomal 30S subunit helps to stabilize the 70S ribosomal complex, which ultimately induces the synthesis of proteins [84].

1.8.2.2 Signaling molecules

Chemical signals like A-factor (γ-butyrolactones) can trigger transcriptional regulators (e.g., master regulator *AdpA*) by binding to receptors that regulate antibiotic production [207,208]. A-factor, for example, promoted streptomycin synthesis in *S. griseus* by binding to a repressor-type receptor protein and separating it from DNA at very low concentrations [209]. Cascade regulators then relay required signals for coordinated modulation of BGCs (e.g., actinorhodin regulator gene *actII*-ORF4 in *S. coelicolor*) [117]. In *Streptomyces*, diffusible signaling molecules could be categorized into five different groups, viz., γ-butyrolactones, furans, γ-butenolides, PI factor (2,3-diamino-2,3-bis (hydroxymethyl)-1,4-butanediol), and *N*-methylphenylalanyl-dehydrobutyrine diketopiperazine [115,210]. Some notable signaling molecules include avenolide (a butanolide hormone; [120]), methylenomycin furan (induces methylenomycin synthesis in *S. coelicolor* [121]), 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids [211], virginia butanolide [121], and trimethylamine [212,213]. Thus, understanding how signaling molecules are controlled and how environmental and physiological cues are detected could provide deeper insights into SMs regulation.

1.8.2.3 Engineering cultivation conditions

Microbes generate a limited number of metabolites in their favourite minimal media. However, different culture media cause the strain to synthesize metabolites with diverse chemical scaffolds, conceptualized as “One Strain Many Compounds” (OSMAC) [214–216]. OSMAC is an efficient method for activating cryptic metabolic pathways [217]. Changes in nutritional content, cultivation conditions (temperature, pH, rate of aeration), addition of enzyme inhibitors, co-cultivation with other strain(s), addition of an epigenetic modifier(s) or biosynthetic precursor(s) can dramatically influence the global physiology of a microbial strain and significantly affect its secondary metabolism [215,218]. For instance, heat-shock treatment of *S. venezuelae* and *S. hygroscopicus* induced the production of jadomycin [219] and validamycin [220–222], respectively. Similarly, nutrient limitation (such as alanine and/or acidic pH as nutrient) increased methylenomycin yield in *S. coelicolor* [223–225]. However, increased concentration of inorganic phosphate (a growth-limiting nutrient), such as dipotassium phosphate (0.001 - 0.2% w/v; ~0.5–115mM) in the fermentation medium suppressed the production of anti-vibrio SMs by *Streptomyces* [226].

Microbial interactions may induce the production of SMs that are not present in axenic cultures [227,228]. Co-cultures may lead to the production of metabolites that influence gene expression in other microorganisms. Co-culturing also alters individual cellular physiology [229], and the co-culturing techniques include either culture of bacterium-bacterium or fungus-fungus species or an interaction of both

[230–232]. The combined culture of bacterium and fungus has allowed researchers to activate several clusters, generating several new compounds [230,231]. For instance, mixed culture of *S. coelicolor* M145 with *Myxococcus xanthus* generated myxochelin (a siderophore) [233].

In addition, chemical elicitors (non-nutrient additives) affect microbial growth and can cause silent cluster activation. As mentioned above, signaling molecules, such as γ-butyrolactone [234], polysaccharides, glycoprotein, lipids, and even enzymes can activate silent clusters. Abiotic (antibiotics, synthetic compounds) and biotic (microbial lysates and soil extracts) elicitors stimulate the production of SMs. Two small-molecule elicitors incredibly successful are ARC2 (a synthetic compound) [235] and godasporin [236]. Even the usage of sub-inhibitory concentrations of jadomycin [237] and monensin [238] effectively induced exogenous production of SMs. In addition, rare earth elements (scandium and/or lanthanum) activated silent clusters in *S. coelicolor* A3(2) [239]. Other elicitors, such as 5-azacytidine, elicited the generation of oxylipins and two new polyketides, lunalides A and B, in *Cladosporium cladosporioides* [240].

1.8.2.4 Phosphopantetheinyl transferase (PPTases)

Many important SMs such as PKSs and NRPSs are produced by large multifunctional modular enzyme complex [241], which requires activation by enzymes of 4'-phosphopantetheinyl transferase (PPTase) superfamily [242,243]. PPTases activate carrier proteins of PKSs and NRPSs by transferring phosphopantetheine moiety to an invariant serine residue of acyl carrier proteins [244]. They carry out the most needed post-translational modifications, converting ACPs and PCPs from inactive apo to active holo forms [242,245–248]. Based on their key sequences and substrate specificities, PPTases are classified into three main classes, viz., (i) bacterial AcpS-type PPTases with a small protein substrate specificity normally associated with primary metabolism, (ii) eukaryotic PPTases used in type I fatty acid synthases, and (iii) Sfp-type PPTases which have a broader substrate choice and are related to bacterial secondary metabolism [246,249].

During SM biosynthesis, the fundamental role of post-translational modification of carrier protein is played by PPTase [250]. Conceptually, the actively transcribed BGCs may have non-functional enzymatic machinery due to the lack of PPTases required for the post-translational modification of PKS and NRPS enzymes [250]. This PPTase enzyme has been discovered to catalytically activate metabolic enzymes, resulting in biosynthetic cluster activation [246]. For example, puromycin A, B, and C in *S. alboniger* are cryptic metabolic pathways that have been activated by using heterologously expressed PPTases [250]. Thus, PPTase is essential for cell viability and post-translational modifications that benefit the discovery of NPs [246,251].

1.8.3 Targeted genome-wide approaches

1.8.3.1 High Throughput Elicitor Screening (HiTES)

High Throughput Elicitor Screening (HiTES) enables rapid screening and identification of NPs generated by bacteria that are otherwise difficult to reproduce in lab [252]. This chemogenetic HiTES approach allows the detection of fluorescent signals generated by cloned reporter genes, the expression of which is triggered by small-molecule elicitors. A library of low-dose antibiotics is created, which acts as the modulators of silent gene clusters in the studied organisms [253,254]. The approach has led to the discovery of a diverse variety of molecular structures with atypical bond arrangements, implying a large and diversified set of metabolic pathways that might be beneficial in the development of novel medications [255].

Several new molecules have been discovered using the HiTES approach. Elicitation by small molecules such as etoposide and ivermectin allowed a generation of 14 novel compounds with antifungal and anticancer properties in *S. albus* J1074 [254]. HiTES was further developed by combining it with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The technology enabled the biosynthesis of cinnapeptin peptide in *S. ghanaensis* that is elicited by amygdalin [256]. Further advancement in HiTES coupled with bioactivity generated cebulantin, a cryptic lantipeptide in *Saccharopolyspora cebuensis* [257]. Having progressed over time, this intriguing method will yield insight into the biological regulation and chemical output of innumerable silent gene clusters in microbes, potentially altering the landscape of antibiotic development.

1.8.3.2 Reporter-Guided Mutant Selection (RGMS)

RGMS is an appealing strain development method that depends on generating a large random mutant library using mutagens such as UV (ultraviolet light), EMS (ethyl methanesulfonate), or NTG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine). The identification of specific mutants where the desired metabolic pathway has been activated can be made directly based on the expression of the reporter genes (Fig. 8). Askenazi et al. [258] pioneered RGMS and characterized lovastatin production in *Aspergillus terreus* using a single reporter gene (*ble*, phleomycin- resistance gene) probed downstream of lovF promoter. Transformants generated via UV mutations were selected based on their tolerance to elevated concentrations of phleomycin. Almost 50% of the sequestered mutants were over-producers of lovastatin [258]. Later, Xiang et al. [259] upgraded RGMS with a double reporter cassette (*xylE* and *neo*), facilitating the rapid selection and production of clavulanic acid (CA) in *S. clavuligerus*. Chemical mutagenesis activated the CA cluster in *S. clavuligerus*, and

the generated mutants were able to tolerate elevated concentration of kanamycin. Surviving cells (*ca*. 90%) produced a higher amount of CA. This double reporter system significantly reduced false positives that afford spontaneous resistance to kanamycin [259]. Other examples include the expression of a silent gaudimycin (*pga*) gene cluster in *Streptomyces* sp. PGA64 and poorly expressed jadomycin (*jad*) gene cluster in *Streptomyces venezuelae* ISP5230 [260].

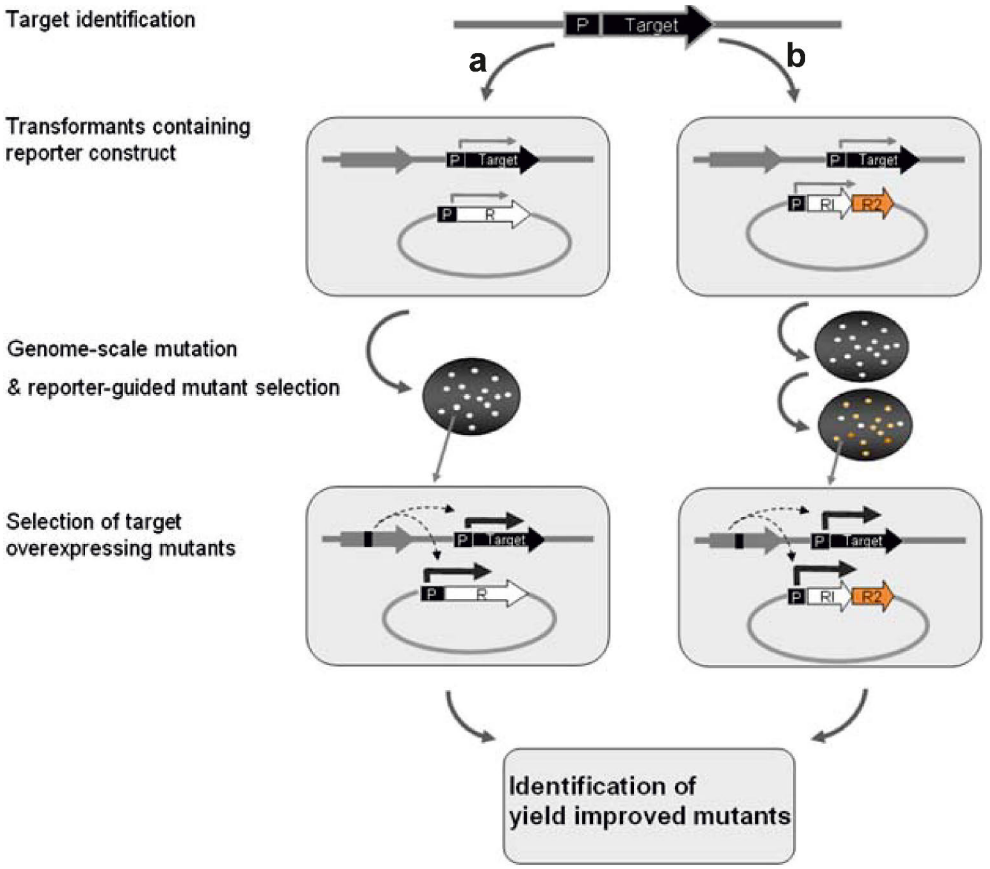

Figure 8. Schematic diagrams of the RGMS principle. **a**) Single-reporter RGMS. Adapted from [258]. **b**) Double-reporter RGMS. Adapted from [259]. (P) target promoter, (R1 and R2) reporters.

Thus, this conventional mutation and screening method tracks the expression level of a biosynthetic gene cluster coupled to the output level of corresponding metabolites, allowing non-producing mutants to be excluded from the experiment [261]. However, mutants arising from random mutations are most often tricky to catalogue for their production efficiency and requires additional screening. Improved

production of compounds generated using RGMS techniques are jadomycin, gaudimycin [260], streptothricin, geosmin, and strevertene [262] in *Streptomyces* (Fig. 9).

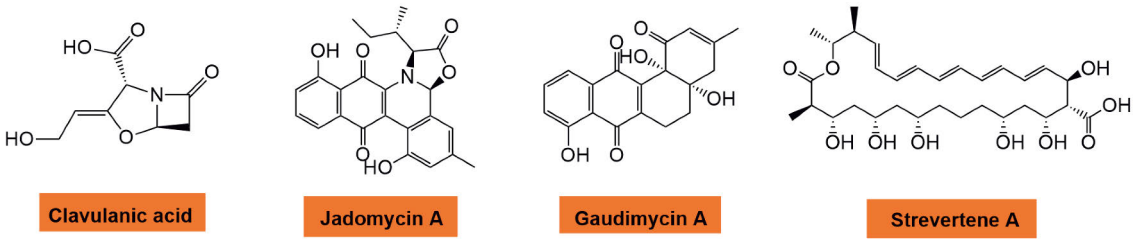

Figure 9. Chemical structure of compounds generated using RGMS technique.

2 Aims of the Study

Bioengineering tools required to activate silent BGC and to manipulate active BGCs are the central focus in this dissertation. Various updated strategies have been adopted in this thesis, such as the RGMS technique for targeted activation of silent gene clusters, co-culture of different organisms (yeast-*Streptomyces*) interactions, CRISPR/Cas9 approach for inactivation of a gene within the pathway, and isolating antibiotics producing organisms from an extreme ecological niche.

In the current study, specifically, I aimed to:

I Develop a single-cell mutant selection platform for target activation of silent metabolic pathways.

II Investigate the use of *Streptomyces*-yeast co-cultures for the production of SMs by *Streptomyces* sp. MBK6.

III Use of CRISPR/Cas9 gene inactivation strategy to alter metabolic pathways directly in producing organism (*S. showdoensis*; showdomycin).

IV Bioprospecting of SMs producing organisms from extreme habitats.

3 Materials and Methods

In this dissertation, various novel approaches (with slight modifications where necessary) were utilized to unveil the functional roles of genes involved in biosynthetic pathways. These utilized methodologies are focused on in activating silent or dormant gene clusters or enhancing the overexpression of cryptic biosynthetic clusters to obtain novel metabolites with promising therapeutic leads.

3.1 Common methodology for all experiments

3.1.1 General DNA techniques (I, II, III and IV)

Streptomyces strains were cultivated in GYM medium (glucose: 4 g/L; yeast-extract: 4 g/L and malt extract: 10 g/L) for a few days (depending upon Actinomycetes strains). Cell cultivation was followed by DNA extraction using the phenol: chloroform extraction method. Similarly, *E. coli* and *Streptomyces* plasmids were extracted separately using commercial plasmid extraction kits according to ThermoScientific and Kieser T et al. (2000) protocols, respectively. Individual manuscripts attached herewith outline DNA and plasmid extraction procedures in detail. The extracted DNA was frozen and stored at -80°C until required.

3.1.2 Culture conditions (I, II, and IV)

Streptomyces and *E. coli* were grown in various media (nutrient-poor and nutrient-rich) depending on their requirements. Usually, *E. coli* was grown in Luria-Bertani and 2×TY medium (tryptone: 16 g/L; yeast extract 10 g/L; NaCl 5 g/L; pH 7.0), while *Streptomyces* was grown in TSB (tryptone: 17 g/L; soy: 3 g/L; NaCl: 5 g/L; K_2HPO_4: 2.5 g/L; glucose: 2.5 g/L) and GYM medium. *Streptomyces* were cultured in various nutrient-rich mediums (such as MS, E1, E10, SC, and YEME) for producing compounds. *E. coli* was grown at 37°C, while for *Streptomyces*, it was 30°C. Media compositions are provided in the appended publications.

3.1.3 Computational analysis (I, II, III and IV)

DNA sequence reads for de novo assembly of *Streptomyces* genome were done using A5-miseq (v20150522), which was then combined with ABACAS (v1.3.1) using *S. albus* NK660 (CP007574.1) as the reference, and gaps were filled using IMAGE (v2.4.1). After the assembly, final annotations were made with RAST and BUSCO (v1.22) was used to check for completeness. Other computational software's were utilized where required, such as Artemis, antiSMASH, Easyfig, Multi-gene blast, MiBIG, and Benchling.

3.1.4 Metabolic profiling (I, II, and III)

The wild-type *Streptomyces* and their respective engineered mutants were grown in different production media for several days (as required). Some notable media used in this study were E1 Soy, MS (solid and liquid), ISP4, YEME, and SS. Experimental analysis was performed in triplicates. Initially, a seed culture was generated by raising engineered clones in TSB media supplemented with appropriate antibiotics (for selecting incoming plasmid). For compounds production, a total of 6L of desired liquid media was prepared, and one mL of inoculum (grown for a few days) was inoculated into every 50 mL of production media supplemented with appropriate antibiotics (50 µg/mL) and were incubated in shakers (30°C; 300 rpm; 7 d). Metabolite extraction was performed either with XAD-resin or with ethyl-acetate (EtoAc; $C_4H_8O_2$) from the centrifuged supernatant. A day before analysis, XAD-7 adsorbent (20 g/L) was added to growing culture and was shaken overnight with slight agitation (120 rpm). Alternatively, 10 mL culture was extracted with EtoAc, dried with rotavapor, and finally extracted with methanol (2 mL). The extracted fraction in MeOH was passed through a filter of size 2 µm and analyzed using analytical HPLC and LC-MS, followed by structure elucidation using NMR. HPLC was performed by CL-10Avp HPLC with an SPD-M10Avp diode array detector (Shimadzu) using a reversed-phase column (Phenomenex, Kinetex, 2.6 µm, 4.6 × 100 mm), while MS analysis were carried out with a low-resolution MS with a HPLC system (Agilent 1260 Infinity 6120 Quadropole LC/MS).

3.1.5 RT-PCR for gene-expression studies (I)

Ascertainment of the pathway activation was performed by gene expression studies. Isolation of total RNA was performed from the engineered mutants grown in liquid MS medium (20 g/L each of agar, mannitol, and soya flour; pH: 7.2) at different time intervals. Reverse transcriptase and other essential components/buffers were used to synthesize cDNA from RNA and utilized as a template for RT-PCR assays. The detailed protocol is provided in the appended manuscript.

3.2 Strategies currently adopted for silent gene activation

Several promising strategies were employed to activate silent BGCs in actinomycetes, which are presented in individual papers appended to this thesis (I-IV). The table 1 summarizes the research design in all the investigations included in this thesis:

Table 1. Research activities performed in different projects that are included in this dissertation.

Experiments done/ approaches utilized	**Study I (SCMS project; FACS usage)**	**Study II (Interaction project; Yeast-*Streptomyces* interaction)**	**Study III (C-nucleosides/ CRISPR-Cas9 usage)**	**Study IV (Bioprospecting; a new marine strain isolated and studied)**
Metabolites generated/studied	Mutaxanthene	Prodigiosin	Showdomycin	Persiamycin A
Genome sequencing and analysis	Whole genome analysis done	Whole genome analysis done	Whole genome analysis done	Whole genome analysis done
BGC cluster selection and expression	Silent cluster chosen for activation	*Red* cluster studied	Showdomycin cluster studied	Persiamycin cluster studied and a hypothetical scheme for its production is shown.
Genes within cluster knock-out/knock-down	--	--	*sdmA* gene knocked out and complemented back	--
Mutations (of any kind)	UV mutation performed for promoter activation	--	--	--
Sorting cells using FACS	Activated single mutants sorted using FACS	--	--	--
HPLC, LC-MS and NMR of the compounds studied	Done	Done	Done	Done
PCR, RT-PCR	Done	--	PCR for amplifying *sdmA* gene performed, while RT-PCR not performed	--
EMS	--	Done	--	--

3.2.1 Brief protocol for silent cluster activation by UV mutagenesis and sorting using FACS (Paper I)

3.2.1.1 Selection of a silent promoter (Paper I)

For target activation of silent BGC, we used *Amycolatopsis orientalis* NRRL F3213 as our model species. A transcriptionally silent cluster encoding Type II polyketide was identified using antiSMASH and other *in-silico* approaches. For activating a silent BGC, I utilized a promoter upstream of the regulatory gene of transcriptionally silent BGC.

3.2.1.2 Preparation of an activation construct (Paper I)

An activation construct pSGKP45 in *E. coli* was prepared by selecting a silent promoter upstream of a regulatory gene (*mutR*) from the studied cluster. This construct was then introduced into *A. orientalis* parental strain through conjugation. For this purpose, pSET152 based *Streptomyces* genome integration plasmid (ΦC31 integrase-based vector) was used. The native terminator in pSET152 plasmid was exchanged with two constitutive terminators (T4 Kurz and ECK120029600). Downstream of the terminators, a silent promoter (promoter from *mutR* regulator) along with ribosomal binding site and two distinct reporter probes (*sf*GFP and Kan^R genes) were cloned. For the termination of the transcription T7 terminator was used. The construct segment for activation (T4+ECK+*mutR*+RBS+*sf*gfp+KanR+T7 terminator) flanked with *Xba*I/*Bam*HI was ordered from Genewiz Company, Bahnhofstr. Leipzig, Germany. For the positive control plasmid, the *mutR* promoter and the RBS were replaced with a constitutive promoter (SP44) and its corresponding RBS (SR41), respectively. These constructed plasmids were then transformed into the parental strain, generating different mutants that served as a control and experimental clones (utilized for their UV activation).

3.2.1.3 Mutagenesis, selection and FACS screening (Paper I)

Millions of spores obtained from exconjugants were exposed to UV-C radiation for 15 min, followed by kanamycin enrichment of positive clones at a dose of 200 µg/mL. Expression of GFP signal was monitored after the cells were fully grown. Followed by the observance of the GFP signal, the kanamycin-enriched positive clones were washed with phosphate-buffered saline, sonicated to break down the mycelial filaments, filtered to remove big mycelial filaments and sorted using FACS (FACSAria Flow Cytometer). *A. orientalis*/pSGKP45 spores (before UV exposure) were used as a negative control for FACS sorting, whereas *A. orientalis*/pSGK was

used as a positive control. Based on the positive and negative controls, gating was done to segregate the positive clones expressing GFP. The FACS sorted single cells were plated on solid media and allowed to regenerate.

4 Results and Discussion (Original publications: I–IV)

Given the worst-case scenario for AMR, innovative drug development is crucial for preventing the spread of antibiotic-resistant superbugs. In such circumstance, microbial biosynthesis of drugs is an excellent alternative to chemical synthesis. However, native hosts typically require extensive modifications to achieve economically viable productivity. Since most species' biosynthetic abilities have already been identified, new engineering tools are needed to manipulate the strains to produce new compounds. Furthermore, designing an optimal method that can drive and determine the expression levels of a wide range of pathways, either in native or heterologous hosts, can significantly aid the drug discovery process.

Here, I report using several new and improvised strategies to activate silent gene clusters and characterize biosynthetic potentials of different pathways to discover new metabolites.

4.1 Single-cell mutant selection (Original publication I)

As described in the literature review (refer to the section 1.8.3.2) and materials and methods section (section 3.2.1), reporter genes have been frequently employed to detect the activation of cryptic metabolic pathways. One of the disadvantages of existing technologies is that reporter genes are either used on cultivation plates or 96-well plates, limiting the techniques' throughput. In order to take advantage of ultra-high-throughput technologies, I wished to convert RGMS to a single cell format and adopt fluorescent-activated cell sorting for use in *Amycolatopsis*. My workflow consisted of (i) a promoter probe double reporter system employing a resistance gene and a fluorescent reporter gene, (ii) mutagenesis to introduce genome-wide disturbances, (iii) enrichment of the mutant library to remove cells where no transcription of the target cluster occurs, (iv) screening of the library by FACS to obtain positive cells with high transcription levels from the targeted pathway, and (v) verification of production profiles of the harvested cells (Fig. 10).

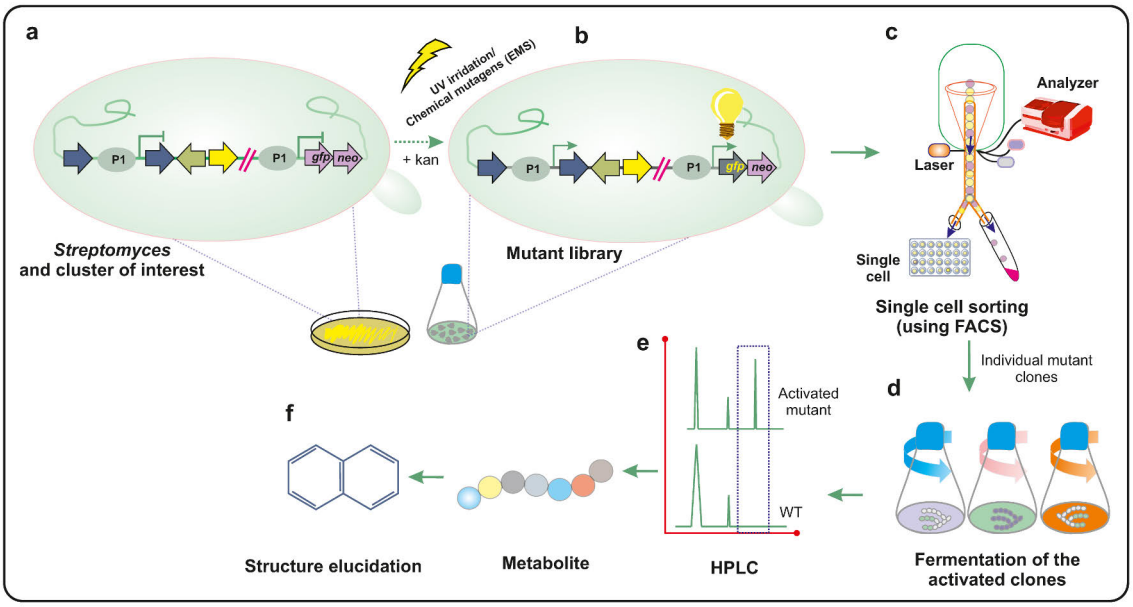

Figure 10. Tentative workflow of single-cell quantification for gene expression in *Amycolatopsis* by flow cytometry. **a**) Selection and cloning of silent promoter in *Amycolatopsis*. **b**) Transformants where UV radiation or EMS are employed to induce the silent promoters and the mutant library of those were created. **c**) Mutant library was subjected to FACS, where *sf*GFP as fluorescence was used to sort the activated mutants. **d**) Metabolic profiling of sorted clones in different media. **e**) HPLC analysis of the grown mutants. **f**) Structure elucidation of the generated metabolites.

The genomes of Actinobacteria were mined by our collaborator for the presence of aromatic type II PKS BGCs and noted a tetracycline-type gene cluster in *Amycolatopsis orientalis* NRRL F3213. The strain was grown in flask cultures, and the metabolites were extracted, but despite the presence of type II PKS clusters, analysis of culture extracts did not reveal any metabolites with distinct UV/Vis spectra associated with aromatic polyketides. Therefore, I surmised that the BGC is silent in the native host and designed an activation construct for the gene cluster. I selected a regulatory gene upstream of KSα of our studied gene cluster and cloned the promoter of this gene along with two reporter genes. The construct was conjugated back to the parental strains before being exposed to UV radiation.

Mutagenesis has been instrumental in the pharmaceutical industry for several decades to improve the yields of microbial natural products. I felt that random mutations could be used to alter the complex regulatory networks that suppressed production of the tetracycline-type compound in *Amycolatopsis* and employed physical (UV radiation) method for the generation of mutant libraries. UV mutation causes dimerization of adjacent thymine bases, which forms a kink in the DNA strand that locally disturbs the base pairing between the DNA strands (Fig. 11). This

thymine dimer disrupts replication and transcription, and is potentially mutagenic, carcinogenic, or fatal to the organism. However, prokaryotes correct this deficiency in the same generation by photolyase before being passed on to the next generation.

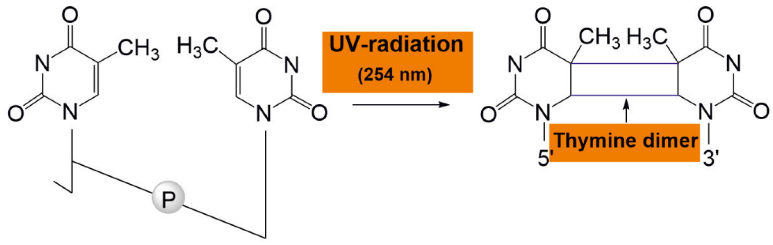

Figure 11. Molecular mechanism of mutation in DNA sequences by UV radiation. UV mutation causes adjacent thymine bases to dimerize, causing a kink in the DNA strand.

In order to prepare a mutagenesis library, the parental strain *Amycolatopsis orientalis* NRRL F3213/pSGK-P45 with reporter genes was grown in MS plates until their sporulation (7-10 d), followed by harvesting the spores. The spores rinsed with water were heat-shocked (55°C; 1 min), allowing dormant spores to loosen their cell walls and germinate. The germinated spores were exposed to UV radiation (UV-C; UV germicidal lamp) kept at a distance of 10 cm for 5 minutes with minimal stirring. One ml of mutant spore suspension was inoculated onto GYM media after UV irradiation and incubated at 250 rpm in the dark. This prevented reverting of photolyase activity. The following day, the addition of kanamycin (100 µg/mL) helped to enrich positive mutants, which were then allowed to grow for a few more days. Analysis of the positive mutants was done by fluorometer based on the GFP expression and their survival in antibiotics.

A key challenge in using FACS with Actinobacteria is the growth of these bacteria in large mycelial networks that do not pass through the nozzles of FACS instruments. *Streptomyces* cell-wall architecture (rigid cell wall) along with its clumping nature (aggregating cells in mycelium) and mycelium-like apical growth was considered a major hurdle for their single-cell analysis and sorting using FACS. So far, just a single method has been devised, which employs a flow cytometry technique with a single-cell (protoplast) resolution in *Streptomyces* [263]. The technique utilizes 10% (w/v) of lysozyme in sucrose to remove the cell wall. The sucrose provides stabilization of the protoplast against osmotic pressure; however, cell viability severely deteriorates in FACS machines. In addition, this technology uses a membrane-permeable dye, propidium iodide (PI), whose differential staining mechanism helps to discriminate between dead and living cells. However, the disadvantage of this methodology is that it requires digestion of mycelia to generate protoplasts and the examination and sequestration of individual protoplasts

depending on the *gfp* expression. An additional downside of this approach is that the fragile protoplasts rupture easily while passing through the FACS instrument during the cell sorting.

We, therefore, put effort into developing a versatile approach whereby the activated mutants can be sorted directly using FACS. The method utilizes sonication of fragment multicellular mycelium and bypasses the need to generate protoplasts. Both positive and negative controls were employed for FACS gating. The wild-type cells and transformants generated before the UV mutation act as negative controls (WT and UV0). A constitutive promoter (pS-GK) with reporter genes cloned downstream of the promoter was employed as a positive control. Flow cytometry was used to examine the mutants, using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Based on the scattered plot of negative and positive controls generated during the flow experiment, we utilized Viikki Sorting Flow Cytometry (University of Helsinki) service and have developed an ideal gating system for FACS (Fig. 12). However, we encountered several challenges while gating the FACS; the precise expression threshold for the activated mutants proved challenging to determine.

Furthermore, *Amycolatopsis* cells remain interconnected as they grow in a mycelium, generating a chain-like structure. It is very unlikely that all the cells in that particular cluster are activated at the same time. FACS examines the signals of all the cells in the chain segment at the same time, which causes issues with downstream processes. For this reason, front scatter (cell/cluster size) could be taken into account. Thus, distinguishing between actual mutants and non-mutants was challenging in the case of *Amycolatopsis* cells. This increased the likelihood of selecting the false positives (cells lacking *gfp* expression). Thus, to get rid of large mycelial clumps, I fragmented mycelium into pieces using a sonicator. The cells, however, rapidly assemble into clumps while standing. Thus, I employed several rounds of sonication for evenly breaking down the cell clumps and employed cell-strainer cap falcon filter to get rid of *Amycolatopsis* filaments. Notably, based on our gating, we were able to sequester the genuine individual mutants and harvest them separately in 96-well plates (Fig. 12).

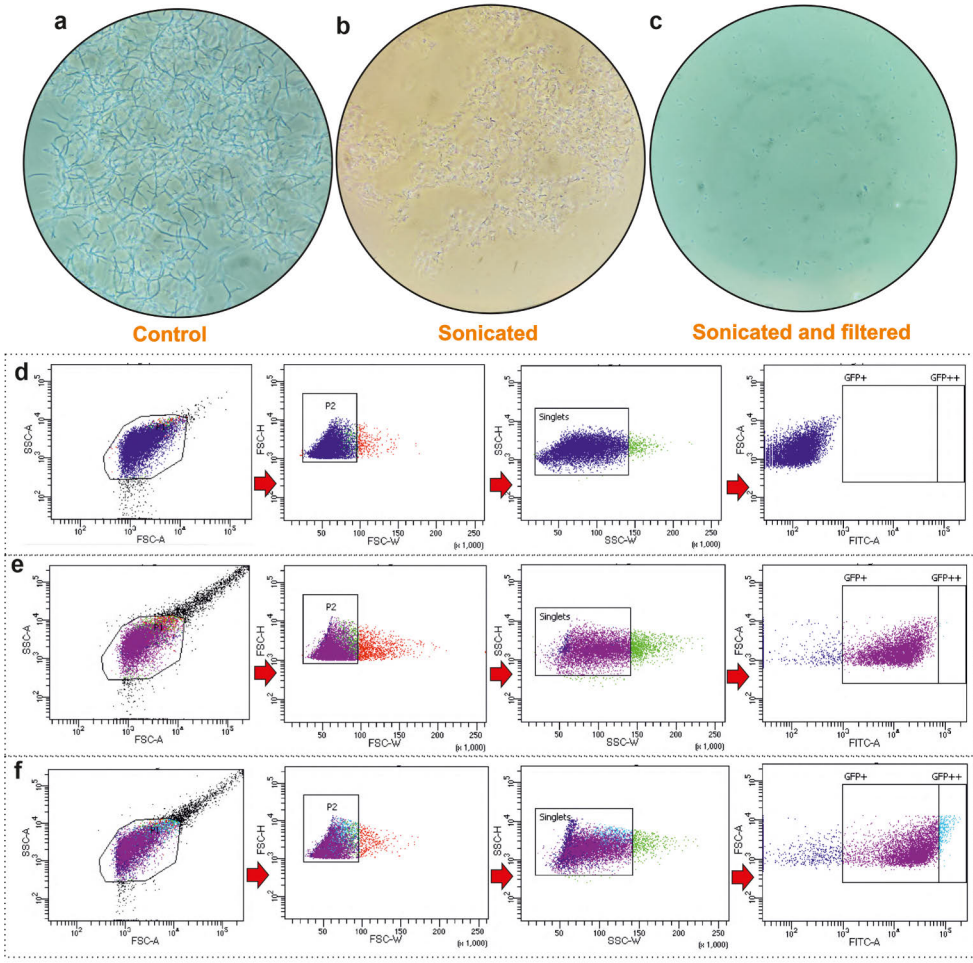

Figure 12. Morphology of *Amycolatopsis orientalis* cells examined under the microscope (100× magnification), and gating strategy used for sequestering activated mutants/cells by using Flow cytometry. a) *Amycolatopsis* mycelium. **b**) Sonicated cells with spores and mycelial filaments intertwined together. **c**) Filtration of sonicated samples through cell-strainer cap falcon filter (Falcon® 12×75mm tube with cell strainer cap) helped us in sequestering the individual cells. FACS gating for screening and sorting activated mutants is represented by d-f. **d**) Silent promoter cloned to the parental strain (*A. orientalis* NRRL F3213 wild-type) before UV mutation (*A. orientalis*/pSGKP45-UV0) was used as a negative control. **e**) A constitutive promoter (pS-GK) cloned to *A. orientalis* NRRL F3213 (labelled as *A. orientalis*/pS-GK) was used as a positive control, and **f**) Promoter activated mutants' library of *A. orientalis*/pSGKP45-UV1(3) were used for sorting best *gfp* expressing clones. Each dot in the scatter plot represents a single cell. The FSC-A versus SSC-A measures the proportion of laser beam that goes around the cell to the amount of laser beam that bounces off particles within the cell. These traits may be used to look at a group of cells and distinguish them differently based on their size and internal complexity. FSC-H versus FSC-W and SSC-A versus SSC-W enables us to distinguish the doublet and identify variations in cell size versus cell signal. (FITC: Fluorescence of singlets; FITC-A: Fluorescence area; FSC: Forward scatter; FSC-A: Forward scatter area; FSC-H: Forward scatter height; P1 and P2 represent population of cells; SSC: Side scatter; SSC-A: Side scatter area; SSC-H: Side scatter height, SSC-W: Side scatter width).

The sorted activated mutants were raised in MS-plates supplemented with kanamycin (200 µg/mL). In a few days, I observed the growth of single cells in individual plates, which were selected for further experimentation. Some phenotypic differences were observed between the parental and activated mutants. I observed that the mutants were darkly pigmented and generated some compounds in MS-agar plates. Moreover, after 4 days of growth in liquid MS media, the mutants produced coloured compounds. I performed comparative metabolic profiling of parental and UV-activated mutants using HPLC and LC-MS and found four unique metabolites not observed in the parental WT strain (Fig. 13). UV spectrum showed absorption maxima at 225, 252, 279, and 409 nm. LC-MS confirmed these compounds to have a mass of 442 and 453 m/z, yielding a chemical formula of $C_{22}H_{18}O_{10}$ (observed mass 442.0900 g/mol; calculated mass 441.0827 g/mol). Structure elucidation by NMR revealed that the compounds detected were known microbial polyketides denoted as mutaxanthenes.

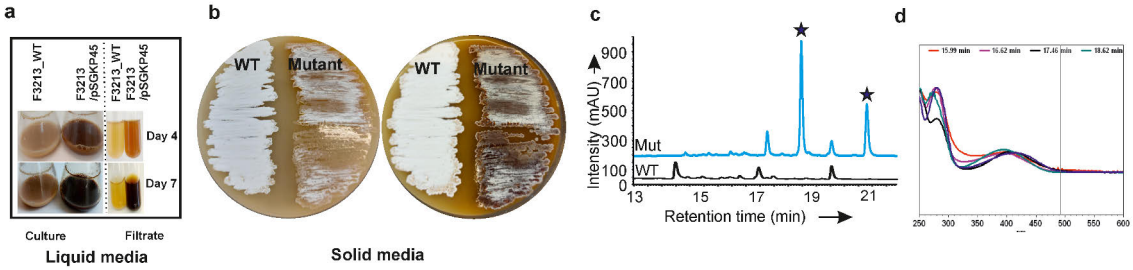

Figure 13. Metabolic profiling of WT and activated mutant in liquid and solid MS medium. **a** and **b**) Comparison of metabolites production by WT and activated mutant in liquid and solid MS medium. **c**) HPLC analysis of metabolites from WT and sorted mutant. d) UV/Vis spectrum (UV-maxima: 225, 252, 279 and 409 nm). Asterisk (*) indicates the production of new metabolites by the sorted mutant compared to the wild-type.

Next, I utilized RT-PCR to determine that the silent mutaxanthene cluster in the sorted mutant strain had been activated. The expression profile of the gene encoding KSα was evaluated from 3-8 days grown mutant by using degenerate primers. The development of band of the size 613 in gel electrophoresis confirmed that the BGC had been turned on in the activated mutant strain, but no mRNA transcripts could be observed from the parental strain used as a control (Fig. 14b-d).

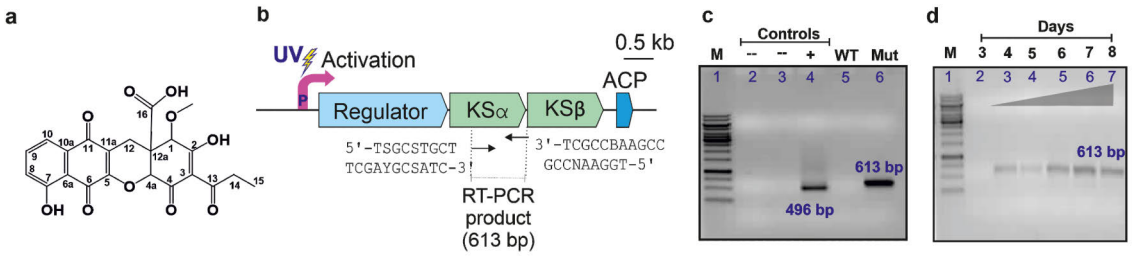

Figure 14. Expression profiling of mutaxanthene cluster. **a**) mutaxanthene chemical structure synthesized by activated mutant. **b**) minPKS of mutaxanthene biosynthetic cluster. **c**) verification of the cluster activation by RT-PCR. Reaction mixtures without reverse transcriptase enzyme and without RNA template were used as negative controls (lanes 2 and 3, respectively). *In vitro*-transcribed human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control RNA (lane 4) was used as a positive control, yielding a product with a size of 496 bp. On day 7, active BGC was detected in *A. orientalis* NRRL F3213/pSGKP45-UV1(3), with the predicted 613 bp band (lane 6). No products were observed in analysis of the wild type strain (lane 5), and **d**) the time course reveals that the BGC is activated on day 4 (lane 3) in *A. orientalis* NRRL F3213/pSGKP45-UV1(3), and transcription proceeds until day 8 (lane 3-7).

My data demonstrated that the mutaxanthene backbone is derived from propionyl-CoA and 9× malonyl-CoA and synthesized by the enzymes encoded by the *mut* cluster of *Amycolatopsis orientalis* NRRL F3213 genome. Based on my results, I hypothesized that this unique scaffold requires numerous skeletal rearrangements of an unreduced decaketide precursor. Given the paucity of comprehensive pathway details, it currently appears that providing thorough mechanistic insights on the process is challenging. However, previous work [264] has postulated the following possibilities for mutaxanthene biosynthesis in *Nocardiopsis* sp. *FU40 ΔApoS8*; (i) uncoupling of two carbons in the decaketide chain at positions C16 and C4a during acetate incorporation experiments suggest that these carbons were once part of an interconnected acetate complex; (ii) existence of an ether and carboxymate moieties, as well as the presence of uncoupled acetate-derived carbons, suggest oxidative cleavage rather than acetate unit cleavage; (iii) occurrence of an acetate-derived carboxylate in the polyketide chain indicates a further carbon skeletal rearrangement involving ring contraction [264].

Based on genes within the BGC, mutaxanthene biosynthesis is possibly initiated by incorporating acetate units to malonyl-CoA and propionyl-CoA, which undergoes condensation reaction (Fig. 15). MinPKS catalyzes the formation of a linear polyketide from malonyl-CoA etc., after which specific cyclases and aromatases give rise to the polycyclic polyketide [264]. The intermediate compound 'C' in the mutaxanthene pathway is related to many polyketides, including polyketomycin and mithramycin pathways (Fig. 15). The product undergoes Baeyer-Villiger oxidation reaction, which adds an *O*-atom to the carbon ring (between 4a and 5-carbon). This intermediate product undergoes a Claisen condensation reaction, followed by the

shuffling of electrons to produce mutaxanthene [264]. A Baeyer-Villiger oxidation reaction has also been observed in mithramycin biosynthesis, where the reaction incorporates an *O*-atom to the carbon ring, followed by ring contraction rearrangement analogous to the Favorskii-like reaction. An oxidative carbonylation sequence 'E' occurs prior to the formation of a cyclopropanone intermediate 'F,' which, when hydrated, rearranges to an internally located carboxylate 'H' in okadaic acids. Finally, an intramolecular Michael extension could complete the xanthene structure [264] (Fig. 15).

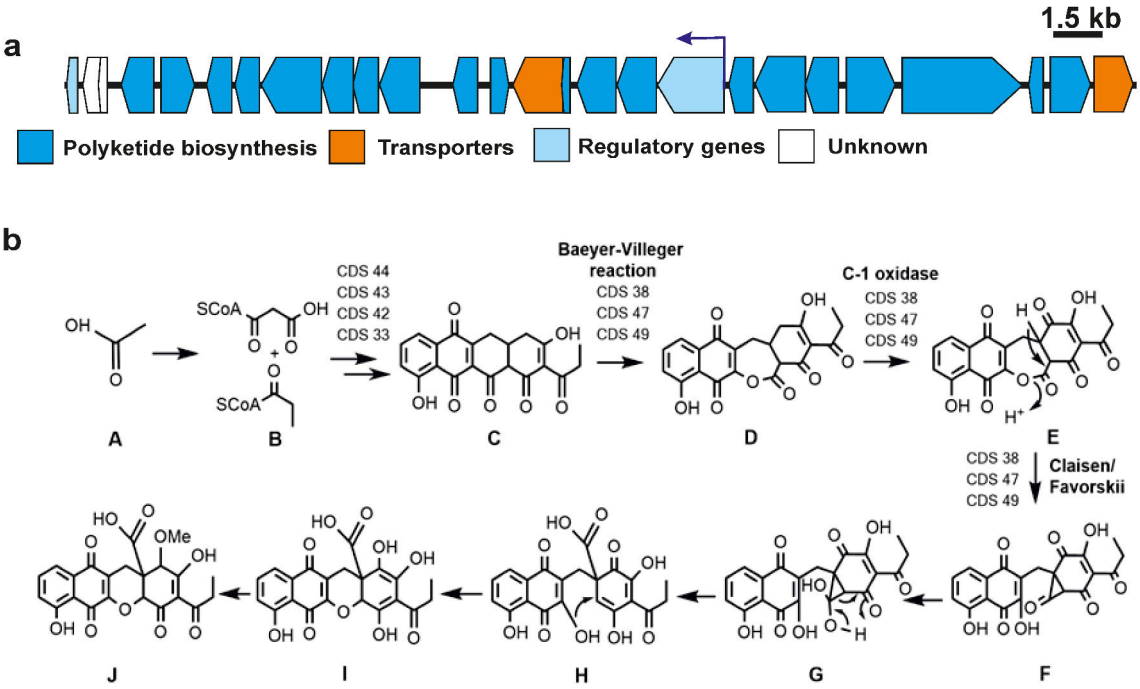

Figure 15. Mechanistic insights of novel polyketide mutaxanthene in *Amycolatopsis orientalis* NRRL F3213. **a**) Mutaxanthene biosynthetic gene cluster revealing the core biosynthetic genes, transporters, and regulatory genes. The arrowhead indicates the selection of the promoter from the corresponding gene for activation. **b**) Putative scheme for the production of mutaxanthene, hypothesizing the putative involvement of different genes within the pathway. Adapted from [264]. Detailed gene name and putative functions of the coding-sequence (CDS) involved in mutaxanthene biosynthesis is provided in the table below.

Table 2. Coding sequences from the silent gene cluster of *Amycolatopsis orientalis* NRRL F3213 and their roles (established / proposed) within the biosynthetic pathway

Gene name	Coding sequence (CDS)	Size (AA)	RAST annotation	Most similar ORF [species](identity %)	Accession no.
mutA	CDS28	113	Transcriptional regulator, PadR family	PadR family transcriptional regulator [*Nonomuraea* sp. WAC 01424](87)	WP_125636118.1
mutB	CDS29	196	Hypothetical protein	Hypothetical protein [*Kibdelosporangium aridum*](91)	WP_037276114.1
mutC	CDS30	90	Hypothetical protein	Predicted protein [*Streptomyces* sp. AA4] (97.78)	EFL10681.1
mutD	CDS31	332	Malonyl CoA-acyl carrier protein transacylase	Acyltransferase domain-containing protein [*Nonomuraea montanisoli*](54)	WP_175592186.1
mutE	CDS32	344	Hypothetical protein	Ketoacyl-ACP synthase III family protein [*Streptomyces* sp. Go-475](57)	WP_114255099.1
mutF	CDS33	257	Hypothetical protein	Cyclase family protein [*Kutzneria* sp. CA-103260](74)	WP_211768124.1
mutG	CDS34	249	Short-chain dehydrogenase/reductase SDR	SDR family oxidoreductase [*Nocardia arthritidis*](59.35)	QIS10038.1
mutH	CDS35	617	Long-chain-fatty-acid--CoA ligase	AMP-binding protein [*Kutzneria* sp. CA-103260](55)	WP_211768122.1
mutI	CDS36	319	Aromatase	Aromatase/cyclase [*Kutzneria* sp. CA-103260](69)	WP_211768121.1
mutJ	CDS37	261	Acetoacetyl-CoA reductase	SDR family NAD(P)-dependent oxidoreductase [*Kutzneria albida*](74)	WP_025361002.1
mutK	CDS38	411	polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases	FAD-dependent monooxygenase [*Kutzneria* sp. CA-103260](63)	WP_211768119.1
mutL	CDS39	252	3-oxoacyl-[acyl-carrier protein] reductase	SDR family oxidoreductase [*Streptomyces thermodiastaticus*](52)	WP_189795586.1
mutM	CDS40	188	*O*-methyltransferase	Ubiquinone/menaquinone biosynthesis C-methylase UbiE [*Kutzneria viridogrisea*](47)	MBA8930871.1
mutN	CDS41	509	Putative integral membrane protein	EmrB/QacA subfamily drug resistance transporter [*Candidatus Frankia californiensis*](52)	SBW23174.1
mutO	CDS42	85	Acyl carrier protein	Acyl carrier protein [*Thermomonospora echinospora*](55)	WP_103936125.1
mutP	CDS43	401	Polyketide chain length factor WhiE-CLF paralog	Ketosynthase chain-length factor [*Streptomyces* sp. CBMA152](65)	WP_188275488.1
mutQ	CDS44	430	Polyketide beta-ketoacyl synthase WhiE-KS paralog	Beta-ketoacyl-[acyl-carrier-protein] synthase family protein [*Kutzneria albida*](75)	WP_030111238.1

Gene name	Coding sequence (CDS)	Size (AA)	RAST annotation	Most similar ORF [species](identity %)	Accession no.
mutR	CDS45	680	Regulatory protein	DNA-binding SARP family transcriptional activator [*Kutzneria viridogrisea*](46)	MBA8926443.1
mutS	CDS46	251	Oxidoreductase, short-chain dehydrogenase/reductase family	Ketoreductase [*Streptomyces argillaceus*](58)	CAK50776.1
mutT	CDS47	516	2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases	FAD-dependent oxidoreductase [*Kitasatospora* sp. NA04385](62)	WP_176190106.1
mutU	CDS48	340	*O*-methyltransferase	Methyltransferase [*Kutzneria albida*](68)	WP_025361012.1
mutV	CDS49	499	Rifampin monooxygenase	FAD-dependent monooxygenase [*Kutzneria* sp. CA-103260](64)	WP_211768107.1
mutW	CDS50	1227	Dihydrolipoamide succinyltransferase component (E2) of 2-oxoglutarate dehydrogenase complex	Alpha-ketoglutarate decarboxylase [*Amycolatopsis regifaucium*](87)	WP_081809737.1
mutX	CDS51	149	Hypothetical protein	Nitroreductase family deazaflavin-dependent oxidoreductase [*Nocardia miyunensis*](69)	WP_084531700.1
mutY	CDS52	412	putative membrane protein	FAD-dependent oxidoreductase [*Pseudonocardia eucalypti*](67)	WP_185062231.1
mutZ	CDS53	399	Nitrate/nitrite transporter NarK	MFS transporter [*Prauserella sediminis*](81)	WP_183785025.1

Industrial usage of microbes necessitates over-producing strains developed through engineering techniques. Among various strategies available, UV-induced mutations have been used to generate microbial strains with significantly higher metabolic yields. With this concept, I performed a second round of UV mutagenesis (UV_C radiation; 5 min) and sorted the best *gfp* expressing clones using FACS. The average amount of mutaxanthene produced by first-round mutants was 11 mg/L. The mutants from the first round were further exposed to a second round of UV mutation and selection was done using FACS. These second-round mutants resulted in a substantial increase in mutaxanthene yield, with an average production of 55 mg/L. *A. orientalis*/pSGKP45-UV2(38), the top performing mutant from the second-round mutation, produced a total of 99 mg/L of mutaxanthene compared to top producing first round mutant *A. orientalis*/pSGKP45-UV1(3) (16 mg/L). Thus, my research confirms that this developed method (UV mutagenesis followed by FACS sorting)

will be highly applicable in pharmaceutical industries for strain development purposes.

Furthermore, the FACS sorting approach had previously been used to sort spores and protoplasts of filamentous organisms like *Streptomyces*, but to a limited extent. My present method of washing the *Streptomyces* filamentous mycelium with phosphate-buffered saline (PBS) followed by sonicating the mycelial hyphae to break them into individual cells before subjecting mycelial clumps to sorting using FACS with a larger nozzle, allowed me to perform studies in a single cell format. Though few earlier investigations such as in *Nocardiopsis* sp. *FU40 ΔApoS8* have been attempted to sort protoplasts using FACS, problems with breaking the cells arose due to the fragile nature of protoplasts [264]. Previously, Cao et al., [265] utilized HTS (high-throughput screening) coupled with FACS to sort *S. avermitilis* spores using propium iodide and fluorescein diacetate to discriminate between dead and living spores. FACS sorted over-producers yielded 20.6 % higher avermectin production compared to the parental strain. However, the severe drawback of this approach involved screening of hundreds of mutants to trace the over producing mutant. Furthermore, FACS was also utilized to sort single biosensor *E. coli* MC4100 cells that produces GFP when induced with tetracycline (50 ng/ml) [266]. However, FACS sorting approach in *Streptomyces* cannot be employed in the same way as for *E. coli* due to the complexities with developing cells in a mycelial network in *Streptomyces*. My present method, which involves washing the cells with PBS, sonication to break down *Streptomyces* filaments into individual cells, and filtration for getting single cells, has significantly reduced the flaws found in this field. This eliminated the difficulties in working with *Streptomyces* at a single-cell resolution by allowing FACS to sort the individual cells directly.

Thus, the beauty of working with SCMS includes a direct correlation between target gene cluster expression and microbial strain survival under selective pressure (elevated concentration of antibiotics). This double reporter method reduces false positives drastically, as was observed previously [267]. In addition, this promising and straightforward workflow bypasses the use of complex molecular genetic methods and can handle several strains in parallel. Most modern methodologies, for example, necessitate the laborious cultivation and sampling of multiple candidate strains to detect positive hits. However, in SCMS, no prior screening is needed, and positive mutants may be chosen directly by using FACS, significantly improving the methodology's throughput. Furthermore, while other approaches restrict modification attempts to the target gene cluster, we could probe the entire genome to drive the expression of the secondary metabolic pathway of choice. The final advantage of SCMS is that it can be used to boost natural product yields in an iterative manner. I believe that this promising strategy might aid in the monitoring

of targeted gene cluster activation and apply them in industries to aid in strain development process.

4.2 Yeast-*Streptomyces* interactions (Original publication II)

Interaction studies between species have been instrumental in unlocking the true potential of cryptic gene clusters [268]. Earlier studies reported overexpression of cholesterol oxidase enzyme by *S. lavendulae*, when co-cultured with yeast cells [269]. This suggested that dead yeast cells could influence overexpression of cryptic gene clusters in *Streptomyces*. To study this interaction further, we isolated several soil isolates and grew them with yeast, and one strain produced a new pigmented metabolite. Using this as a guide, we could demonstrate by NMR and LC-MS that the soil isolate produced prodigiosins upon contact with yeast cells. Data obtained from genome sequencing revealed that the interactions led to overexpression of a cryptic *RED* cluster (undecylprodigiosin; size *ca*. 31 kb) [270,271] from *Streptomyces* sp. MBK6 in a medium containing dead yeast cells.

The model organism *S. coelicolor* also produces prodigiosins. I was eager to see if the production of prodigiosin by *S. coelicolor* and undecylprodigiosin by *Streptomyces* sp. MBK6, are regulated in a similar manner. Remarkably, *S. coelicolor* and MBK6 responded differently in cultured medium, and a marked distinction was observed in their growth in yeast media. Unlike *S. coelicolor*, which grows homogeneously, MBK6 was found to sequester yeast cells in its mycelial network during its development, which also increased undecylprodigiosin production (Fig. 16). Yeast cells, however, induced prodigiosin production both in *S. coelicolor* (30×) and *Streptomyces* sp. MBK6 (75×). With these notable differences in production profile, I speculated that the discrepancies might be due to a regulatory gene *redZ* located in the BGC.

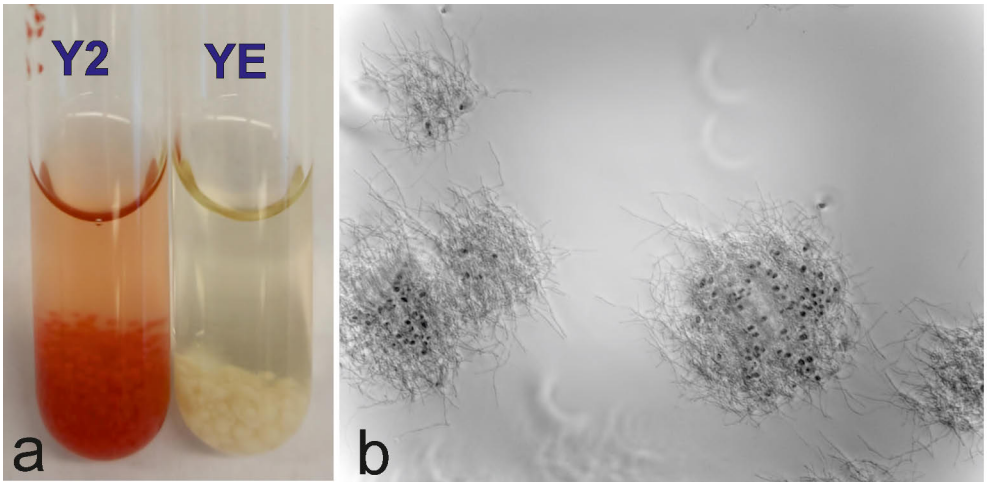

Figure 16. Growth comparison of MBK6. a) Four days old culture of MBK6 in Y2 media with yeast and YE medium (without yeast). MBK6 generated red compound when cultivated in yeast media (Y2) but not in YE medium, and the produced compounds were localized intracellularly. **b**) Microscopic picture (100× magnification) of MBK6 growing in Y2 medium. Dark spherical yeast cells were sequestered at the periphery of MBK6 mycelial filaments.

The genome of *Streptomyces* sp. MBK6 was sequenced, and a cluster expressing undecylprodiogsin was discovered to be colinear with the red cluster of *S. coelicolor* (Fig. 17). However, there were several noticeable differences, such as the lack of a *redF* homolog and the translational fusion of *redL* (type I polyketide synthase) and *redK* (type II polyketide synthase) (oxidoreductase). A significant difference in the N-terminal of *redZ* (a cluster-situated master regulator belonging to the orphan response regulator) was identified when the prodigiosin synthesizing cluster in *S. griseoaurantiacus* and *Streptomyces* sp. MBK6 were examined (Fig. 17A). The undecylprodiogsin cluster of *Streptomyces* sp. MBK6 was compared to reference genomes in the NCBI database, and a highly similar (98.91%) cluster with *S. griseoaurantiacus* MO45 was discovered. A 16S rRNA analysis between *Streptomyces* sp. MBK6 and *S. griseoauranticus* revealed only a base change.

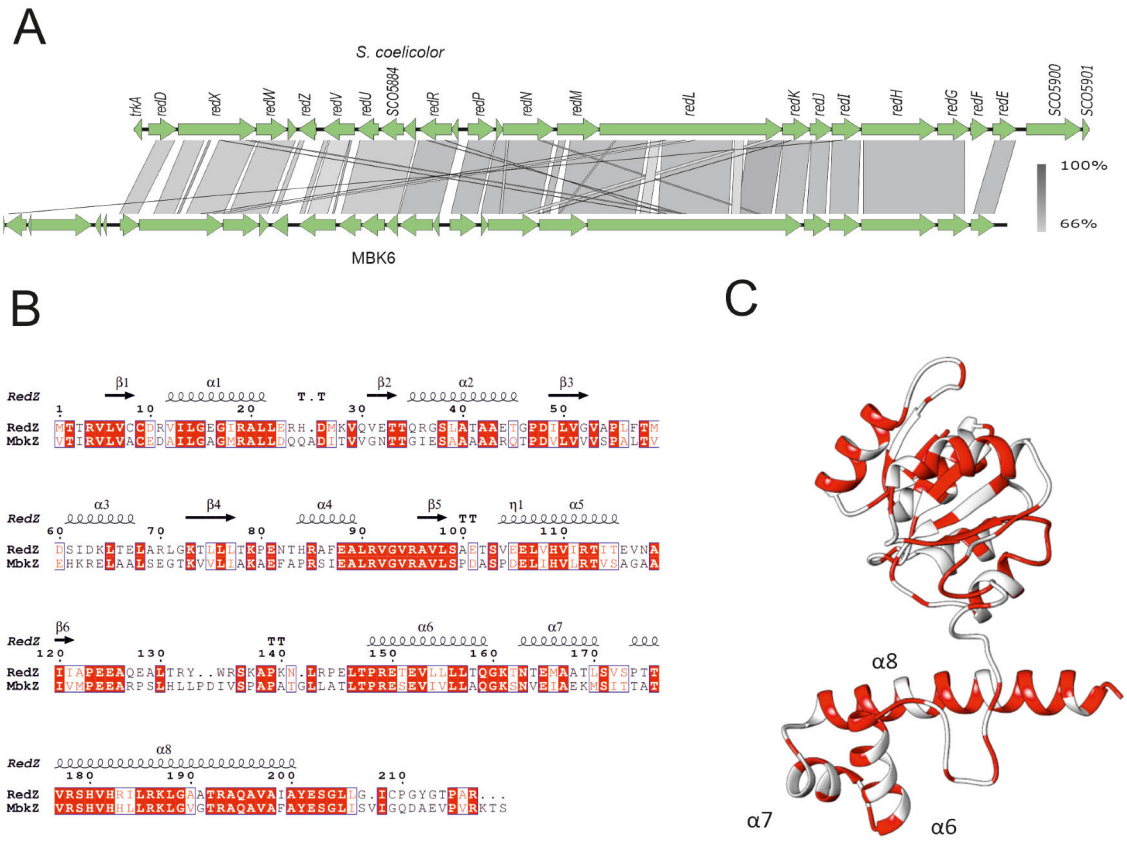

Figure 17. Prodiginine cluster comparison and a pathway-regulator (*RedZ* and *MbkZ*) comparison in *S. coelicolor* and *Streptomyces* sp. MBK6. A) Comparative analysis of prodiginine biosynthetic gene cluster in *S. coelicolor* and *Streptomyces* sp. MBK6. **B**) Alignment of RedZ and MbkZ. **C**) Homology model of *RedZ*, with amino acids conserved with *MbkZ* are colored in red.

Constructs with different combinations of *mbkZ* promoter were developed. Synthetic gene *mbkZ* with its own promoter was ordered and cloned into the plasmids pIJE486 (*ermE*p-*mbkZ*p), pIJ486 (*mbkZ*p), and pIJE486 without its own promoter (*ermE*p). These promoters were first cloned in *E. coli*, then transformed into an intermediate host, *S. lividans* TK24, before being transformed into *Streptomyces* sp. MBK6 and *S. coelicolor*. The mutants were then cultured in a range of different culture media. Introducing *mbkZp* promoter to *S. coelicolor* stimulated prodigiosins production in yeast and yeast-extract medium. However, *S. coelicolor* mutants, which have a strong-constitutive promoter (wild type *ermE* preceding the *mbkZ* promoter) dramatically suppressed metabolite production in both mediums, which contrasted with *Streptomyces* sp. MBK6. In *S. coelicolor*, the repression was also observed with a construct containing only the *ermE*p promoter. This indicates differential

expression of regulatory genes in two separate hosts under two different conditions (Fig. 18a,b). Having observed these marked differences, an electrophoretic mobility shift assay (EMSA) was performed to investigate MbkZ binding to the promoter regions of *mbkD*. We discovered that heterologously produced MbkZ protein binds to promoter regions of *mbkD* and *S. coelicolor redD* (Fig. 18c).

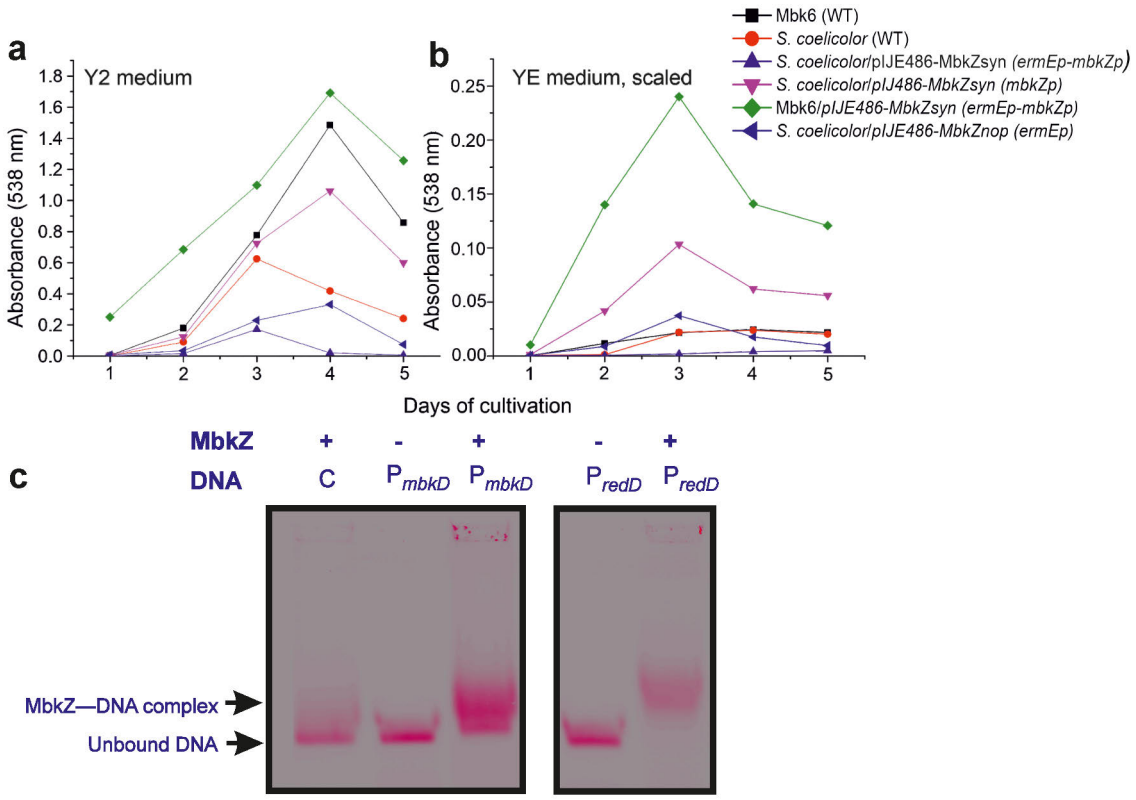

Figure 18. Comparative analysis of prodigiosin production by MBK6 and *S. coelicolor*. **a**) Production of prodigiosin in autoclaved yeast-media (Y2). **b**) production of prodigiosin in yeast-extract medium (YE), and **c**) Electrophoretic mobility shift assay (EMSA) to investigate MbkZ binding to the promoter regions of *mbkD*.

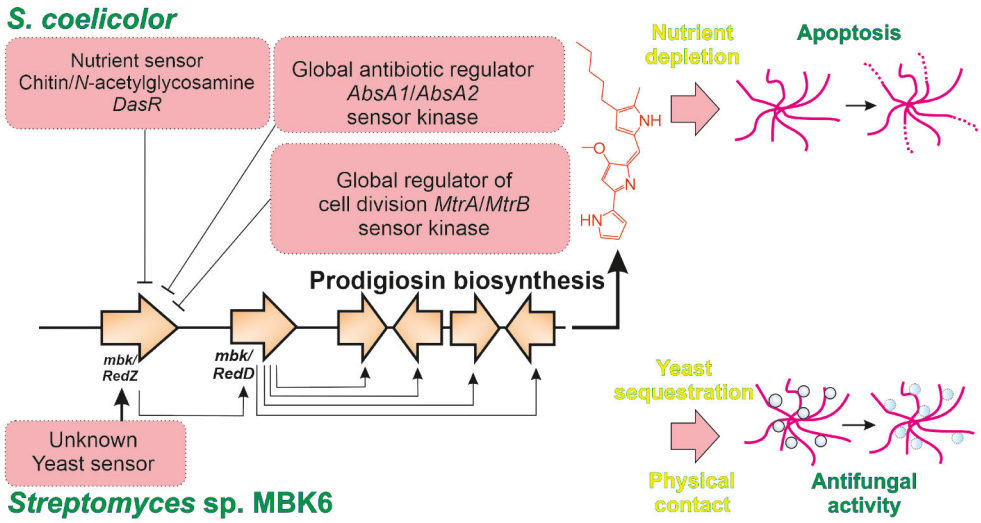

Figure 19. Regulation of prodigiosin biosynthesis in *S. coelicolor* and *Streptomyces* sp. MBK6.

I argue that this discrepancy in prodigiosin production may be due to the use of the compound for different purposes. Production of prodigiosin in *S. coelicolor* is tightly co-regulated by two pathway-specific activators, *redZ* [272] and *redD* [171]. Several mechanisms, including sensor kinases, antibiotic synthesis, and nutrient supply, inhibit *redZ* transcription affecting the prodigiosin biosynthesis in *S. coelicolor* [130,273–276]. Also, in addition to two-pathway regulators (*redZ* and *redD*), global transcriptional regulators, such as two-component systems AfsQ1/Q2 [277], DraR/K [278] and AbsA1/A2 [279], TetR-family regulator RrdA [280], LysR-type regulator StgR [281] and ROK family protein Rok7B7 [282] are known to regulate prodiogsin production in *S. coelicolor*. Furthermore, in *S. coelicolor*, intracellular localization of prodigiosin is toxic, resulting in programmed cell death [130,276]. This apoptosis has been attributed to the lack of nutrients required for mycelial growth, causing some mycelium to sacrifice themselves to ensure the survival of their fellow members (Fig. 19).

Streptomyces sp. MBK6, on the other hand, behaves differently, sequestering yeast cells within their mycelial network and generating prodigiosin in large quantities. As *Streptomyces* species are efficient nutrient explorers, they have evolved diverse adaptive responses to thrive in various ecological niches and nutrient-limited environments. Such a phenomenon for nutrient exploration occurs with an intricate association and metabolic trade-off between inter-kingdom species *Streptomyces* and yeast, which triggers a change in developmental behaviour in bacteria, allowing *Streptomyces* to biosynthesize new compounds or overexpress

specific cryptic clusters (e.g., prodigiosin biosynthetic cluster in *Streptomyces* sp. MBK6).

My experiments revealed that the production of prodigiosin differs between organisms and is affected by various factors. Prodigiosin is a common secondary metabolite, and it may be that it serves even more diverse roles in other *Streptomyces* species. The production of prodigiosin is regulated differently, but typically stringent control exists by transcriptional regulatory cascades (e.g., global and pathway-specific regulators, and their feedback regulation) [25]. Prodigiosin production in *Streptomyces* is affected by several factors, including inorganic phosphate abundance, media, temperature, pH [283], and interactions with other species. *Bacillus subtilis* living or dead (autoclaved) cells boosted undecylprodigiosin production in *S. coelicolor* by 256%, possibly due to *B. subtilis* dead cells supplying growth and undecylprodigiosin stimulants, which could act as precursors for generating the product [284]. Two complex mechanisms, viz., *N*-acyl-L-homoserine lactone quorum-sensing-dependent and-independent pathways, are known to regulate prodigiosin production in *Serratia* [131,285]. However, the same regulator responds differently to yeast cells in different expression hosts, such as *S. coelicolor* and *Streptomyces* sp. MBK6. Also, it is worth noting that MBK6 mutants may respond differently to yeast-surface proteins, which could explain why prodigiosins were overexpressed in my study. Despite the failure to access other mutants, it would have been a rewarding discovery if I had been able to observe how mutants of MBK6 with other constructs behave in culture with dead yeast. Finally, I conclude that systematic rewiring of these regulatory networks, such as the induction of positive regulators, as in my experiment, could largely facilitate the development/ overexpression of new metabolites and their production in the future.

4.3 CRISPR/Cas9 system for characterizing a showdomycin cluster (Original publication III)

Among several methods currently used for genome engineering, we used a robust and advanced tool, CRISPR/Cas9, to characterize an involvement of a gene within the biosynthetic pathway. CRISPR/Cas9-based genome editing toolbox has revolutionized genome engineering and is now widely used in all domains of life (archaea, bacteria, and eukarya) [286,287]. This system utilizes a transcribed synthetic guide RNA (sgRNA), which directs Cas proteins (mostly Cas endonucleases, including the *Streptococcus pyogenes*-derived Cas9) to any desired site in the genome [288]. Because of its precision, handiness, and rapidity, CRISPR allows quick engineering of individuals or multiple genes within a pathway [289]. However, a built-in technological constraint of this system involves the need for G-rich protospacer-adjacent motif (PAM) sequence (5'-NGG-3') that is recognised by

the sgRNA, which is necessary for proper Cas9 binding and target sequence recognition. Since *Streptomyces* genomes have high-GC regions (>70%) [290], PAM sequences are found commonly dispersed in *Streptomyces* genomes (e.g., 260 targets per 1000 bp in *S. coelicolor*), which can result in off-target effects [291]. Furthermore, AT-rich DNA sequences may or may not contain the PAM series (Fig. 20).

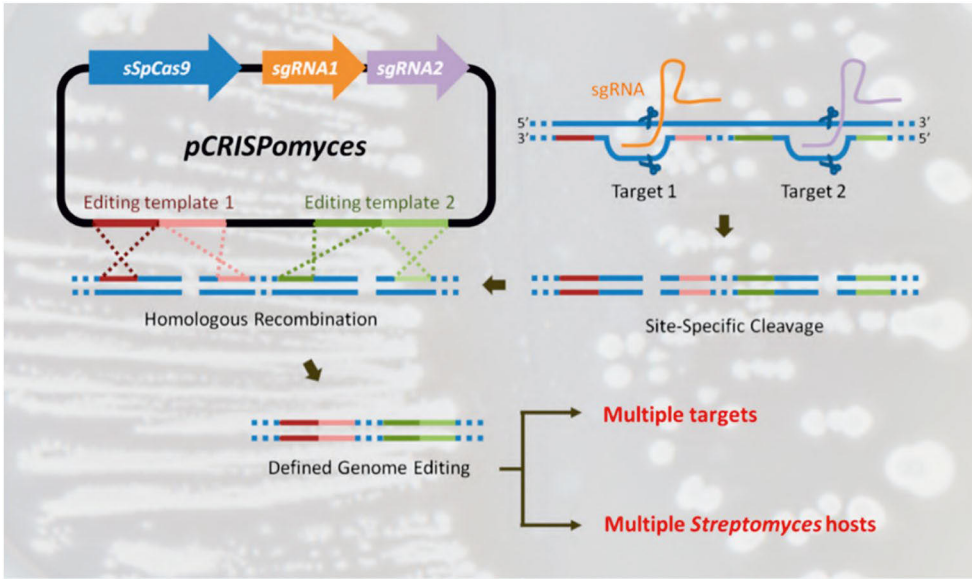

Figure 20. Genome editing in *Streptomyces* species using pCRISPomyces plasmid. Adapted from [292].

S. showdoensis is the native producer of showdomycin (a *C*-glycosyl nucleoside), the production of which we have confirmed using metabolic profiling experiments [293]. Showdomycin structurally resembles uridine and pseudouridine, with the exception that the nucleobase is substituted with an electrophilic maleimide moiety in showdomycin [294]. In *C*-nucleosides, the nucleobase is bound to C-1' of the sugar ring by a stable C-C bond instead of a C-N bond, making the *C*-nucleotides more stable [295]. Furthermore, as nucleosides possess antitumor, antiviral, and antibiotic properties [296], this has piqued my interest. *N*-glycosyl-related purines and pyrimidines have limitations in RNA and DNA chemistry due to their lower stability. However, the *C*-nucleosides resolve the stability issues shown by N-nucleosides, making the variants more stable with impressive biological prowess [297]. Despite their significance, the enzymes that generate *C*-ribosides and *C*-glycosides have received little attention. In current experiment, I have utilized CRISPR/Cas9 technique to characterize the involvement of *sdmA*, a member of the

pseudouridine monophosphate glycosidase family, in the showdomycin pathway. I have discovered that *sdmA* catalyzes *C*-glycosidic bond formation by disrupting the gene using the CRISPR/Cas9 technique and then complementing back with its full copy, which restored the compounds' biosynthesis.

For experimental analysis, a gene inactivation construct (CRISPR/Cas9 pCRISP-2_*sdmA*_2kb) was first created. Golden Gate assembly was used to insert the protospacer sequence into a vector (pCRISPomyces2). The *sdmA* gene within the showdomycin pathway was chosen as the target gene to be knocked out. The Golden gate reaction was carried out with annealed protospacers (detail protocol in the supplement of paper III). The 1 kb flanking regions of the target gene (*sdmA*) to be knocked-out were PCR amplified and cloned using the Gibson cloning method, which promoted homologous recombination. The plasmid pCRISP-2_*sdmA*_2kb was produced and cloned into methylation deficient *E. coli* ET12567/pUZ8002 before being conjugated into *S. showdoensis* ATCC 15227.

The single cell conjugants that appeared were analysed for the deletion of the target gene. This was accomplished using genomic DNA and PCR (designed to anneal beyond the area of the homologous template regions in pCRISPOmyces-Δ*sdmA*), resulting in a 2 kbp fragment in the right clones. Before being cultivated to observe the production, the correct clone was subjected to plasmid eradication. Complementation of the *sdmA* gene was achieved by amplifying the gene using PCR, cloning it into pUC18 and then transferring it to pIJE486 plasmid, which was then inserted to the knocked-out mutants using protoplast transformation method (detail protocol in the supplement of paper III).

Showdomycin biosynthetic cluster comprises 16 different genes associated with aglycone synthesis, C-glycosylation, resistance, and some genes yet to be characterized. Homologs of seven genes from the *S. showdoensis* showdomycin cluster were also discovered in *S. globisporus* and *N. alba*, although their functions are yet to be determined (Fig. 21a). Using antiSMASH, the analysis of showdomycin gene cluster revealed an unusual combination of genes. Only two genes, *sdmC* and *sdmD*, were found to encode for amino acid adenylation and peptidyl carrier protein domains, respectively. The genome sequence revealed that clusters of genes identical to *sdmABCD* are extremely rare in actinobacteria, however two examples where the homologs of this cluster were found are *S. globisporus* C-1027 and *Nocardiopsis alba* ATCC BAA-2165. *S. showdoensis* grows very rapidly and produces highest level of showdomycin on the first day following inoculation. Because of its unstable nature, showdomycin progressively degrades after prolonged culture (Fig. 21d). The extract ion chromatogram traces of showdomycin **(3)**, revealed the production of showdomycin by *S. showdoensis* WT, and the production was abolished when a crucial gene *sdmA* was knocked-out (*S. showdoensis* Δ*sdmA*) using CRISPR/Cas9 technique. The production was restored when an intact copy of the gene was

complemented back to the knocked-out strain (*S. showdoensis* Δ*sdmA/*pIJE486-*sdmA*) (Fig. 21e).

The formation of aminopyrrole moiety in *C*-nucleosides had long been a mystery. The aminopyrrole moiety in the kosinostatin pathway, which shares genes with the showdomycin biosynthetic cluster, has recently been revealed to be exceptional [298]. *In vitro* evidence suggests that the aminopyrrolinic acid (used as a substrate) was generated through the cyclization of L-glutamine by an L-ectoine synthase-like enzyme, KstB3 [298]. Transglutaminase and FAD-dependent dehydrogenase have also been linked to aminopyrrole production [298].

Despite prior findings suggest that the maleimide ring of **(3)** is generated from carbons 2-5 and the nitrogen atom of L-glutamate, software projections demonstrate that SdmC favours L-glycine as a substrate. The substrate specificity of recombinantly generated and purified histidine tagged SdmC protein was evaluated against all proteinogenic amino acids and ATP. However, none of the amino acids examined produced pyrophosphate, indicating that SdmC does not use proteinogenic amino acids as a substrate. As a result, we proposed that the cyclization of L-glutamine by either SdmE or SdmG is the first step in the biosynthesis of **(3).**

According to our propositions and available literatures, the showdomycin pathway potentially initiates with L-glutamine, which then undergoes cyclization probably encoded by SdmE or SdmG. SdmE catalyzes the cyclization of L-glutamine to an intermediate (2-amino-1-pyrroline-5-carboxylate), which is eventually placed onto the phosphopantetheine arm of peptidyl carrier protein SdmD [299]. Updated information reveals that the early steps in showdomycin biosynthesis utilizes the involvement of nonribosomal peptide synthetases that synthesizes nonribosomal peptide encoded by the genes *sdmC* and *sdmD* [299]. SdmM and SdmF can deaminate and desaturate enamine, while SdmG (a transglutaminase-like protein) catalyzes the hydrolysis of the thioester linkage to yield 2-amino-1H-pyrrole-5-carboxylate as a crucial step [299]. The flavoenzyme SdmF, on the other hand, is unable to catalyze the oxidation of the SdmE-catalyzed product [299]. This has however been discovered to be different in a kosinostatin biosynthesis pathway, which is a common evolutionary ancestor of the showdomycin pathway and contains homologs to several showdomycin pathway genes (*sdmECDFG*). In addition to oxidation, the kosinostatin BGC requires additional NRPS catalyzed modifications [300]. The activity of SdmF and SdmG releases a free acid which acts as the substrate for the *C*-glycosidase SdmA [299]. The *C*-glycosylation of the reaction product (free acid) is catalyzed by SdmA, while SdmB dephosphorylates it further. SdmH and SdmP are likely to maintain the proper stereochemistry and catalyze the decarboxylation at C-1, respectively, while SdmN is likely to form the ultimate maleimide ring (Fig. 21c).

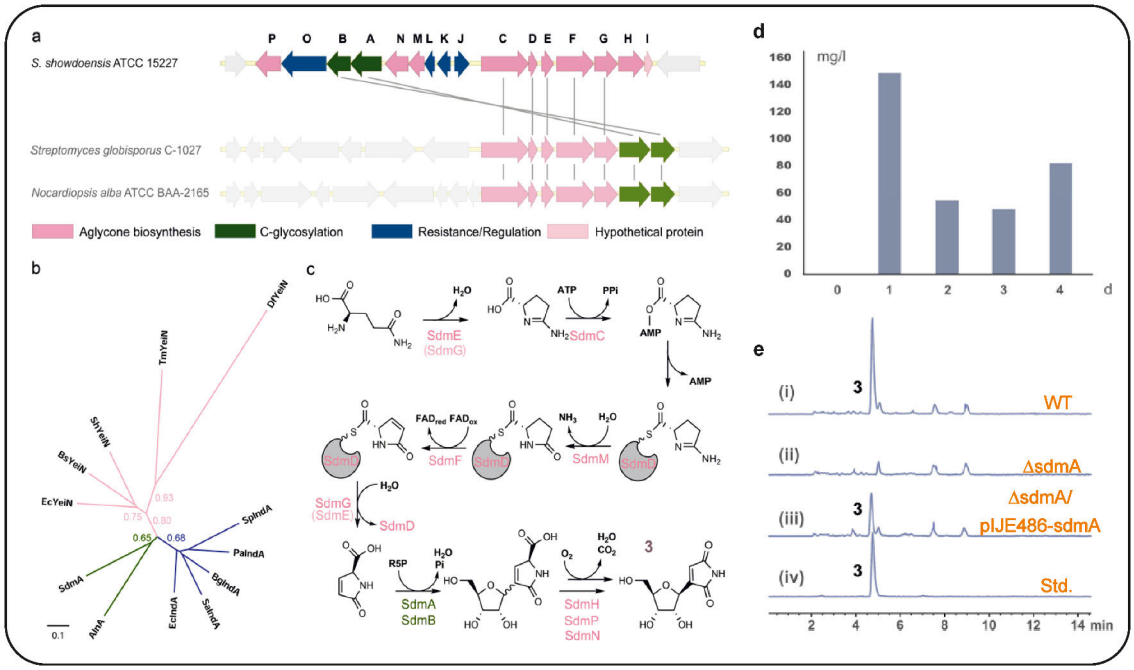

Figure 21. Discovery of showdomycin gene cluster and the biosynthesis of showdomycin. **a**) Related cluster comparison between *S. showdoensis* ATCC 15227 (top panel on 'a'), *S. globisporous* C-1027, and *Nocardiopsis alba* ATCC BAA-2165. Similarity between the genes is illustrated by gray lines that connect them. **b**) Phylogenetic analysis of proteins belonging to the pseudouridine glycosidase family, which includes monophosphate glycosidases, indigoidine pigment genes, and C-glycosynthases involved in antibiotic synthesis. **c**) Proposed scheme for the biosynthesis of showdomycin. **d**) Showdomycin production by *S. showdoensis* over time. Owing to the strain's rapid growth, the production of showdomycin was found to be maximum on the first day following inoculation. After extensive cultivations, the compound degraded due to its unstable nature. **e**) Extract ion chromatogram traces of showdomycin **(3)** from cultivation samples (i) *S. showdoensis* WT, (ii) *S. showdoensis* Δ*sdmA*, (iii) *S. showdoensis* Δ*sdmA*/pIJE486-*sdmA*, (iv) standard that was commercially available.

Thus, the product generated by the C-C linkage between C3 of 2-amino-1H-pyrrole-5-carboxylic acid and C1' of ribose 5-phosphate in presence of molecular oxygen is highly unstable and is a crucial step in the biosynthesis of showdomycin [299]. Furthermore, high-sequence homology between SdmA and YeiN (pseudouridine monophosphate *C*-glycosidase) indicates that they are both ribose 5-phosphate dependent rather than phosphoribosyl pyrophosphate-dependent, implying that a comparable C-glycosidation process for showdomycin production might occur [301,302]. Moreover, our analysis using CRISPR/Cas9 to knock-down a crucial gene (*sdmA*) further verifies that SdmA catalyzes the process.

Finally, CRISPR/Cas9 toolkit is widely used in *Streptomyces* to reveal cognate biosynthetic pathways [292], particularly showdomycin in my current research, a

novel pentangular type II polyketide [303], and auroramycin [304]. The technique's high efficiency (70 to 100%) spurred biochemical and mechanistic investigations in the field of *Streptomyces* [292], which includes remodeling organisms to direct the metabolic flux [305] and pathway engineering for eliminating the competing pathways [292] and provide product diversification [306].

4.4 Bioprospecting underexplored microbial lineages (Original publication IV)

Prolific microbial lineages from harsh environmental niches may contribute to the development of novel metabolites. Such productive species can be isolated from symbiotic organisms (plants or animals), soil-dwelling organisms, virgin lands, or deep-sea organisms. Several microbial species with a capacity to metabolize a variety of organic molecules or synthesize new metabolites in unusual environmental settings has been discovered everyday [307]. However, culture-independent studies have unveiled that working with marine actinomycetes from sediments and sponges is challenging because these actinomycetes are not recovered by cultivation-based methods [308,309]. Thus, it is apparent that the marine-derived organisms are very less studied for their potential to generate novel metabolites.

Isolation of marine sponge associated Actinobacteria was performed from the Gulf of Persia. The minPKS gene amplification of all 45-marine sponge-associated species unveiled that KSα genes involved in antibiotic biosynthesis were scarce in the isolated organisms. However, sequences responsible for producing spore pigments were abundant and clustered together. 16S rRNA sequencing confirmed an isolate with high bioactivity to be *Streptomonospora* sp. PA3, which displayed broad activity against *S. aureus* and *P. aeruginosa* (Fig. 22). Using a genotyping-guided strategy, two novel bioactive aromatic polyketide compounds, Persiamycin A and 1-hydroxy-4-methoxy-2-naphthoic acid, were isolated from sponge-associated halophilic *Streptomonospora* sp. PA3. Persiamycin A, a newly identified aromatic polyketide, was discovered to have modest effectiveness against *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*. The compound was discovered to have antiproliferative activities against breast carcinoma MDA-MB-231 tumor cell lines. The persiamycin A ($C_{20}H_{13}O_6$) was the first polyketide discovered from *Streptomonospora*. Structurally, persiamycin A possesses four aromatic rings, two methyl groups, and four hydroxyl groups.

According to my findings, the *Streptomonospora* sp. PA3 strain found in marine sponges could be a source of bioactive compounds that could be turned into therapeutic drugs. A similar study on the marine-derived rare actinobacteria *Nocardiopsis* sp. SCA21 revealed two compounds: 4-bromophenol, a bromophenol derivative, and bis(2-ethylhexyl) phthalate, a phthalate ester. Similar to persiamycin

A, these newly discovered compounds displayed inhibitory activity against *B. subtilis* ATCC 6633, *K. pneumoniae* ATCC 13883, and *S. aureus* ATCC 12600 [310].

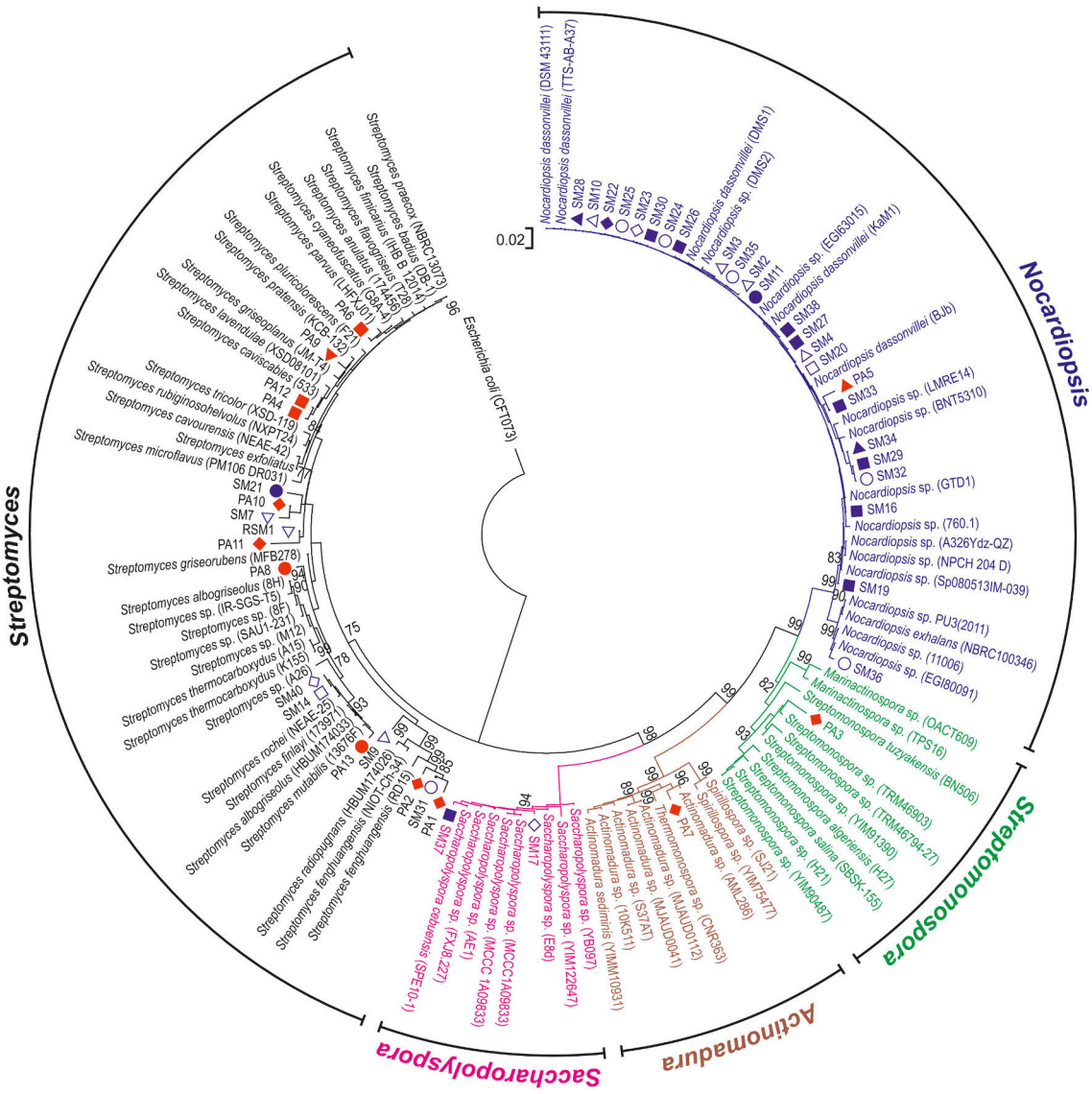

Figure 22. Phylogenetic study of actinomycetes using 16S rRNA sequences from the studied sponge isolates and sequences obtained from NCBI. The tree was constructed using *E. coli* as an outgroup. At the nodes, bootstrap values greater than 75% are displayed, based on 1000 replicates. The scale bar represents 0.02 nucleotide substitutions. The sponge host from which bacterial isolates were obtained is represented with different symbols (red and blue) in the figure.

Based on 16S rRNA gene sequences, all 45 strains had a high level of sequence similarity (96-100%) with strains available in databases. The isolates fall under five families and six genera, with the *Nocardiopsis* (53%) and *Streptomyces* (38%) clades accounting for most of the isolates. Some minorities in the rare actinomycetes clade included *Streptomonospora* (2%) and *Actinomadura* (2%). My experimental species, namely *Streptomonospora* sp. PA3 belong to *Streptomonospora* clade, which is shared with other *Marinactinospora* sp.

I investigated the presence of ketosynthase alpha (KS_α) genes in marine *Streptomyces* and rare actinomycetes species. Both the KS_α and KS_β subunits are involved in the synthesis of aromatic polyketides. I employed degenerate PCR primers to amplify and sequence the KS_α gene from 23 isolates, followed by the construction of a phylogenetic tree using these 23 sequences as well as KS_α sequences retrieved from the MIBiG database. The majority of KS_α genes clustered with those that generate spore pigments, indicating that antibiotic producing KS_α was rare. Four isolates were found to be clustered with *whiE* ORFIII gene from *S. coelicolor*, while 14 others were found to be clustered with *Streptomyces* spore pigments and antibiotic synthesis genes. According to my findings, the potential clusters in *Nocardiopsis* indicated a collection of four different genes (KS_α, KS_β, ACP and a cyclase).

In my experiment, the antibiotic biosynthetic genes are separated into distinct branches based on the type of antibiotics generated (Fig. 23). Additionally, the antibiotic biosynthesis genes are segregated from the spore pigment production genes according to the chemical scaffold and biosynthesis starter unit. Surprisingly, PA3 was clustered together with the angucycline class of compounds. This misclassification may have been due to the long evolutionary distance between terrestrial *Streptomyces* and sponge-associated *Streptomonospora*.

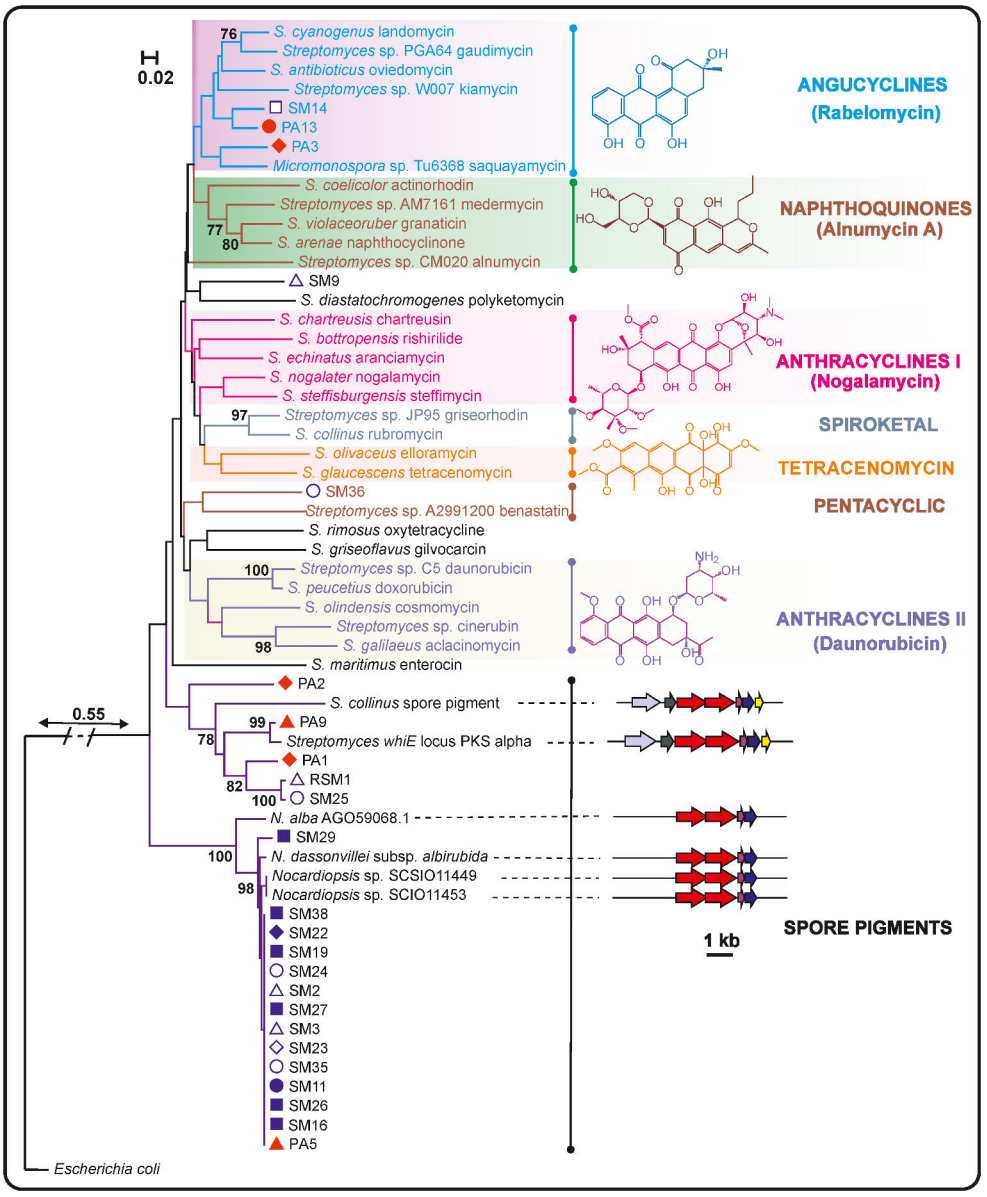

Figure 23. Genotyping the ability of sponge associated Actinobacteria to produce aromatic polyketides. Using aligned KS_{α} domain amino acid sequences, a neighbour-joining tree was built. The tree was constructed using *E. coli* as an outgroup. The discovered or anticipated compounds of the gene cluster are listed next to the organism's name from which the KS_{α} sequence was acquired. At the nodes, bootstrap values greater than 75% are displayed, based on 1000 replicates. The scale bar represents 0.02 nucleotide substitutions.

Genome analysis of *Streptomonospora* sp. PA3 revealed seven distinct BGCs involved in the generation of secondary metabolites. Terrestrial *Streptomyces* typically possess 20-40 different BGCs, therefore having seven BGC in this species is remarkable. Identification of an aromatic polyketide gene cluster (*per* cluster) that encodes enzymes that synthesize persiamycin A was performed. The *per* cluster harboured 12 and 8 genes with their possible involvement in the polyketide assembly and glycosylation. The *perHIJ* was involved in forming malonyl-CoA by an acetyl-CoA carboxylase complex, and the minimal polyketide synthase encoded by *per*123 is responsible for the biosynthesis of the polyketide backbone. Besides, a dedicated set of aromatases/cyclases *perABCD*, resembling those to tetracenomycin biosynthetic pathways such as TcmI, TcmJ, and TcmN, were discovered. Three genes *perEFG* were identified for post-PKS tailoring steps that could lead to the development of persiamycin A chromophore. MFS transporters involved in transport functions were also readily visible in the per cluster (Fig. 24).

The remarkable survival strategy of *Streptomonospora* sp. PA3 under high osmotic stress motivated us to investigate its genome, which we did by comparing the genomes of *Streptomonospora* sp. PA3 and *S. coelicolor* A3(2). *S. coelicolor* A3(2) was chosen for the comparison because it is well-characterized and appears to be well-adapted to saline settings [311]. Previous investigations provide hints that the salt stress induces major changes in the primary and secondary metabolism of bacteria [312], including the Gram-positive *Streptomyces*. Comparative analysis of gene products utilizing osmolyte L-ectoine were found to be similar [313], while *Streptomonospora* sp. PA3 possess greater capability of uptaking and synthesizing betaine-type osmoregulatory compounds [314]. Furthermore, in *Streptomonospora* sp. PA3, four genes associated in choline uptake were found, and choline dehydrogenase and betaine aldehyde dehydrogenase could convert choline to trimethylglycine. Additionally, *Streptomonospora* sp. PA3 may encode a second pathway that is absent in *S. coelicolor* A3(2) and is responsible for the synthesis of betaine via successive methylation of glycine by dimethylglycine *N*-methyltransferase and glycine *N*-methyltransferase.

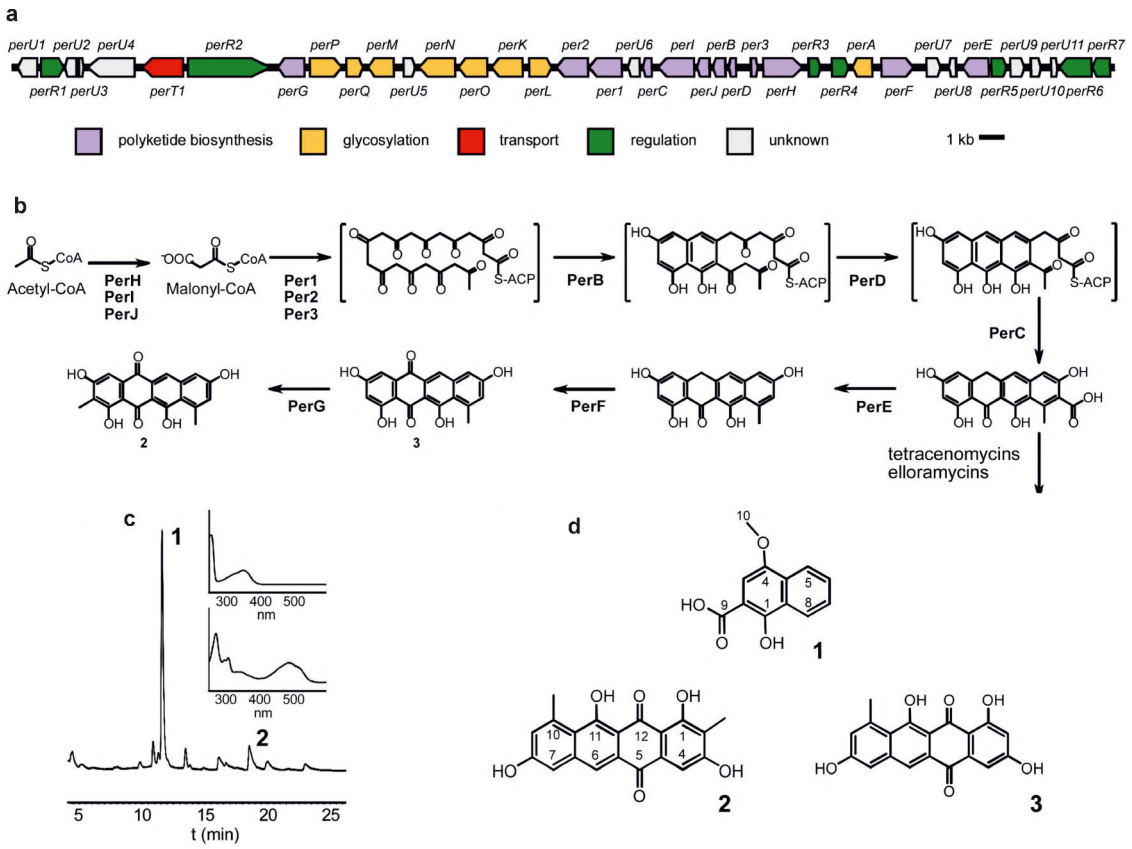

Figure 24. Putative biosynthetic pathway of persiamycin metabolite. a) Cluster responsible for the formation of persiamycin A. **b**) Scheme for the formation of persiamycin A. **c**) HPLC and UV-spectrum of the isolated compound. **d**) Chemical scaffold of **(1)**, **(2)** and related compounds. In panel 'c', the compound eluting at 11 minutes is 1-hydroxy-4-methoxy-2-naphthoic acid **(1)**, while eluting at 18 minutes is Persiamycin A **(2)**. The compound **(3)** mentioned in panel 'd' is a related compound, known as tetracenomycin D.

Finally, based on limited metabolic profiling studies conducted in these understudied Actinomycetales relative to other species, I concluded that these species tend to produce novel metabolites. As these organisms live in close harmony with sponges, the metabolic trade-off between these partners complements each other and enables them to thrive in adverse conditions (hypersaline and Salt lake) [307,315]. The 16S rRNA and PKS analysis confirmed that marine actinomycetes are an underexplored species harboring novel biosynthetic pathways and can generate new chemical scaffolds. In addition, although *Streptomonospora* has fewer biosynthetic pathways than terrestrial *Streptomyces*, the evolution and diversification of these pathways are believed to give rise to a distinct chemical space [316]. Furthermore, because cultivating

marine actinomycetes is challenging, detailed characterization of these unique species could help researchers better understand their ecological roles while also providing a valuable source for the discovery of novel metabolites.

5 Conclusions and Future Perspectives

Given the rising threat of antibiotic-resistant bacteria, it is crucial to accelerate drug research and development while also boosting manufacturing productivities utilizing various potential alternatives. Several new methods being adopted, discovering the novel techniques that exploit the ability of microbes to generate novel compounds will increase significantly.

Different model organisms, such as *Streptomyces*, which we have exploited extensively in our research, could prove fertile ground for generating novel bioactive compounds. *Streptomyces*, as prolific antibiotic biofactories, have an extraordinary proclivity for producing a broad spectrum of SMs. Antibiotics are one of the many NPs produced by these tiny organisms, and they have long been used in therapeutic practice to treat a variety of diseases. Several new species with the potential to generate new metabolites have been discovered. This, however, is insufficient, and a hunt for new strains from exotic habitats may replenish the dried antibiotics pipeline.

Apart from the search for new species for new antimicrobials, other advanced technologies, such as robust whole-genome sequencing conjugated with different activation approaches, may help us to generate novel antibiotics. In addition, the plummeting cost of whole-genome sequencing provided a glimpse that microbes harbour far more potential to generate novel metabolites. Thus, the work on identifying silent clusters and induce their expression using various strategies allowed us to generate bountiful of bioactive metabolites.

I postulated that the target-activation of these silent biosynthetic gene clusters might lead to the induction of transcript formation and, in turn, to the production of novel compounds that were not known before. Some of the cryptic clusters may be transcribe at such low basal level that they lead to production of only trace amounts of metabolites, which necessitates different approaches to dramatically enhance production to allow detection of the compounds from cultures. The new unknown products that are detectable in tiny amounts may have profound antimicrobial activity if their production is enhanced. Keeping such things in mind, we have prioritized our work in activating such silent/cryptic gene clusters using various

approaches. Better than before, we now have advanced technologies that help us to achieve our dreams.

Among multiple strategies that have been presented in current work, my prime focus was to perform genome mining and activate silent gene clusters of *Streptomyces* sp. using a novel molecular technique called SCMS. The technique helped to sort an activated mutant that generated many derivatives of the same compound. Based on antiSMASH prediction and metabolic profiling studies, a putative silent biosynthetic cluster from *Amycolatopsis orientalis* NRRL F3213 was chosen for UV activation. After the identification of appropriate silent pathway, the silent promoter from that cluster was activated using UV radiation and sorting using FACS. The SCMS technique does not require advanced cloning techniques and is highly flexible, allowing it to be used in the endogenous host. Because of two reporter genes conjugated downstream of silent promoters, the number of false positives can be kept to a minimum, which significantly increases the robustness of this technique. However, the disadvantage of SCMS in conjunction with FACS is that it is difficult to distinguish between activated and non-activated false mutants in *Streptomyces* because these organisms develop in chain-like structures. This might make sorting the mutants more difficult. Furthermore, contemporary FACS devices have a tiny nozzle that makes passing the individual spores problematic. However, by using severe sonication, filtration via specific filters and a larger size nozzle, we were able to overcome these flaws.

Other techniques I have employed have facilitated the development of potent compounds with promising therapeutic potential. This includes cultivation with dead yeast cells, where the dead yeast triggered *Streptomyces*' scavenging behaviour, ultimately enhancing the overexpression of a red compound. This project utilizes a laboratory-isolated strain, which we named as *Streptomyces* sp. MBK6, the genome of which was sequenced by us with Illumina sequencing. The strain generates a red pigment called prodigiosin in a defined medium supplemented with dead yeast. The genome sequence led to the identification of a *red* cluster, which is known to be involved in producing compounds actinorhodin and prodigiosin. Further genome analysis deciphers the fact that the *red* cluster in the genome seems to be conserved since it shows high similarity to the *red* cluster in *S. coelicolor* and *S. lividans* (except for some notable differences). Surprisingly, we obtained evidence that the regulation of *red* clusters in these two organisms differ. The expression of *redZ* using the constitutive promoter in *Streptomyces* sp. MBK6 and *S. coelicolor* was found to be divergent, i.e., positive and negative regulation in *Streptomyces* sp. MBK6 and *S. coelicolor*, respectively. This finding was further supported by the electrophoretic mobility assay (EMSA), where DNA and proteins' interactions were observed. The work demonstrates that interaction studies between species help us overexpress cryptic gene clusters, which generated inspiration in working with interwoven

transcriptional regulatory cascades. Thus, we overexpressed a cryptic gene cluster, namely undecylprodigiosin from *Streptomyces* sp. MBK6 by co-culturing it with dead yeast cells.

The third part of this thesis is concentrated with genetic manipulation of gene clusters directly in the producing host using CRISPR-Cas9. We selected *S. showdoensis* ATCC 15227, a prolific producer of showdomycin antibiotics, as our target. We started the work with the genome sequencing of *S. showdoensis*, which led to unravelling a cluster responsible for producing the nucleoside antibiotics, namely showdomycin. We identified a gene within the cluster, namely *sdmA*, which encodes an enzyme of the pseudouridine monophosphate glycosidase family. We have proven that this gene catalyze the formation of the *C*-glycosidic bond by gene inactivation experiments using CRISPR/Cas9, followed by the complementation of an intact copy of the same gene to the knockout mutants. Upon gene disruption, no product was observed. Furthermore, the production was restored once the gene's intact copy was complemented back to the knockout mutants. This confirmed that the studied gene *sdmA* of the locus was involved in the production of showdomycin. CRISPR/Cas9 toolkits allow biochemical and mechanistic investigations in *Streptomyces*, including remodeling organisms to direct the metabolic flux and pathway engineering. Although this technique is extremely useful for knocking in/out/down of target genes, its off-target consequences are often regarded as a serious drawback. For example, we utilized CRISPR/Cas9 toolkit to characterize a naphthocyclinone cluster (NCN; a distinctive red pigment belonging to the isochromane quinone antibiotics) from *S. arenae* DSM 40737. In order to characterize the NCN cluster, I aimed to knock-out three key genes encoding monooxygenase (*ncnJ*, *ncnO*) and dimeraze (*ncnN*). However, despite several attempts, no transformants were obtained, which I believe was due to the Cas9 enzyme's off-target activity. Cas9 toxicity has also hindered its widespread use in *Streptomyces* and *E. coli* due to its substantial intracellular expression, prohibiting these organisms from executing genetic changes. However, Cas9s' toxicity, on the other hand, may be considerably lowered by modulating its endogenous nuclease activity [317]. Recently, theophylline-dependent riboswitch (for lowering the protein expression) and blue light inducible Cas9 reconstitution have been used to resolve Cas9's activity in *Streptomyces* [318].

Apart from genetic engineering of microbes, underexplored organisms from exotic habitats are prolific producers of SMs, and the metabolic trade-off between these partners (sponges and *Streptomyces*) complements each other to thrive in adverse conditions. Bioprospecting of these microsymbionts (*Streptomyces*) can generate new chemical scaffolds that could help evolve and diversify the biosynthetic pathways, ultimately giving rise to distinct chemical space. However, the drawback of working with soil isolates is that even if we identify a novel strain,

the odds of discovering a new potent metabolite are slim. Our discovery of persiamycin A demonstrates that the likelihood of finding new chemistry has increased by working with rare Actinobacteria.

Because unculturable microbial communities latent in soil possess BGCs that may encode new compounds, improved cultivation strategies for these bacteria may generate new products. Alternatively, their biosynthetic potential could be tapped by capturing the entire BGC and expressing it in a heterologous surrogate host. In addition, knowing the particular factors triggering the activity can be difficult at times. Furthermore, isolating and characterizing extremophiles from atypical habitats could be beneficial to the pharmaceutical industries. Recently, synthetic biology has garnered significant popularity in re-shuffling and assembly of genes, either from the same pathway or unrelated pathways. Interactions between multiple strains in a co-culture has been shown to stimulate production of novel compounds. Meanwhile, a high throughput bio-reporter system, in conjunction with a library of signaling molecules, could be used to characterize hundreds of novel strains simultaneously for their silent / cryptic cluster activation. Also, the expression of reporter genes could provide information about the need for signaling molecules that can activate particular BGCs.

In conclusion, this dissertation presents potential guidelines for future engineering of *Streptomyces* chassis to improve the production titer of novel chemical scaffolds. To summarize; i) Emerging new approaches have their own set of benefits and drawbacks and are likely to aid in the discovery of novel compounds. (ii) The direct-cluster capture approach (currently not included in this thesis) was deemed promising among various techniques I used for manipulation of BGCs, because it utilizes homologous recombination technique to directly fish the cluster of interest and express it in the heterologous surrogate host. I have found this to be a convenient and straightforward approach. (iii) In addition, technical advancements in the field will keep natural products at the forefront of antimicrobial therapy. With all these technological advances, the future of microbial natural products appears bright and appealing to me.

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