MASTERARBEIT | MASTER'S THESIS

Titel | Title

Expression, purification and characterisation of selected marfuraquinocin biosynthetic enzymes

verfasst von | submitted by

Laura Maria Groschedl BSc

angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Magistra pharmaciae (Mag. pharm.)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 605
Studienrichtung lt. Studienblatt | Degree pro- gramme as it appears on the student record sheet:	Masterstudium Pharmazie
Betreut von | Supervisor:	Univ.-Prof. Sergey Zotchev PhD
Mitbetreut von | Co-Supervisor:	Dr. Olha Schneider B.Sc. MSc

Acknowledgements

Completing my degree in Pharmacy at the University of Vienna has been a challenging yet rewarding journey, and I am deeply grateful to those who have accompanied me along the way.

I would like to express my sincere gratitude to Univ.- Prof. Dr. Sergey B. Zotchev for giving me the unique opportunity to join his research group and work on a fascinating topic that enriched my experience in pharmaceutical biotechnology. I also thank the Department of Pharmaceutical Sciences at the University of Vienna for providing an excellent academic environment and the resources that made this research possible.

I am very grateful to Dr. Olha Schneider for her mental support and insightful advice throughout this time. Her expertise and encouragement were invaluable to me, both academically and personally.

I thank every single member of the Pharmaceutical Biotechnology group. The welcoming and supportive atmosphere made each day feel enjoyable and inspiring. I truly appreciated the friendly interactions, the collaborative spirit and the valuable advice I received.

Finally, I would like to express my deepest gratitude to my family and friends for their patience, honest words and support throughout this time. Their encouragement, especially during moments when I felt overwhelmed and stressed, grounded me and gave me the strength to overcome the most challenging phases of this journey.

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1. Zusammenfassung

Der Anstieg antibiotikaresistenter Mikroorganismen betont die dringende Notwendigkeit neuer antiinfektiver Wirkstoffe. Naturstoffe gelten weiterhin als vielversprechende Quelle für bioaktive Verbindungen, ihre industrielle Nutzung wird jedoch häufig durch hohe Wiederentdeckungsraten oder begrenzte Produktionsmöglichkeiten erschwert. Diese Masterarbeit konzentriert sich auf meroterpenoide Naturstoffe marinen Ursprungs, insbesondere Marfuraquinocine, die von *Streptomyces* Arten produziert werden und deren Biosynthese, welche bislang noch nicht aufgeklärt wurde. Zur Aufklärung dieser Wege wurden Methoden der functional genomics eingesetzt – der Ansatz der heterologen Expression und CRISPR/Cas9- basierte Genom-Editierung.

Sechs biosynthetische Gene wurden in *Escherichia coli* BL21 als heterologem Wirtsystem exprimiert und anschließend mittels Nickel-NTA-Affinitätschromatographie aufgereinigt. Für fünf der sechs Zielproteine konnte eine erfolgreiche Expression und Aufreinigung erzielt werden, was eine weiterführende funktionelle Charakterisierung dieser Proteine ermöglicht. Zur Validierung der Genfunktion wurden gezielte Gen-Deletionen mithilfe von CRISPR/Cas9 durchgeführt. Nach Fermentation der Kulturen wurden die Extrakte zweier Konstrukte mittels LC-MS analysiert und mit dem Wildtyp verglichen. Die Ergebnisse zeigten unterschiedliche Metabolitenprofile, was auf eine Beteiligung der deletierten Gene in der Marfuraquinocin-Biosynthese hinweist.

Diese Arbeit demonstriert den Nutzen von Genome Mining und synthetischer Biologie zur Aufklärung komplexer Biosynthesewege und zur Optimierung der Naturstoffforschung. Die gewonnenen Erkenntnisse tragen zu einem tieferen Verständnis der Marfuraquinocin-Biosynthese bei und ebnen den Weg für die zukünftige Entwicklung neuartiger Meroterpenoid-derivatisierter Wirkstoffe.

2. Abstract

The rise of antibiotic-resistant microbes emphasises the urgent need for novel anti-infective agents. Natural products remain a promising source of new bioactive compounds; however, their industrial exploration is often challenged by high rediscovery rates or limited product scalability. This thesis focuses on marine-derived meroterpenoids, specifically marfuraquinocins produced by *Streptomyces* species, whose biosynthetic pathways remain incompletely understood. To address this, functional genomic methods were employed, combining heterologous expression approaches and CRISPR/Cas9- mediated genome engineering.

Six biosynthetic genes were cloned and expressed in *Escherichia coli* BL21 as heterologous host, followed by nickel-NTA affinity chromatography. Successful expression and purification were achieved for five of six target proteins, enabling further functional characterisation. To validate gene function, CRISPR/Cas9 was used to generate targeted gene deletions. After fermenting the cultures, the extracts of two engineered constructs were analysed by LC-MS and compared to the wildtype. This revealed diverse metabolite profiles, indicating the involvement of the deleted genes in marfuraquinocin biosynthesis.

This work demonstrates the utility of genome mining and synthetic biology tools in elucidation of complex biosynthetic pathways and optimising natural product discovery. The findings contribute to a deeper understanding of marfuraquinocin biosynthesis and pave the way for future engineering of novel meroterpenoid derivative drugs.

3. Introduction

3.1. Natural products in drug discovery

Natural products are of particular importance for drug discovery, due to their remarkable diversity in bioactivities and chemical structures. Extracts derived from plants, fungi, bacteria or algae contain complex compounds with a wide range of biological effects, including antibacterial, antifungal or cytotoxic properties and have been used in traditional medicine for centuries to treat various diseases, demonstrating their therapeutic relevance. These natural extracts mostly consist of secondary metabolites, which are not essential for the organism's survival, but often provide ecological advantages for defence, communication and signalling.

As highlighted by Atanasov and colleagues, natural products are crucial in the development of modern pharmaceuticals, especially in the fields of oncology and infectious diseases. Their structural complexity, characterized by a high degree of stereochemistry and diverse functional groups, sets them apart from synthetic compounds. Although this complexity often promotes high specificity and interaction with biological targets, it causes difficulties in terms of product isolation, characterisation and synthesis (Atanasov et al. 2021).

Since the early days, many prominent drugs have been isolated from natural sources. A breakthrough in the treatment of infectious diseases was the discovery and isolation of penicillin - an antibiotic derived from the fungus *Penicillium notatum* – which demonstrated the immense potential of microbes as source of novel antibiotics. Another example is paclitaxel, an antimitotic agent originally extracted from the bark of *Taxus brevifolia* and is widely used in cancer chemotherapy. Due to the low natural yield paclitaxel is now produced semi-synthetically and in plant cell cultures. However, the challenges associated with isolating sufficient quantities of bioactive compounds and the complexity of their total synthesis have contributed to a declining interest in natural product research within the pharmaceutical industry (Beutler 2009).

To address the difficulties associated with discovery of novel metabolites and minimize re-isolation of known structures, modern technologies such as high throughput screening, genome editing, bioinformatics and artificial intelligence are increasingly used (Corbett and Duncan 2019).

3.2. *Streptomyces* spp. as source for novel drugs

A major producer of bioactive compounds are *Actinomycetes*, a family of gram-positive bacteria living in soil or marine environments and other ecological niches. They tend to form symbiotic relationships with plants, fungi, insects or animals, which drives chemical interaction

and production of secondary metabolites. *Streptomyces* is a genus of sporulating, filamentous actinomycete bacteria, are the origin of numerous antibacterial compounds, including tetracycline, streptomycin and tobramycin. As antibiotic resistance continues to rise, these bacteria remain promising candidates for discovery of new antibiotics. Furthermore, their products exhibit antifungal, antiviral, anticancer and immunosuppressive activities, emphasising their significance in pharmaceutical and biotechnological applications. The biosynthesis of these metabolites is encoded by genes, co-localised in biosynthetic gene clusters (BGCs). The main challenge researchers face, is that the majority of BGCs require specific stimuli to be transcriptionally active, limiting the access to undiscovered compounds (Tarasova et al. 2023; van der Meij et al. 2017; Yang et al. 2020).

3.3. Biosynthetic gene clusters (BGCs)

The genes encoding the biosynthetic pathways of secondary metabolites are arranged in BGCs (Figure 1). The genes encode enzymes that assemble the core molecule or add functional groups to chemically modify the metabolite. Also, genes encoding enzymes involved in export of compounds, import of building blocks and secretion of the final product are coordinated within a BGC. Adenosine triphosphate (ATP) binding cassette (ABC) transporters are a superfamily of transport proteins, build of two membrane integrated domains and two domains facing the cytoplasm, where the ATP binding and hydrolysis, that drives the conformational changes necessary for translocation of substrates, occurs. These proteins mediate the cellular export of toxic or antibiotic compounds to protect the host and create self-resistance, but also import essential components for the biosynthesis of the final compound (Miethke and Marahiel 2007).

Each BGC is tightly regulated by transcription factors, that facilitate upregulation (activators) or downregulation (repressors) of the gene expression. By understanding the machinery of a BGC, it is possible to predict the biosynthetic pathway and modify the intermediates, to improve their yield or bioactivity, reduce toxic and off-target effects or even create novel compounds, which is of huge interest in drug discovery (Monteiro et al. 2025).

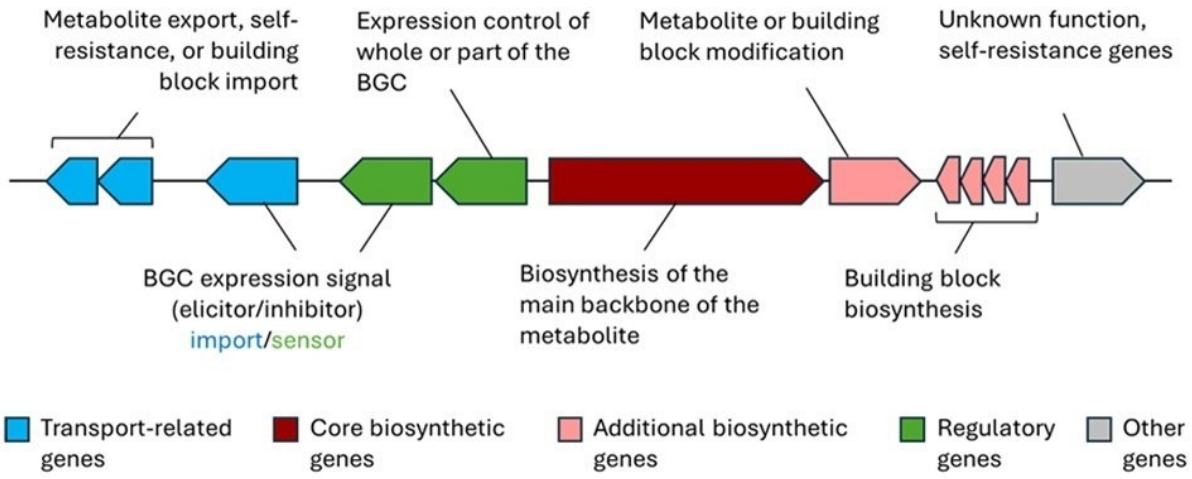

Figure 1: Schematic illustration of a BGC (Monteiro et al. 2025).

Unfortunately, many BGCs remain silent under laboratory conditions, making their products inaccessible through conventional cultivation methods. Different approaches for the activation of silent BGCs are developed to overcome this challenge and to uncover their full potential for the discovery of new metabolites (Figure 2) (Zhang et al. 2019).

3.3.1. Genome mining

Genome mining sums up several strategies for discovering natural products. Advanced bioinformatic tools (antiSMASH, ClusterFinder, PRISM etc.) were developed to identify and analyse cryptic BGCs, responsible for production of secondary metabolites. Putative structures of products can be predicted after comparing the BGC to similar BGCs from well-studied genomes and linking the biosynthetic genes to already known compounds. By combining molecular biology techniques with metabolomics data, such as mass spectra, genome mining enables the systematic analysis and deciphering of the biosynthetic pathways and structures of predicted natural products (Scherlach and Hertweck 2021; Yang et al. 2020).

3.3.2. Strategies to activate silent BGCs

Most BGCs need specific conditions to be transcriptionally active in laboratory environments, which challenges discovery of potentially novel natural products specified by them. Various approaches to activate silent BGCs have been developed (Figure 2).

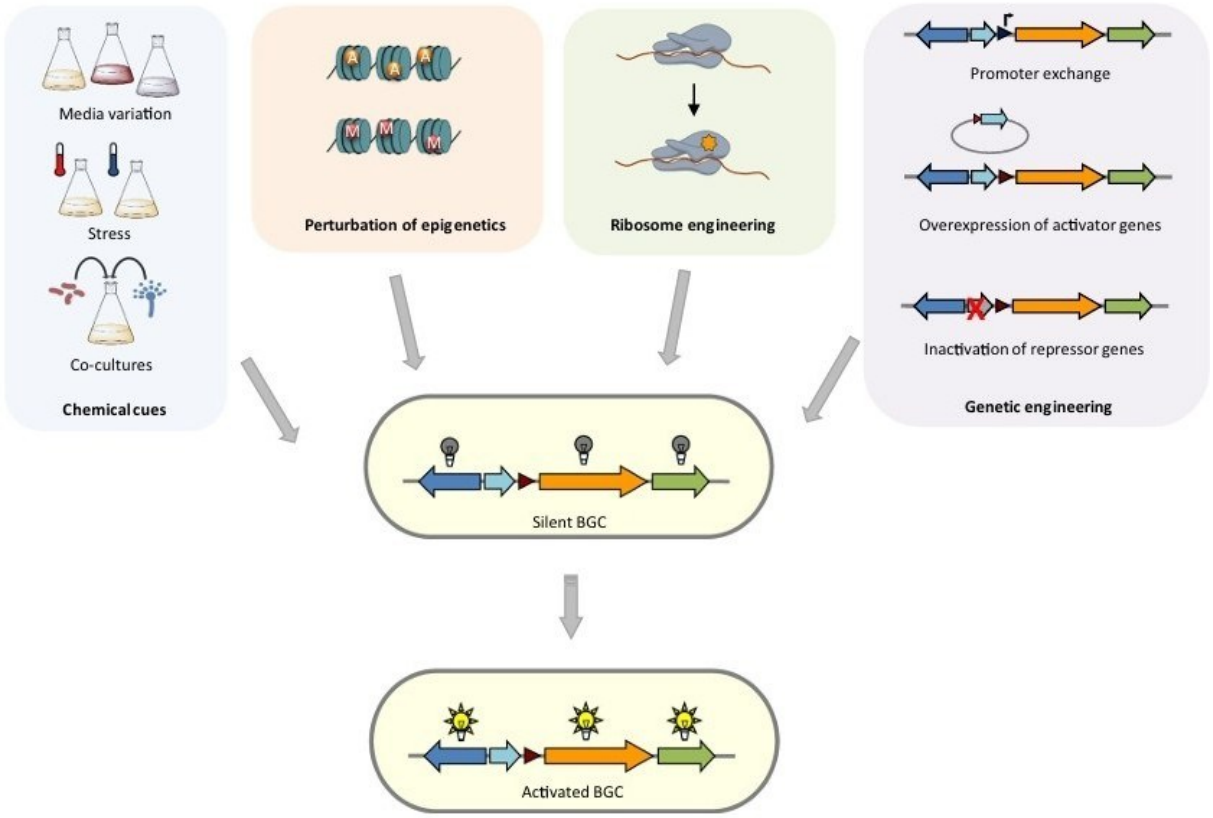

Figure 2: Strategies for activation of silent BGCs in the native host modified from (Scherlach and Hertweck 2021)

Secondary metabolites are produced as defence mechanism against other species, signalling molecules or as adaption to foreign environment, therefore chemical variation of the growth conditions, the use of elicitors or co-cultivating with other microbes are believed to trigger the activation of silent BGCs.
Epigenetic modulation targets the regulatory mechanisms that control gene expression, without changing the DNA sequence. These modifications include DNA-methylation, histone-acetylation/deacetylation and remodeling of chromatin structure or non-coding RNAs.

Ribosome engineering was developed in *Streptomyces* species and is now widely used to modify the ribosomes and create mutants with unique gene expression patterns (Hosaka et al. 2009).

Genetic engineering strategies for BGC activation are applied to the cultivable native host or heterologous host strains alternatively. A common approach is promoter exchange, where the native promoter is replaced by an inducible or constitutive, stronger promoter to enhance transcription activation.

Overexpression of endogenous transcriptional activators facilitates increased frequency of transcription factor binding to target DNA sequences and leads to gene activation, which could promote cluster transcriptional activity (Scherlach and Hertweck 2021).

The Streptomyces Antibiotic Regulator Protein (SARP) family is a prominent example for transcriptional activators in *Streptomyces spp.,* that typically initiate expression of genes responsible for secondary metabolite biosynthesis (Yan and Xia 2024). Therefore, their overexpression has become a powerful tool in biotechnology, allowing the activation of BGCs and inducing secondary metabolism. Manipulation of *ccaR* in *Streptomyces clavuligerus* ATCC 27064 increased the yield of clavulanic acid and cephamycin to 200-300 % (López-Agudelo et al. 2021). The work of Ma and colleagues from 2018 identified *BulY* and *BulZ* as important activators in the production of the immunosuppressant tacrolimus in *Streptomyces tsukubaensis* NRRL18488 (Ma et al. 2018).
Conversely, inactivation of repressor genes can relieve transcriptional suppression. Repressor genes encode repressor proteins, that inhibit gene expression by binding to specific DNA sequences. A prominent example of such repressor protein is the lac repressor, regulating the lac operon in bacteria by binding to the lac operator and thereby inhibiting the transcription of genes encoding for the lactose- metabolism. The transcription inhibition is released, when for example lactose or allolactose are present, following the conformational change of the repressor protein (Lewis 2005).

Genome editing is often done using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 nuclease (CRISPR/Cas 9) method, which will be explained in chapter *CRISPR/Cas9 method*. This system was first adapted for genome editing by Doudna and Charpentier and is now widely used in the fields of environmental sciences for precisely targeted genetic modifications (Doudna and Charpentier 2014; Richardson et al. 2023).

3.3.3. Heterologous expression to activate silent BGCs

Not all microbial strains are responsive to genetic manipulation and are difficult to cultivate. Heterologous expression of full or engineered BGCs is a strategy to enable efficient activation of silent BGCs, compared to the transcriptional activity in the native producer strain. Essential aspects, that increase the chance of successful heterologous expression, are the choice of a stable vector carrying the BGC and a compatible host strain, that allows genetic manipulation and can be cultivated easily. The process of heterologous expression typically begins with the isolation of DNA from the native host, followed by its cloning into suitable vectors. Depending on the size of the insert, cosmids (45 kb), fosmids or bacterial artificial chromosomes (BACs) (200 kb) are used to construct genomic libraries, that can be screened to identify clones harbouring the BGC of interest (Huo et al. 2019).
For cloning large DNA inserts between 100- 300 kb phage P1-derived artificial chromosomes (PACs) are applied. This approach was demonstrated by Jones and colleagues in 2013, when they successfully expressed the 83,5 kb FK506 BGC from *S. tsukubaensis* NRRL 18488 –

responsible for production of tracrolimus - in *Streptomyces coelicolor* derivatives (Jones et al. 2013).

The assembly of the complete BGC is often achieved through homologous recombination in yeast using shuttle vectors. After that, the BGCs are introduced into a heterologous host, which may undergo further genetic manipulation to enhance expression. Finally, compound production is optimised by variation in growth conditions of the recombinant strains (Huo et al. 2019).

3.3.4. CRISPR/Cas9 method

CRISPR/Cas9 is a powerful and precise tool for targeted genetic modifications, with its background lying in the adaptive immunity of bacteria. Clustered regularly interspaced short palindromic repeats (CRISPR) are short sequences of DNA with unique spacer sequences, originally derived from bacteriophages or foreign genetic elements. CRISPR-associated protein 9 (Cas 9) is a nuclease, derived from *Streptococcus pyogenes*, cleaving double strands at precise target sites.

In 2007 Barrangou and colleagues were one of the first to demonstrate the natural function of CRISPR-Cas systems in *Streptococcus thermophilus* strains, after they found short DNA spacer sequences in the CRISPR loci of the bacterial genome. CRISPR Cas acts as genetic memory in adaptive immunity against viral infections (bacteriophages), by integrating spacer sequences from the phage genome. This mechanism would lead to the development of phage-resistance. To proof this, the researchers inserted new spacer sequences into the CRISPR locus of *S. thermophilus*, which turned resistant to the corresponding phages, confirming the role of CRISPR in adaptive immunity(Barrangou et al. 2007).

The new inserted spacers are transcribed into CRISPR-RNA (crRNA), which allows the bacteria to target matching viral DNA and direct Cas proteins to the cleavage site. For the activation of Cas, the transactivating-RNA (tracrRNA) is essential, as it binds to crRNA and forms a complex guiding Cas nuclease. The single guide RNA (sgRNA) is a combination of crRNA and tracrRNA and designed to simplify the construction of target-complementary sequences in the laboratory. However, Cas nuclease also needs a nucleotide sequence, the protospacer adjacent motif (PAM), located next to the target sequence to successfully bind and cut the DNA at the target site (Doudna and Charpentier 2014).

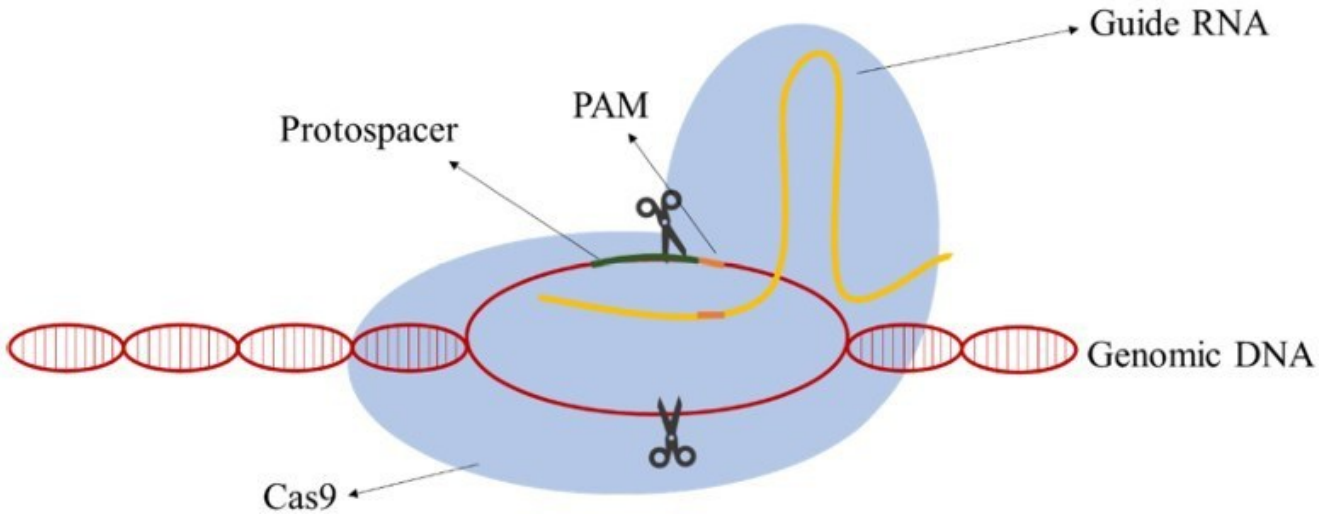

Figure 3: CRISPR/Cas9 mode of action. The guide RNA complex (gRNA) directs Cas9- endonuclease to the target site 3-4 bp upstream of the protospacer adjacent motif (PAM) sequence. Cas9 induces a double strand break, activating cellular DNA repair mechanism, like NHEJ or HDR (Rainha et al. 2020).

Inducing a double strand break activates one of two main cellular DNA repair pathways: Non-Homologous End Joining (NHEJ) or Homology-Directed Repair (HDR) (Yeh et al. 2019). NHEJ often results in small insertions or deletions (indels), making it useful for disrupting gene function in gene knock-out experiments (Kang et al. 2019). In contrast, HDR enables precise genetic modifications by using a homologous donor DNA template, making it ideal for gene knock-ins but can also be used in knock out experiments by inserting a stop codon or frame shift mutation (Cui et al. 2018; Jakočiūnas et al. 2015).

3.4. Meroterpenoids derived from *Streptomyces* spp.

Meroterpenoids are a diverse group of secondary metabolites predominantly produced in plants, fungi, algae or bacteria. Especially marine microbes are believed to be a rich source of unique, yet undiscovered compounds, as they adapt to the ecological stress conditions of their environment. Due to their hybrid origin, combining terpene moieties with polyketides, phenols, amino acids or alkaloids, the structural diversity of meroterpenoids implies a great range of bioactivities. Therefore, they are of major interest in research for a broad spectrum of drugs, including antibiotics, anticancer drugs or anti-inflammatory drugs. (Murray et al. 2020)

Streptomyces spp. are prolific producers of bioactive meroterpenoids:

Naphthomevalin (Figure 4), produced by *Streptomyces* sp. Gö 28, belongs to the family of napyradiomycins and has weak antimicrobial activity against Gram positive bacteria (Henkel and Zeeck 1991)

Napyradiomycin A1 (Figure 4), isolated from *Streptomyces kebangaanensis* WS-68302 possesses growth inhibiting properties against *Staphylococcus aureus* MS870 and MS9610 (Zhang et al. 2023)

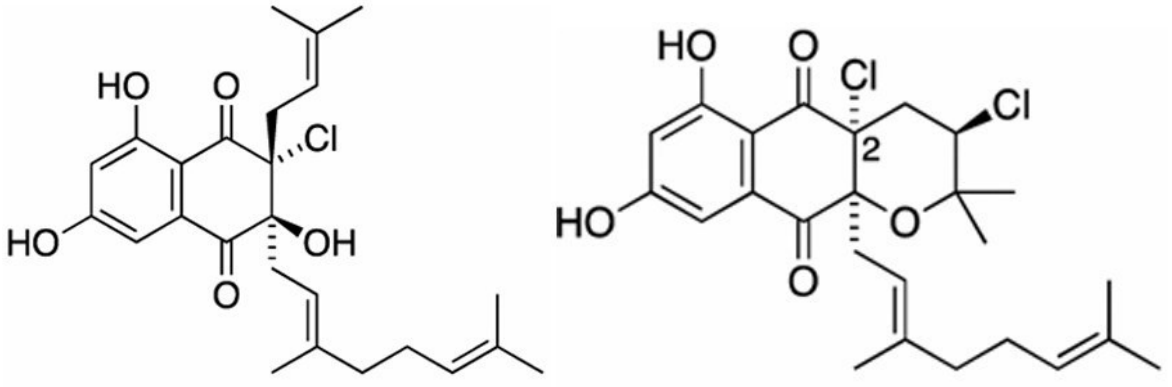

Figure 4: Naphthomevaline (left) isolated from *Streptomyces* sp. Gö 28 and napyradiomycin A1 (right) produced by *Streptomyces kebangaanensis* WS-68302 (Henkel and Zeeck 1991; Zhang et al. 2023)

Marinone and debromomarinone (Figure 5) both show strong antibacterial activity against Gram positive bacteria and were isolated from *Streptomyces* sp. CNB-632 in 1992 (Pathirana et al. 1992)

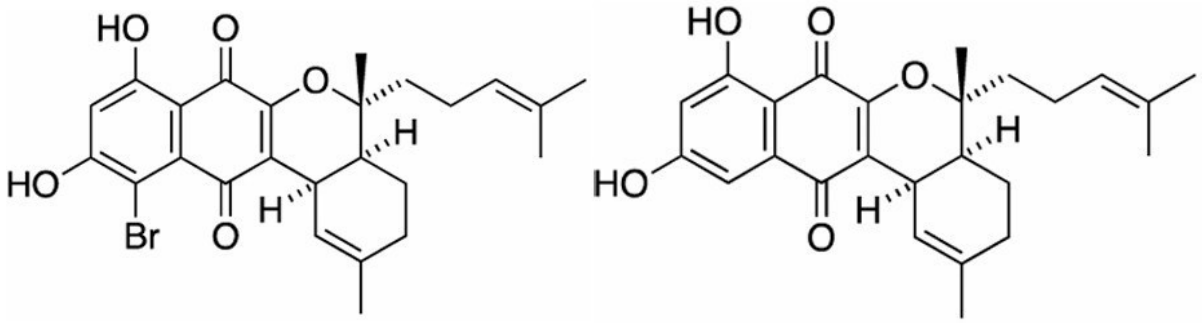

Figure 5: Marinone (left) and debromomarinone (right), produced by *Streptomyces* sp. CNB-632 (Pathirana et al. 1992)

Furaquinocins A and B (Figure 6) are derived from *Streptomyces* sp. KO-3988 and showed cytotoxicity against HeLa S3 cells in vitro (Komiyama et al. 1990)

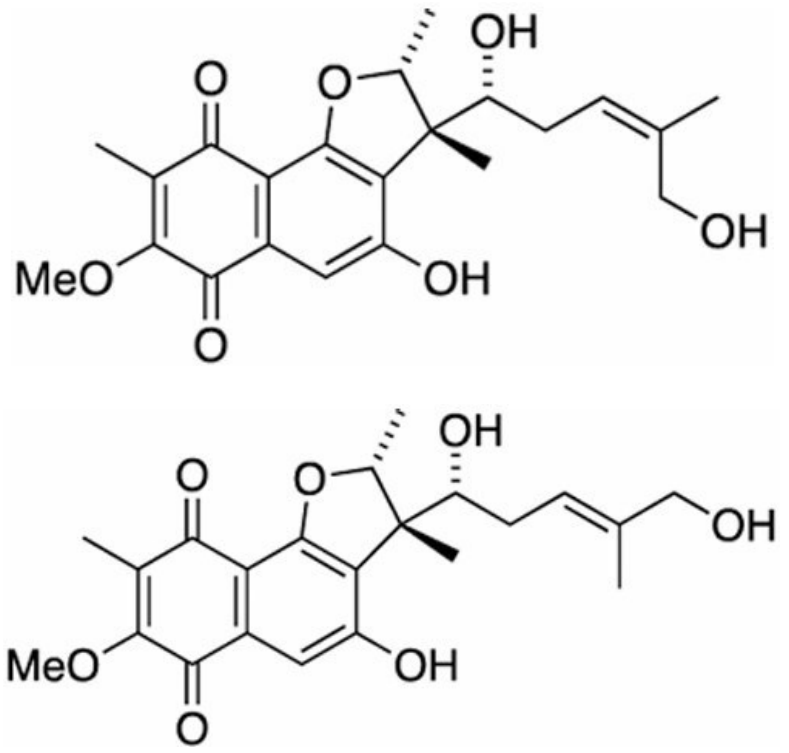

Figure 6: Furaquinocin A and B, produced by *Streptomyces* sp. KO-3988 (Komiyama et al. 1990)

3.5. Meroterpenoid biosynthesis

The biosynthesis of meroterpenoid derived from marine microbes was investigated in recent studies (Isogai et al. 2012; Song et al. 2013; Moore 2018; Murray et al. 2020; Noguchi et al. 2025). Meroterpenoids like furaquinocin, naphterpin and furanaphthoquinone are produced by various *Streptomyces* species. These compounds share a common polyketide precursor, 1,3,6,8-tetrahydroxynaphthalene (THN), and exhibit diverse biological activities, including antibacterial and antitumor effects. Despite their structural diversity, their BGCs often contain conserved elements, suggesting a shared core biosynthetic logic. The full biosynthetic pathways of these molecules are not yet fully elucidated. The recent study by Noguchi et al. provides a detailed look into the *fur* BGC, for the biosynthetic pathway of furaquinocin, a THN-derived meroterpenoid.

THN is biosynthesised by Fur1 (type III polyketide synthase) out of five malonyl coenzyme A units. Notably, the function of Fur1 is similar to that of NphC, NapB1 and Fnq6, in the biosynthesis of other meroterpenoids, such as naphtherpin, napyradiomycin and furanaphthoquinone (Murray et al. 2020; Noguchi et al. 2025).

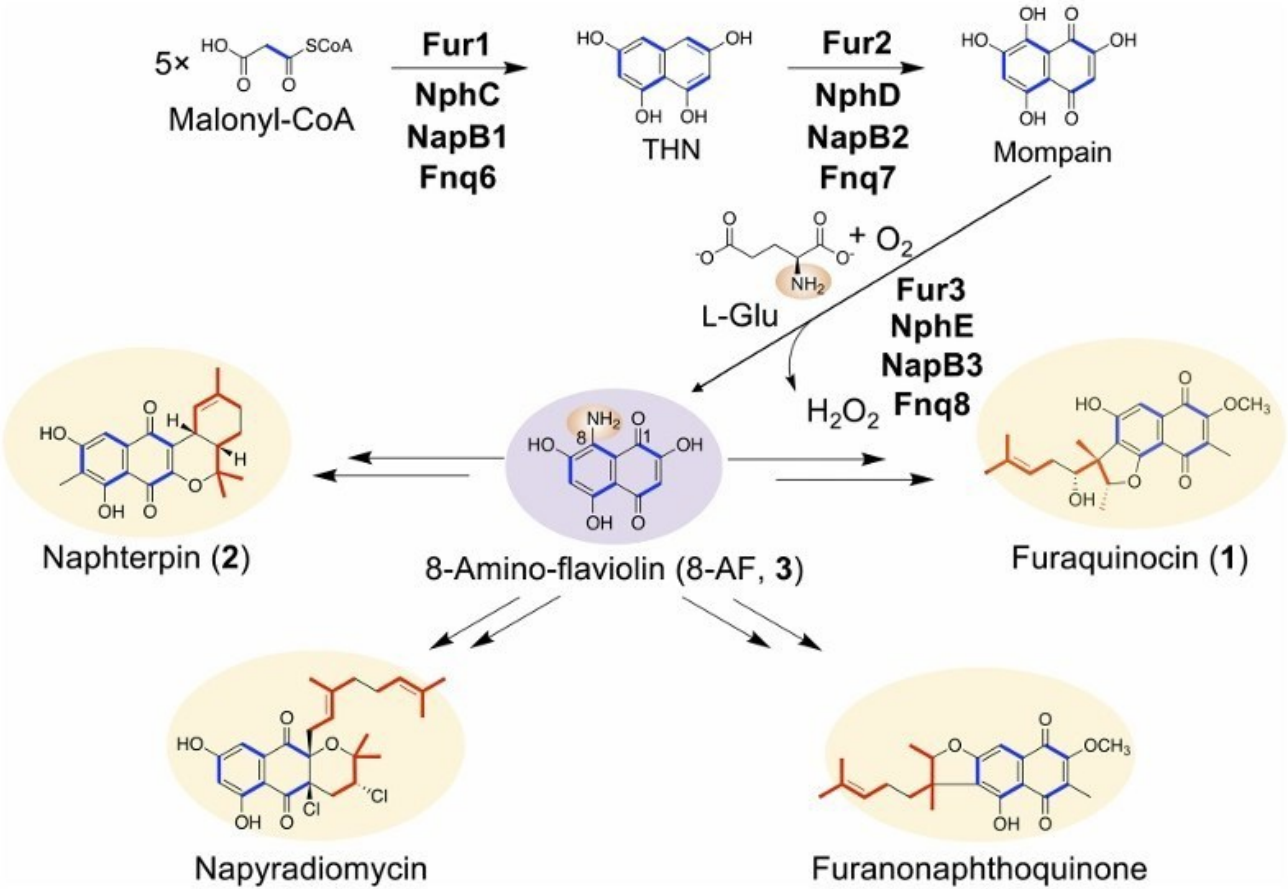

Figure 7: Common biosynthetic steps for meroterpenoids produced by *Streptomyces* species. Five malonyl- coenzyme A units form THN, that is transformed to 8-amino-flaviolin, a precursor molecule shared by naptherpin, napyradiomycin, furanonapthoquinone, furaquinocin and derivatives. Blue: polyketide moiety. Red: terpene parts (Noguchi et al. 2025)

THN undergoes enzymatic transformation via Fur2 and Fur3 to yield 8- amino flaviolin (8-AF), a key intermediate across THN-derived meroterpenoids. A critical step involves the removal of the amino-group from 8-AF, which is achieved by transient diazotization facilitated by nitrite producing enzymes Fur 16 and Fur 17, taking the amino group of L-aspartate. The diazo group is removed non enzymatically as nitrogen gas, which leads to the formation of 1,2,4,5,7-pentahydroxynaphthalene (PHN), a hydroquinone intermediate. PHN undergoes two methylation steps, catalysed by S- adenosylmethionine (SAM)- dependent methyltransferases Fur6, Fur4. Next it is reduced to the intermediate 2- methoxy-3-methylflaviolin (MMF), on which prenyltransferase Fur7 introduces the terpene moiety leading to a hybrid structure. To yield the furaquinocins, an intramolecular hydroalkoxylation of the terpene side chain occurs to form the characteristic furan ring. This reaction is catalysed by Fur21, a homolog of SAM-dependent methyltransferase, that is notably independent of SAM when doing a cyclisation (Noguchi et al. 2025).

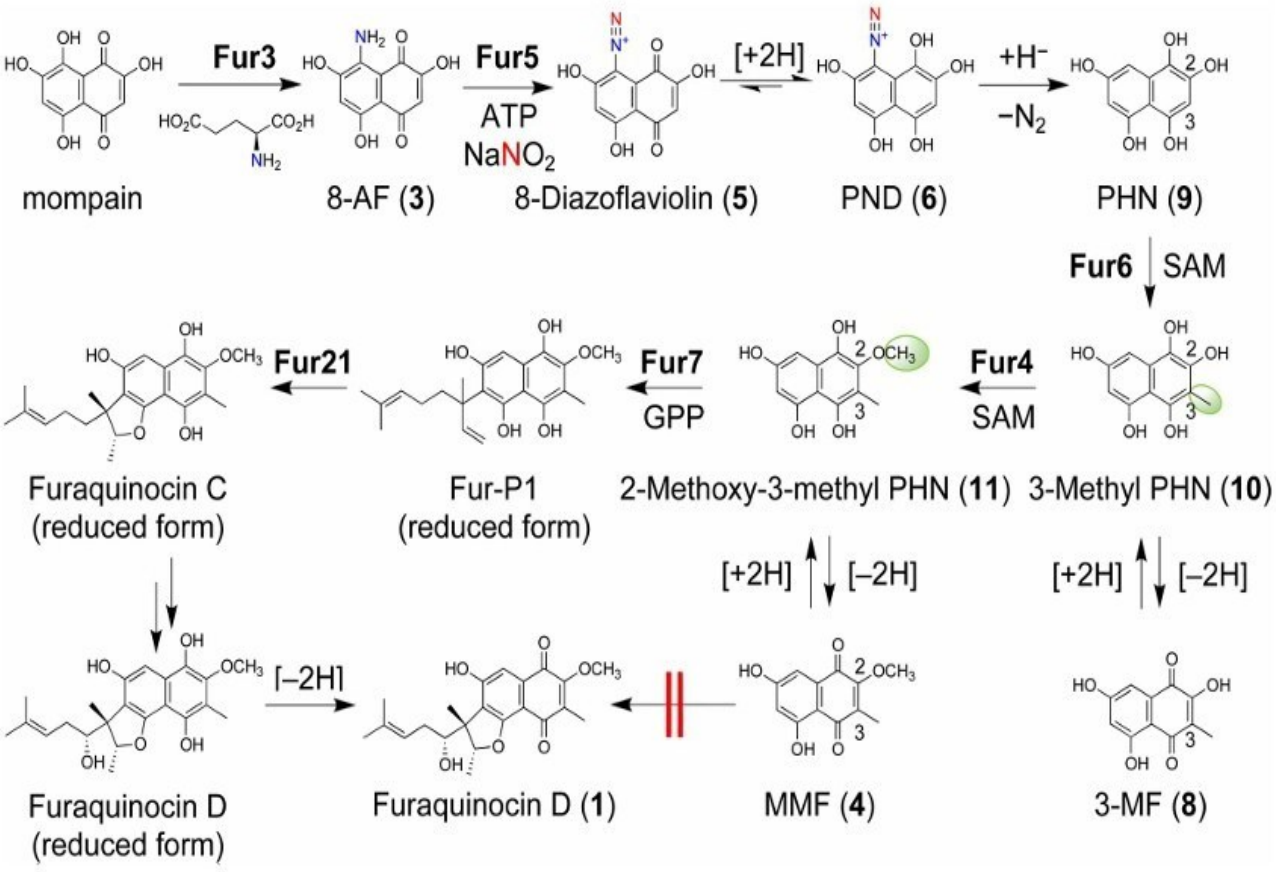

Figure 8: Proposed biosynthetic pathway for furaquinocins, according to Noguchi et al. 2025. THN is converted to mompain, which undergoes amination to yield 8-AF. To remove the amino group, diazotization occurs, leading to PHN in the next steps. After two methylation reactions, in presence of SAM, followed by reduction, MMF is the next key- intermediate to be prenylated and yield a hybrid molecule. The following intramolecular hydroalkoxylation leads to the cyclisation of the terpene side chain and builds the furan ring of furaquinocins (Noguchi et al. 2025).

3.6. Approaches and tools for studying gene function

Studying gene function is essential for understanding the biological significance of the corresponding genes, their expression regulation patterns and interactions within the cellular environment. The combined approaches of genomics, transcriptomics, proteomics and metabolomics are used to explore biological relationships and generate hypotheses about gene functions (Figure 9).

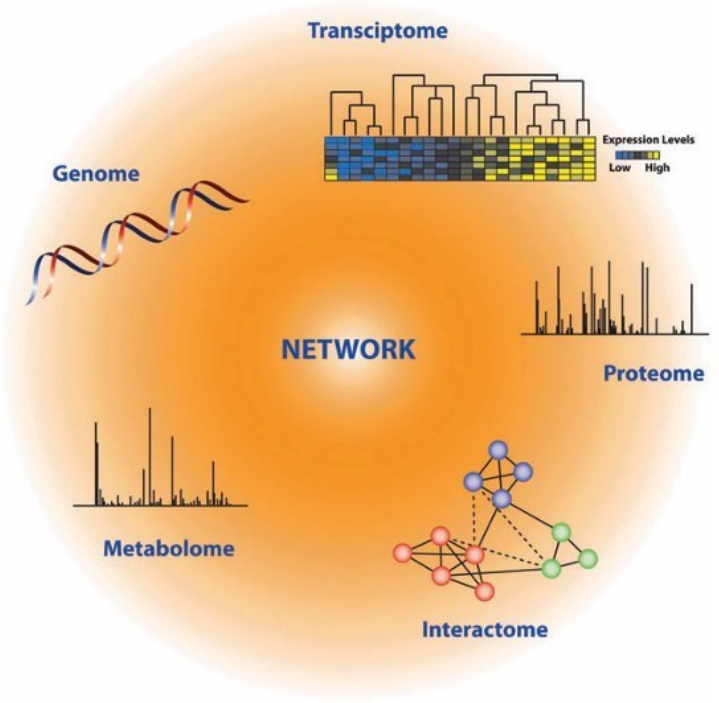

Figure 9: Schematic overview of network analysis approaches as tool for gene function studies (Bunnik and Le Roch 2013).

3.6.1. Genomics

Sequencing technologies from first generation Sanger-sequencing to modern Next-Generation sequencing (NGS) have revolutionised the ability to explore genomic data in detail. While Sanger sequencing laid the foundation, NGS enables high-throughput detection of single nucleotide polymorphisms (SNPs), gene deletions or duplications and supports genomic and epigenomic studies. These technologies allow researchers to identify coding and non- coding sequences, as well as epigenomic data, including DNA-methylation patterns, nucleosome location and transcription factor binding positions. Chromatin immunoprecipitation sequencing (ChIP-seq) provides insight on histone modification. Epigenomics are essential to understand the complex regulatory network of the transcription (Bunnik and Le Roch 2013; Gasperskaja and Kučinskas 2017).

Gonzales-Silva and colleagues illustrated the workflow of genomics to analyse three *Streptomyces* sp. strains (A1, J25, and J29 ori2) with antifungal activity. Initially, 16S rRNA gene sequencing was used to identify and classify the strains based on these conserved regions, for phylogenomic analysis. This step confirmed that the isolates were distinct strains of *Streptomyces albidoflavus*. Following this, genomic DNA (gDNA) was isolated from each strain, and high throughput screening (HTS) was performed. After genome assembly and annotation, comparative genomics was then applied to explore similarities and differences among the strains. Finally, the antiSMASH tool was used to predict biosynthetic gene clusters

(BGCs), providing insights into each strains' potential to produce bioactive secondary metabolites, such as antifungal compounds (Gonzalez-Silva et al. 2024).

3.6.2. Transcriptomics

Especially transcriptomics is used to understand gene regulation and elucidate functional pathways. Early methods like Northern blotting and reverse transcriptase polymerase chain reaction (RT-PCR) were limited to known transcripts, while serial analysis of gene expression (SAGE) introduced a more quantitative but low throughput approach. DNA microarrays achieve broader transcript profiling is by hybridization of labelled complementary DNA (cDNA) to arrays, enabling comparative expression analysis and clustering co-expressed genes. A NGS technique, RNA sequencing, has largely replaced these methods due to its ability to link transcript reads to the genome, quantify expression levels and detect alternative splicing patterns and non-coding RNAs. Additionally, reporter gene assays using green fluorescent protein (GFP) or luciferase help identifying regulatory elements by linking them to measurable gene expression changes (Bunnik and Le Roch 2013).

3.6.3. Proteomics and interactomics

While transcriptomics gives insight into gene expression, it does not always correlate with the protein abundance or activity, as post -transcriptional and post- translational modifications can significantly alter protein function. Techniques such as immunoassays (for example enzyme linked immunosorbent assay, ELISA), antibody free methods like 2D gel electrophoresis and mass spectrometry (MS) are widely used for protein detection and quantification. For complex samples, as it is often the case with natural extracts, advanced methods like multidimensional protein identification technology (MudPIT) and liquid-chromatography MS (LC-MS) are applied as high-throughput approach.
Understanding protein function also requires studying their interaction with other proteins, affecting signalling pathways and transcriptional activation or repression. The two-hybrid system, developed in yeast and bacteria, is an approach to identify interactions between target proteins, that must be validated via tandem affinity chromatography (TAP), including two rounds of affinity purification coupled to MS, and NGS techniques (Gasperskaja and Kučinskas 2017; Bunnik and Le Roch 2013).

Jayapal and colleagues employed a combined transcriptomics, proteomics, and interactomics approach to investigate mRNA-protein dynamics in *Streptomyces coelicolor* M145 during growth in liquid $R5^-$ media. Samples were collected at eight time points (7 to 38 hours), and RNA was extracted for microarray-based transcriptome profiling. The resulting data were processed to calculate expression ratios relative to the initial time point (7 h), generating time-series profiles of mRNA abundance. To gain proteomic data, the labeled peptides were

separated using cation exchange chromatography and analysed via LC-MS. Transcriptomic profiles were then compared to proteomic data. Principal Component Analysis (PCA) was applied to both transcriptomic and proteomic datasets to analyse expression dynamics giving insight into the secondary metabolism and stress response (Jayapal et al. 2008).

3.6.4. Metabolomics

Metabolomics focuses on the analysis of metabolites, small molecules such as amino acids, sugars or fatty acids, that undergo enzymatic catalysed chemical reactions during metabolism of a cell and therefore provide direct insight into the cell's biological health status. The goal of metabolomics is to analyse these molecules for better understanding of cellular metabolism and link that information to the cellular phenotype. Key analytical techniques are nuclear magnetic resonance (NMR) spectroscopy and LC-MS, as they enable sensitive, high-throughput detection of a wide range of metabolites, structure elucidation and assist in identifying novel metabolic pathways (Bunnik and Le Roch 2013).

Tangerina et al. investigated the metabolomics of *Streptomyces* sp. BRB081 via one strain many compounds (OSMAC) strategy. This was done by growing the strain under varied growth conditions, by changes in media composition, temperature, pH, light intensity or oxygen levels. Comparing the metabolite profile data from different extracts, which were obtained via LC-MS/MS, lead to the identification of divergent secondary metabolites including siderophores, fatty acid derivatives and cyclic polypeptides (Tangerina et al. 2020).

3.7. Marfuraquinocins and phenaziterpenes from marine *Streptomyces* sp.

In 2013, Song and colleagues explored deep-sea living *Streptomyces niveus* SCSIO 3406 and discovered six novel metabolites, some of them providing promising antibacterial and cytotoxic properties. Marfuraquinocins A-D (Figure 10), sesquiterpenoids with naphthoquinone core and phenaziterpenes A and B (Figure 11), two geranylated phenazines where isolated and tested for activity against several cancer cell lines and different microbes, including *Staphylococcus aureus* ATCC 291213 and methicillin-resistant *Staphylococcus epidermis* (MRSE). Marfuraquinocin A and C showed potent cytotoxicity against the human lung cancer cell line NCI-H460. In addition to their anticancer properties, marfuraquinocin A, C and D demonstrated antibacterial activity against *Staph. aureus* ATCC 29213. Furthermore, marfuraquinocins C and D were effective against MRSE. Phenaziterpene A exhibited moderate cytotoxicity against human glioblastoma cell line SF-268. The discovery of these compounds expands the chemical diversity of marine-derived natural products and proves the therapeutic potential of

deep-sea actinomycetes as source of new anticancer and antibacterial agents (Song et al. 2013).

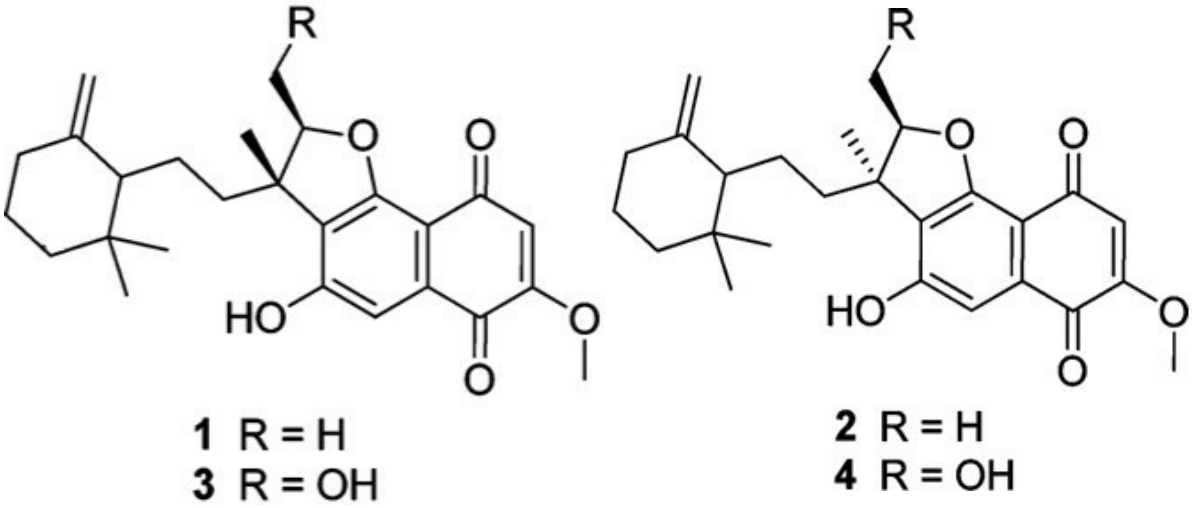

Figure 10: Marfuraquinocin A (1), marfuraquinocin B (2), marfuraquinocin C (3) and marfuraquinocin D (4) (Song et al. 2013).

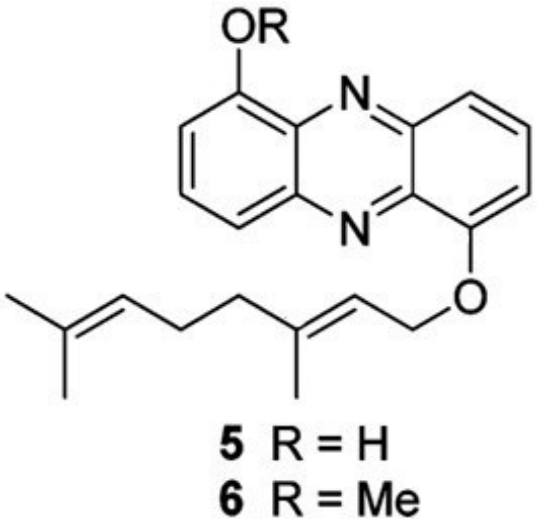

Figure 11: Phenaziterpene A (5) and phenaziterpene B (6) (Song et al. 2013).

3.7.1. Biosynthesis of marfuraquinocins and phenaziterpenes

Marfuraquinocins, furaquinocins and neomarinone all feature a naphthoquinone core, linked to a dihydrobenzofuran ring system build from terpenoid units. In the case of marfuraquinocins, this ring system is constructed from farnesyl units, in contrast to the geranyl derived system found in furaquinocins. The variable terpenoid side chain in marfuraquinocins undergoes cyclisation into a cyclohexane ring, which is catalysed by terpene-cyclases. Marfuraquinocin A-D (Figure 10) only diverge in the configuration of C-13 and oxidation state at C-11. Oxidoreductases mediate oxidation and reduction steps, including the formation of the quinone

moiety and the oxidation state of specific carbon positions (Isogai et al. 2012; Murray et al. 2020).

Phenaziterpenes are fused of a phenazine core with a terpene moiety. Their biosynthesis involves the enzymatic activity of geranyl diphosphate synthase, creating the terpene backbone geranyl group, that is attached to the phenazine scaffold via prenylation by prenyltransferases (Guttenberger et al. 2017; Zhu et al. 2021).

The biosynthetic genes encoding enzymes to produce marfuraquinocins and phenaziterpenes are organised within the *mfq* BGC (Figure 12). For the work in this thesis, the cluster was cloned from *Streptomyces* sp. S4.7 derived from the rhizosphere of *Leontopodium nivale* ssp. alpinum, Edelweiss (Oberhofer et al. 2019).

The corresponding biosynthetic pathway (Figure 13) could only be predicted based on the presumed gene functions and needs to be validated experimentally (Zotchev et al.).

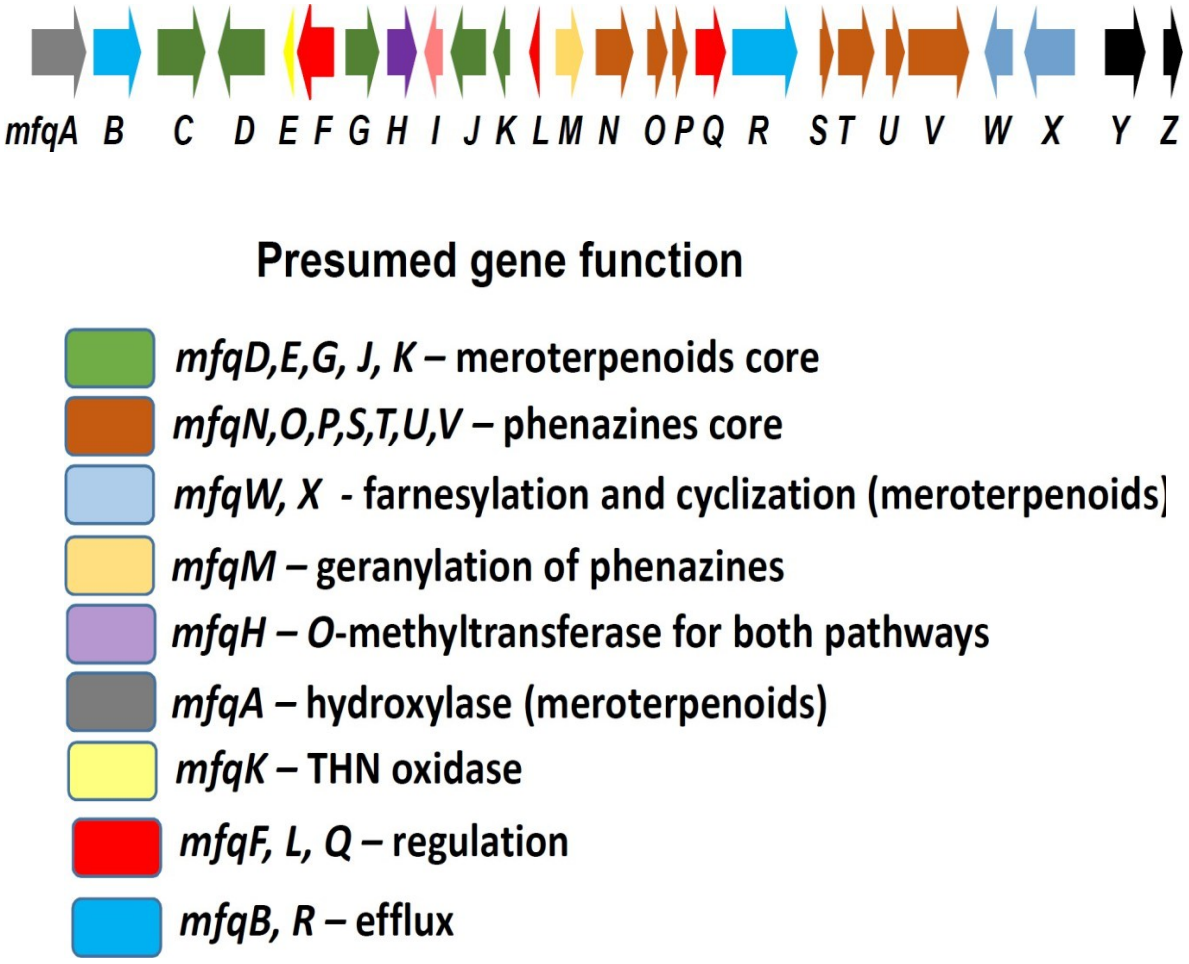

Figure 12: Organisation of the *mfq* BGC and presumed gene functions (modified from Zotchev et al.).

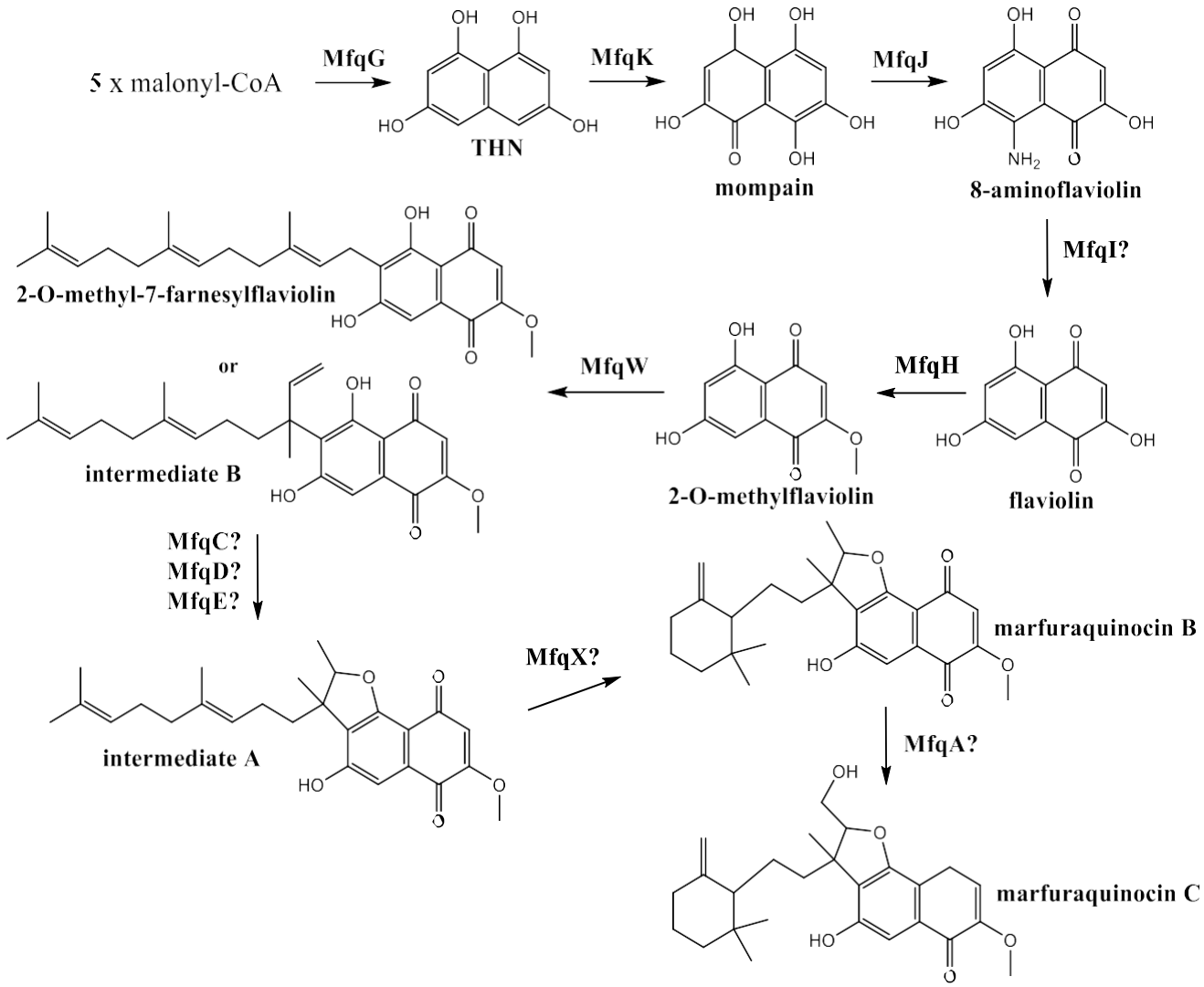

Figure 13: Putative biosynthetic pathway of marfuraquinocins in Streptomyces sp. S4.7 (Zotchev et al.)

4. Aim of the work

The class of meroterpenoid natural products produced by *Streptomyces* spp. represents a promising source for drug discovery and development. Within this group are marfuraquinocins and phenaziterpenes, that showed notable biological activities, including antibacterial and cytotoxic effects. This makes them attractive candidates for structural modification aimed at enhancing their pharmacological properties. The key to designing novel drug derivatives of marfuraquinocins and phenaziterpenes, is the understanding of the underlying biosynthetic pathways, of which the biosynthetic genes are organised on the *mfq* BGC (Figure 12). The proposed biosynthesis of marfuraquinocins (Figure 13) shows the enzymatic steps catalysed by Mfq enzymes including MfqA, MfqC, MfqD, MfqE, MfqI and MfqX. Experimental validation of this pathway is essential in order to perform targeted modifications and generate derivatives with improved pharmacological activities and reduced off- target effects. In this thesis the function of six genes within the *mfq* BGC was investigated. The aim was to analyse the function and elucidate their roles in the biosynthetic process and thereby contribute to the prediction of the pathway.

5. Materials and methods

The following chapter lists the equipment and techniques that were used in this project.

5.1. Strains, plasmids and primers

5.1.1. Microbial strains

Table 1: Microbial strains used in this work

Strain	Characteristics	Source
Streptomyces sp. S4.7	Isolated from the rhizosphere of *Leontopodium nivale* ssp. alpinum; source of the BGC	(Oberhofer et al. 2019)
Streptomyces coelicolor M1154	Heterologous host for expression of natural products	(Gomez-Escribano and Bibb 2011)
Escherichia coli XL1-Blue MR	Engineered host strain used for transformation	('XL1-Blue MR Competent Cells, XL1-Blue MRF Competent Cells | Agilent')
Escherichia coli DH5 α F'	Engineered host strain used for transformation	('NEB® 5-Alpha Competent E. Coli (Subcloning Efficiency) | NEB')
Escherichia coli BL21	Heterologous host for protein expression	(Studier and Moffatt 1986)
Escherichia coli EPI300	Electrocompetent *E. coli* strain for high replication	('TransforMax EPI300 | LGC, Biosearch Technologies', n.d.)
Escherichia coli C3020 (NEB® 10-beta)	Electrocompetent *E. coli* strain for transformation of large plasmids	('NEB® 10-Beta Electrocompetent E. Coli | NEB', n.d.)
Escherichia coli ET12567	Carries the vector pUZ8002 promoting mobilization function during conjugation	(Choi et al. 2004)
Saccharomyces cerevisiae TC3	Strain for CRISPR-Cas9 modifications, carries Cas9 protein (Δ*trp*)	(Jakočiūnas et al. 2015)

5.1.2. Plasmids

Table 2: Plasmid vectors

Plasmid vector	Application/Characteristics	Source
pUC57	Subcloning vector, Amp^R	BioCat GmbH
pET30	Protein expression vector, Kan^R	Novagen, USA
pSH	BGC *mfq* expressing vector, based on pCLY10 (Δ*leu*)	Zotchev Group
pTJOS	sgDNA-expressing vector (Δ*ura*)	(Jakočiūnas et al. 2015)
pUWLoriT	pSH expressing vector, Amp^R, $Thio^R$	(Luzhetskyy et al. 2005)

5.2. Antibiotics

All antibiotics were purchased from Sigma Aldrich.

Table 3: Antibiotics and concentrations used for *Streptomyces spp.* and *E.coli*

Antibiotic	Solvent	Used concentration *E. coli* (µg/mL)	Used concentration *Streptomyces spp. (µg/mL)*
Ampicillin (Amp)	ddH_2O	100	-
Apramycin (Apra)	ddH_2O	100	50
Chloramphenicol (Cml)	EtOH 96%	25	-
Kanamycin (Kan)	ddH_2O	25	-
Nalidixic acid (Nal)	0,1 M NaOH	30	-
Thiostrepton (Thio)	DMSO	-	30

5.3. Glycerol stocks and spore suspensions

To store the strains, cells were cultivated overnight in 2 mL of liquid medium containing the appropriate antibiotic or selective marker in the incubator. Depending on microbial strain different temperatures were used (for yeast 30 °C, *E. coli* 37 °C). The next day, the culture was

centrifuged at 14000 rpm for 1 minute at room temperature, and the pellet was resuspended in 1 ml of 20% glycerol and stored at -70 °C.

For spore suspensions of *Streptomyces spp.* plates with corresponding growth medium were grown at 30 °C for >7 days until spores were forming (actual time depends on microbial strain/ mutations). If the optimal growth of spores was achieved, 2 mL of 20 % glycerol were pipetted onto the plate and spores were carefully scraped off with the tip. The suspension was filtered through a cotton filter to separate the spores from mycelium. The filtered suspension was transferred into sterile cryo-tubes and stored at -70 °C.

5.4. Media for microbial strains

All *E. coli* strains were cultivated in Luria Bertani (LB) medium. For the preparation of solid media 1 g/L agar was added before autoclave. In case of the protein expression, a triple-concentrated LB medium was mixed to re-feed the protein-producing culture. For *Saccharomyces cerevisiae* two different media with supplements (Y0750 and Y1771) were prepared according to Sigma-Aldrich protocol. For the cultivation of *S. coelicolor* Soy-flour medium (SFM), 2xYT medium and MYM were prepared.

Luria Bertani medium:

10 g/L	Tryptone
5 g/L	Yeast extract
5 g/L	NaCl

2xYT medium (conjugation medium):

16 g/L	Tryptone
10 g/L	Yeast extract
5 g/L	NaCl

SFM:

20 g/L	D-Mannitol
20 g/L	Soy-flour
20 g/L	Agar
10 mL/L	$MgCl_2$ added after autoclaving

MYM medium:

4 g/L Maltose

4 g/L Yeast extract

10 g/L Malt extract

0,5 L Tap water

0,5 L ddH_2O

pH was adjusted to 7,3 before autoclaving

2 mL/L R2 trace elements added after autoclaving

Y0750 medium (- leu, - trp):

1,54 g/L yeast synthetic drop-out medium supplement

6,7 g/L yeast nitrogen base without amino acids

40 mL/L Glucose 50% (w/v) added after autoclaving

Y1771 medium (- leu, - trp, - ura):

1,46 g/L yeast synthetic drop-out medium supplement

6,7 g/L yeast nitrogen base without amino acids

40 mL/L Glucose 50% (w/v) added after autoclaving

5.5. Generating chemically competent cells

To incorporate the expression vector into *E. coli* BL21 cells, the cells were made competent (Mandel and Higa 1970). $CaCl_2$ was used for this purpose, as its cationic charge binds to negatively charged molecules, thereby enabling the cells to incorporate foreign DNA more efficiently. Each step was worked under sterile workbench:

Growing preculture overnight at 37 °C in LB medium without antibiotic

Growing the culture for competent cells:

0,5 mL of preculture were transferred into a 200 mL flask containing 50 mL LB medium. This culture was shaken at 37°C until its OD_{600} measured between 0,4 – 0,5. Then the cells were transferred on ice for 20 min before transferring them into a sterile 50 mL falcon tube and collecting them by centrifugation for 8 min at 4000 rpm and 4°C.

Treatment with Calcium chloride:

Cells were resuspended in ice-cold $CaCl_2$ (50 mM) and incubated on ice for 20 min. After centrifugation the cell pellets were resuspended in 2,5 mL of an ice- cold solution of $CaCl_2$ (50 mM) with 10% glycerol.

The competent cells were separated in aliquots of 100 µL into cooled microfuge tubes, frozen in liquid nitrogen and stored at -70°C for later use.

5.6. Transformation

5.6.1. Heat shock method for DNA transfer into *Escherichia coli*

Transformation is a process of gene transfer in bacteria. Applying heat to the cells is linked to a more likely DNA- uptake, therefore this technique is in common use.

100 µL of chemically competent cells were thawed on ice, mixed with 1 µL of plasmid DNA per 20 µL ligation sample, then kept on ice for 30 minutes. The heat shock was performed by transferring the tubes onto a 42 °C heating block for 45 seconds. followed by 5 minutes on ice. After adding 900 µL LB-medium into the tube, the cells were incubated at 37 °C for 50 minutes. Under the sterile bench, 100 µL of cells were spread on agar plates supplemented with the corresponding antibiotic and incubated overnight at 37 °C.

5.6.2. Electroporation method for DNA transfer into the *Escherichia coli* and *Saccharomyces cerevisiae* strains

Transformation of *Escherichia coli* via electroporation method:

In this project, *E. coli* EPI300 cells were also used for electroporation-transformation. After resuspending the cells in 10 % ice-cold glycerol, 2 µL of DNA was added and the mixture was incubated for 5 minutes in a 2 mm electroporation cuvette. Electroporation was then performed at 1,5 kV; 25 µF and 200 Ω. Afterwards, the cells were resuspended in 900 µL of ice-cold LB-medium and incubated at 37 °C for 1 hour. Finally, 150 µL of culture was plated on LB-agar plates containing apramycin and grown overnight at 37 °C.

Transformation of yeast cells with lithium acetate pre- treatment:

Applying high voltage to cell membranes, enhances their permeability in reversible or irreversible manner and leads to higher uptake of foreign DNA. For yeast transformation, the cells were pre-treated with Cleland's reagent (also known as 1,4- Dithiothreitol/ DTT) and lithium acetate, which were shown to increase the efficiency of transformation (Thompson et al. 1998). The process of electroporation can be divided into four steps:

1. Preparation of preculture:

100 µL of TC3 cells were plated on agar-plates with Y0750-medium (-Leu/-Trp) and incubated for two days at 30 °C, then one colony was inoculated into 10 mL liquid Y0750-medium and incubated at 30 °C overnight. 200 µL of overnight preculture were re-inoculated into 10 mL liquid Y0750- medium before incubation at 30 °C overnight again.

2. Preparing culture for electroporation:

3 mL overnight preculture were transferred into 50 mL liquid Y0750- medium and shaked at 30 °C until the OD_{600} reached 1-2, which took about 4 hours. Then cells were collected by centrifugation (4000 rpm, room temperature, 5 min) and the supernatant fluid was discarded. The cell pellets were resuspended with the electroporation reaction mix and incubated for 30 minutes at room temperature.

<u>Electroporation reaction solution (per reaction):</u>

0,012g	Cleland's reagent
800 µL	lithium acetate 1 M
80 µL	sorbitol 1,2 M
4 mL	Tris-HCl buffer 1 M; pH 8

Filled to 8 mL with ddH_2O

After centrifuging and removing supernatant reaction solution, the cell pellet was resuspended in 1,5 mL of ice-cold 1M Sorbitol and centrifuged again (4000 rpm, 5 min, 4 °C). This step was repeated 3 times, and the cells were kept on ice from this time until the end of the protocol.

3. Transformation/ electroporation step:

Finally, the cells were resuspended in 100 µL of ice-cold 1M sorbitol. For each transformation reaction 65 µL of pre-treated cells were mixed with 1 µL of vector DNA and 5 µL of ds-Oligonucleotides into a 0,2 cm gap electroporation vial and incubated on ice for 5 minutes. To validate efficiency and success of the process, a negative sample without DNA was also electroporated and plated an agar- medium.

The electroporation was carried out using the BIO-RAD Gene Pulser Electroporation System at 1,5 kV; 25 µF and 200 Ω. After the electro- impulse, the cells were immediately resuspended with 900 µL 1M Sorbitol.

4. Growing transformants:

500 µL of cultures were plated on agar-plates with Y1771- medium and incubated at 30 °C for 2 days. Transformants that successfully incorporated the correct DNA insert, were identified by colony PCR, which is described in chapter 2.9.

5.7. Conjugation into *Streptomyces* strains

Conjugation is a form of horizontal gene transfer in bacterial species, which is widespread in nature. (Flett et al. 1997) In this project this phenomenon was used to exchange the engineered plasmids of knocked out *mfq*-genes between *Escherichia coli* ET12567/pUZ8002 and *Streptomyces coelicolor* M154 according to following steps:

2 µL of pDNA were heat shock transformed into *E. coli* ET12567 and transformants were cultivated on LB-agar plates with ampicillin (for clones with SARP-regulator for pSH expression) or apramycin (for selected clones with deleted *mfq*-genes) at 37 °C overnight. For the latter, the best 4 clones of these colonies were picked with a loop and plated on LB-agar plates with apramycin (100 µg/mL), chloramphenicol (25 µg/mL) and kanamycin (25 µg/mL) and grown overnight at 37 °C.

The next day, a *Streptomyces* spore suspension was prepared, before preparing the *E. coli* cells. 50 µL of spore suspension, previously thawed on ice, were mixed with 350 µL of 2xYT medium in 1,5 mL tubes and incubated at 50 °C for 7 minutes, then cooled to room temperature for at least 5 minutes.
Meanwhile, the best grown colony of *E. coli* ET12567 was selected and scraped of the agar plate with a sterile loop and transferred into 500 µL 2xYT in a 1,5 mL tube. The cells were gently resuspended and 100 µL of *E. coli* suspension was added to 400 µL of the heat-shocked *Streptomyces* spores. The mixture was centrifuged at 5000 *rpm* for 1 minute. About 250-300 µL of supernatant was removed and the pellet was resuspended in the remaining liquid. The suspension was plated on SFM plates supplemented with 1M $MgCl_2$ and incubated for at least 20 hours at 28 °C.

For the selection of clones, a mix of 1 mL ddH_2O, 30 µL nalidixic acid and the corresponding antibiotic was prepared for each plate. When the surface of the plates appeared matte, 1 mL of this mix was poured onto each plate and spread until the surface was dry. Plates were then incubated at 28 °C for an additional 2-3 days. Once sufficient colony growth was observed, the most prominent colonies were picked and transferred to new SFM plates containing the corresponding antibiotic and incubated until sporulation occurred. Once the *Streptomyces* strains had sporulated sufficiently, spore suspensions of the different strains were prepared as described in section 2.3.

5.8. Fermentation and Extraction

5.8.1. Fermentation of *Streptomyces*

For the stimulation of the biosynthesis and production of secondary metabolites, the *Streptomyces coelicolor* strains containing the modified *mfq*-BGC were fermented in two different fermentation media SFM and MYM. First, a seeding culture of 50 µL spore suspension was put into 10 mL of 2xYT medium with antibiotics for 3 days at 28 °C. A control with 2xYT medium and antibiotics was prepared in the same way and should serve as baseline for the liquid-chromatography and mass-spectrometry (LC-MS) of the extracts. 2 mL of preculture were transferred into 30 mL of fermentation medium with antibiotic. The fermentation was carried out for 7 days at 28 °C.

5.8.2. Extraction of compounds

The fermented samples were centrifuged at 1000 *rpm* for 20 minutes. Supernatants and cell pellets were processed differently, but all stored at -20 °C before and after the extraction protocol, to ensure the stability of the extracts and prevent precipitation. All extracts were analysed with LC-MS at the Mass Spectrometry Centre in the Faculty of Chemistry of the University of Vienna by Mag. Dr. Martin Zehl.

Extraction of pellets

Pellets were resuspended with 20 mL of 80% acetone and kept at 4 °C overnight in 50 mL falcon tubes. Next day, the extracts were centrifuged at 1000 *rpm* for 20 minutes and filtered into round bottomed flasks under the hood. The acetone samples were dried on the rotavapor, resuspended in 1,5 mL of methanol and transferred into 2 mL Eppendorf Tubes®. If it was not able to dry the samples enough, they were frozen to -80°C and freeze dried for 3 days instead.

Extraction of supernatant

Supernatants were freeze dried for 3 days. 30 mL of methanol were used to extract the compounds by shaking them at room temperature for 2 hours. The samples were filtrated and dried on the rotavapor. As with the pellets, all samples were resuspended in methanol (3 mL) and stored at -20 °C until the analysis.

5.9. Molecular genetic techniques

5.9.1. DNA-plasmid isolation

For the plasmid isolation of bacterial strains, cells were cultivated overnight in 2 mL LB medium containing a certain antibiotic, at 37°C. After centrifugation for 1 minute at room temperature one cell pellet was processed according to the Promega Miniprep Wizard® Plus SV protocol.

The yeast cells had to be prepared separately with Longlife™ Zymolyase® (Cat. # 786-914) from G-Biosciences according to the manufacturers' protocol (taking ddH_2O in step 1), before using the Promega Miniprep Wizard® Plus SV kit to extract the plasmids.

5.9.2. DNA restriction

DNA digestion with endonucleases was used to prepare DNA for ligation (preparative reaction) or to analyse the correct bands following plasmid preparation (analytical restriction) to confirm the accuracy of the final product. Each restriction process was carried out under incubation for 1,5h at 37°C. Enzymes and buffers were purchased from New England BioLabs NEB.

reaction mixture for preparative restriction:

20 µL pDNA

4 µL rCutSmart™ buffer

2 µL Enzyme

filled to 40 µL with ddH_2O

reaction mixture for analytical restriction:

3 µL pDNA

1,5 µL rCutSmart™ buffer

1 µL Enzyme

filled to 15 µL with ddH_2O

5.9.3. DNA ligation

The ligation reaction was operated at room temperature for 1h, using enzymatic catalysis of T4 DNA Ligase and T4 DNA Ligase buffer. In this project, ligation of sticky ends by forming phosphor-diester-bonds came to use for *mfq*-genes with pET30-vector and the circulation of pTJOS-PCR-constructs.

reaction mixture for mfq/pET30- *mfq* derivative:

0,8 µL vector

7 µL insert

2 µL T4 DNA Ligase Reaction buffer

1 µL T4 DNA Ligase

filled to 20 µL with ddH_2O

reaction mixture for pTJOS:

2 µL	PCR product
2 µL	T4 DNA Ligase Reaction buffer
1 µL	T4 DNA Ligase

Filled to 20 µL with ddH_2O

5.10. Polymerase chain reaction (PCR)

PCR is a technique used to selectively amplify a specific DNA template. The reaction mixture includes a DNA template, forward and reverse primers, desoxy-Nucleotide-Triphosphates (dNTPs), DNA-Polymerase and its corresponding buffer. The Q5® High-Fidelity 2X Master Mix from NEB (M0492S) contains dNTPs, the polymerase enzyme and a suitable buffer. Each reaction mix is pipetted into PCR tubes and amplification is performed in PCR-Cyclers, which allow customised temperature settings and cycling conditions.

The PCR process begins with an initial denaturation step at 98°C for at least 3 minutes, to separate the DNA strands. This is followed by cycles of primer annealing, polymerisation and denaturation. Annealing temperatures depend on the specific primers but generally range between 50- 72 °C. The DNA-Polymerase in the Q5® High-Fidelity 2X Master Mix functions optimally at 72 °C, which should be maintained for 20-30 seconds per kilobase, with the duration depending on the product size. After 25- 30 cycles a final extension step at 72 °C is held for 10 minutes before cooling the reaction to 4 °C.

reaction mix per PCR:

12,5 µL	Q5® High-Fidelity 2X Master Mix
1,25 µL	forward primer
1,25 µL	reverse primer
Variable	DNA template

Filled to 25 µL with nuclease free water

5.10.1. SOEing PCR

Splicing by overlap extension PCR is a method to assemble double stranded oligonucleotides into larger fragments of DNA and was used in this project to perform selected gene knockouts in the *mfq*-BGC. To address this, each gene of *mfqA, mfqC, mfqD, mfqE, mfqI* and *mfqX* was flanked by two fragments of Oligonucleotides (*flank1* and *flank2*) with specific primers, of which primer *2fw* had an overlap of 19 base pairs with *1fw* at the 3' end. This overlap of 19 bp was

removed by splicing and the joined sequences of *flank1* and *flank2* were amplified via PCR. The final products served as oligonucleotide inserts for the homologous recombination to excise the targeted gene. (Ho et al. 1989)

5.10.2. Touchdown PCR

To increase specificity and product yield, touchdown PCR protocol was applied in this project. The difference to regular PCR is the temperature setting for primer annealing in touchdown mode. At first, it is set higher than needed for optimal primer annealing and is then progressively decreased until the specific annealing temperature is reached and hold for the last cycles. This ensures less unspecific priming, leading to higher amount of correct PCR-product. (Don et al. 1991)

5.10.3. Yeast Colony PCR

To verify the presence of the correct DNA-insert, clones from the big Y1171-medium plates were selected for colony PCR protocol. Under the sterile bench, the clones were picked with a pipette tip and transferred into 20 µL of ddH_2O. The cells were lysed at 100 °C for 45 minutes to obtain the genomic DNA and *mfq*-carried plasmid. After centrifuging at 10,5 *rpm* at room temperature for 10 minutes, 2µL of supernatant were used as template for the following PCR. The PCR reaction mix was the same as seen above in section “PCR” and should include the *fw1* and *rev2* primers of the SOEing-products and the vector pSH was used for positive control.

5.11. CRISPR/Cas

To investigate the function of the individual Mfq proteins within the BGC, the following genes were selected for deactivation: mfqA, mfqC, mfqD, mfqE, mfqI, mfqX. This was achieved with CRISPR-Cas9 technology in *Saccharomyces cerevisiae* TC3 strains, as described by (Jakočiūnas et al. 2015).

For the primer design of the flanks and single-guide RNAs (sgRNAs), the softwares Clone Manager and CRISPy-web (Blin et al. 2016) were used. The vector pSH contained the assembled *mfq*-BGC and served as template for the flanks for spliced overlap extension PCR (SOEing PCR). The vector pTJOS was used for expression of sgRNAs. The primers with phosphorylated 5’ ends were synthesized by Eurofins.

5.12. DNA validation via gel electrophoresis

Gel electrophoresis separates negatively charged DNA fragments based on their size, allowing for the assessment of DNA quality by verifying fragment size and concentration in each sample. Loading dye (B7024A) from NEB was added to the sample before application to the gel, and a DNA ladder (N3232L) was used to analyse fragment sizes. As for analytical restriction, the

bands belonging to the genes were cut out of the gel. If, the DNA was extracted according to Monarch® DNA Gel Extraction Kit from NEB.

0,8% Agarose-Gel:

0,8 g/100 mL Agarose

filled to 100 mL with Tris-Borat-EDTA buffer

10 µL GelRed

5.13. Protein expression, extraction and purification

The production of Mfq-enzymes expressed from the pET30a+ vector occurred in *E. coli* BL21 cells. Purification of the His-tagged proteins was carried out by affinity chromatography using Nickel-Nitrilotriacetic acid (30210) from Qiagen.

5.13.1. Buffers for protein extraction and purification

Each buffer had a pH of 8, which was calculated using AAT Bioquest (Potassium Phosphate (pH 5.8 to 8.0) Preparation and Recipe | AAT Bioquest) and Cryptographica (Henderson-Hasselbalch Calculator for Tris Buffers (cytographica.com)).

Lysis buffer:

50 mM potassium phosphate buffer

300 mM NaCl

10 mM imidazole

1 mg/mL lysozyme (Cas: 12650-88-3) from Sigma-Aldrich added before using

Washing buffer:

50 mM potassium phosphate buffer

300 mM NaCl

20 mM imidazole

Elution buffer:

50 mM potassium phosphate buffer

300 mM NaCl

250 mM imidazole

Dialysis buffer:

50 mM TRIS-HCl buffer pH 8

50 mM NaCl

5.13.2. Heterologous production of Mfq-enzymes in *E. coli* BL21

E. coli BL21 cells were used for the heterologous expression of MfqA, MfqC, MfqE, MfqI and MfqX, as this strain can produce proteins in higher amounts, as they harbor the T7 expression system with the inducible *lac*UV5 promoter. Overproduction is activated by Isopropyl β-d-1-thiogalactopyranosidase (IPTG). (Miroux and Walker 1996; Studier 1991)

For the overnight cultivation at 37°C the cells were inoculated into LB medium containing 25 µg/mL kanamycin. 5 mL overnight culture were re-inoculated into a 1 L flask containing 200 mL of triple- concentrated LB medium with 25 µg/mL Kanamycin and the incubation at 37°C continued until the optical density at 600 nm reached a value between 0,9 – 1,1. At that point 0,5 mM of IPTG was added. The incubation process carried on for 2h at 25°C and the overnight cultivation was carried out at 15°C.

5.13.3. Protein extraction and purification

To obtain the cells, cultures were centrifuged for 15 minutes at 4000 *rpm* and 4°C. This cell pellet was resuspended in 10 mL lysis buffer. As the proteins were not secreted into the medium, sonification (amplitude 30%, cycle 3) was applied to destroy the cell membrane. To remove cell remnants, the samples were centrifugated for 30 min at 10000 rpm and 4°C and filtrated through a 2 µm filter membrane.

Nickel-NTA was used as a binding reagent, because of its high affinity for His-tagged proteins. For equilibration step, 1 mL of Ni-NTA needed to be washed two times with 10 mL lysis buffer per step and centrifugated for 10 min at 700 rpm and 4°C beforehand.

After adding the filtrated samples to the washed Ni-NTA, the samples were incubated for 1 h at 4°C and inverted every 5-10 min.

The Ni-NTA mixed samples were added onto a column and their flowthrough was saved in a 50 mL flask and stored at 4°C for SDS-PAGE. The samples were washed with 4 mL washing buffer twice and each wash- flowthrough was kept for SDS-PAGE as well.

The eluates were collected using 4 mL of elution buffer each. The dialysis tubes Amicon Ultra – 15 with regenerated cellulose membrane (UFC9010; UFC9050; SigmaAldrich) were prewashed with dialysis buffer by centrifugation at 4°C. Dialysis of eluates was done three times with 4 mL of dialysis buffer. The process occurred at 4°C; the *rpm* and time varied between samples. The aim was to reach a volume of 1500 µL, then the purified samples were aliquoted into 1,5 mL tubes and instantly frozen with liquid nitrogen, then stored at -70°C.

5.13.4. SDS-PAGE

To determine the molecular weight and purity of the extracted products, they were compared to different stages of their purification process by Sodium-dodecylsulfate polyacrylamide-gelelectrophoresis (SDS-PAGE). SDS is an amphiphilic denaturation agent, as it binds the hydrophobic regions in the proteins' tertiary structure. The overall charge of the protein-SDS-complex is negative and creates a constant charge to mass ratio, which is important for accurate separation.

30 µL of each sample were mixed with 30 µL of Laemmli buffer (S3401-1VL) from Sigma Aldrich and denatured at 95°C for 3 min. Aside from SDS the buffer contained β-mercaptoethanol to break disulfide bonds. 12 µL of samples were loaded on the ready to use Precast Mini-Protean TGX Gels (4561096) from Bio-Rad. Precision Plus Protein Unstained Protein Standard (161-0363) from Bio-Rad was used as size marker.

After filling the tank with the right amount of Tris-Glycine-SDS- buffer (1610732) from Bio-Rad, the gel ran at 150 V for about 1,5 h and was stained with Bio-Safe™ Coomassie Stain (#1610786) from Bio-Rad afterwards. The destaining was performed with Coomassie-destaining solution for 20 min and overnight in distilled water.

Coomassie destaining solution:

400 mL ddH_2O

100 mL acetic acid

500 mL methanol

6. Results

6.1. Heterologous protein expression and purification

6.1.1. Construction of *E. coli* BL21 expressing Mfq proteins

The His-tagged codon-optimized *mfq*-genes were received in pUC57 subcloning-vector from BioCat. *E. coli* XL1-Blue and *E. coli* DH5α- F' were transformed with these plasmids to amplify the constructs. Both vectors pUC57 containing *mfq* genes and pET-30a expression vector were cut with *Bam*HI and *Nde*I, to linearize the vectors and to purify the DNA fragments with His-tagged *mfq* genes. After the successful ligation of His-tagged *mfq* genes into pET30a, the assembled constructs were introduced into *E. coli* BL21 for the heterologous protein expression. A schematic visualisation of the restriction-cloning process for His-*mfqA* is shown in Figures 14 and 15.

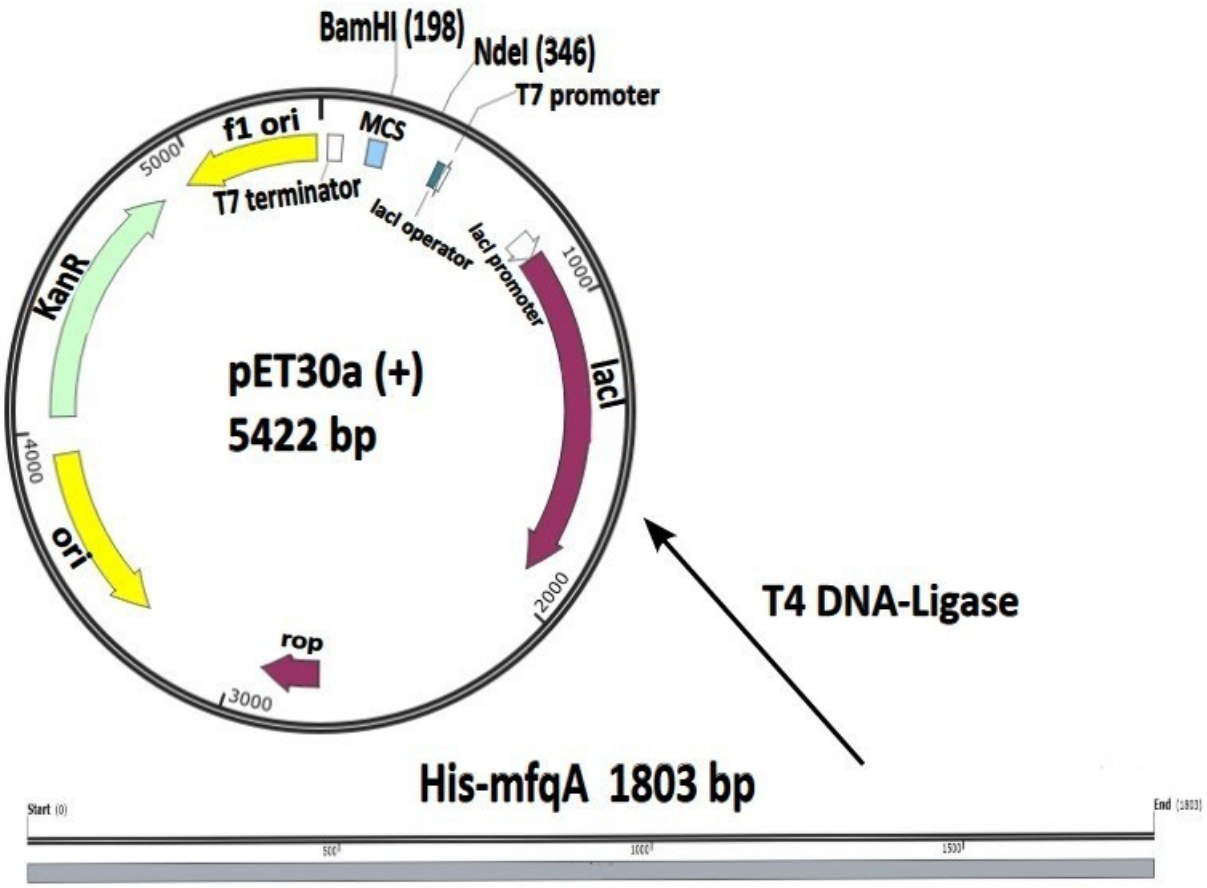

Figure 14: Restriction cloning scheme used in this project for His-*mfqA* as example. Subcloning-vector pUC57_His-*mfqA* (not shown) and expression vector pET-30a were both cut with *Bam*HI and *Nde*I,to linearize both vectors and isolate the His-tagged gene. Then His-*mfqA* was ligated into the linearized

pET-30a vector with T4-DNA Ligase. The assembled pET-30a_His-*mfqA* was then transferred into *E. coli* BL21 for protein expression

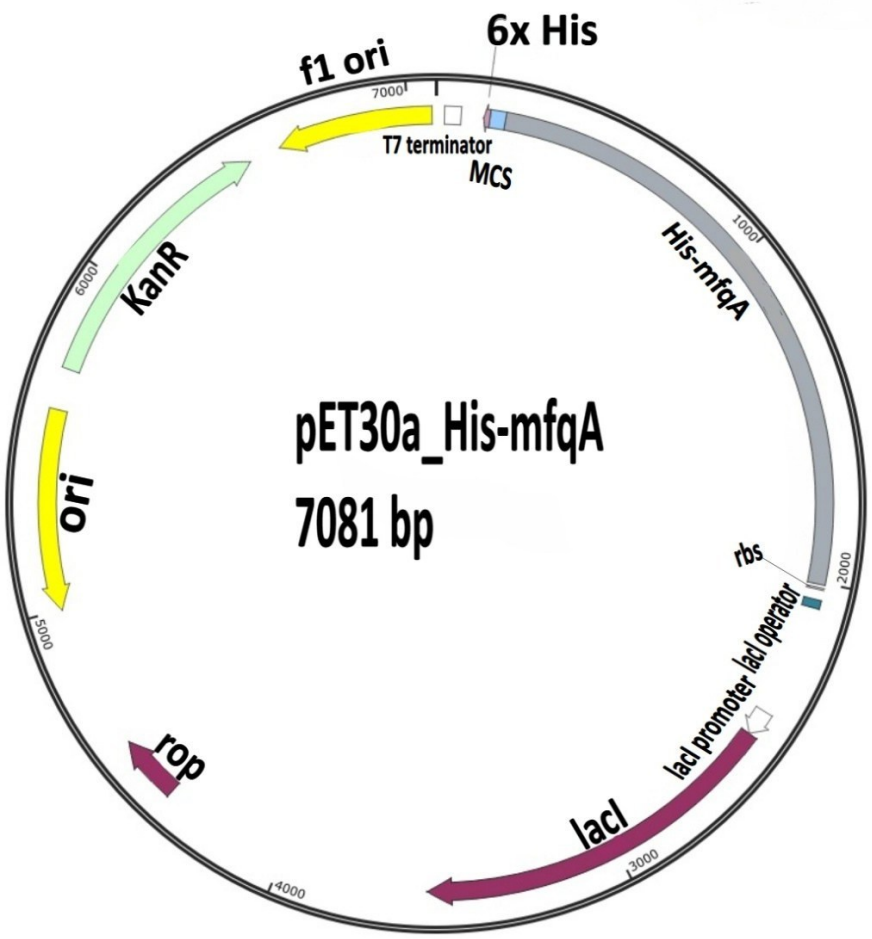

Figure 15: Expression vector pET30a after ligation with His-mfqA, which was transferred into *E. coli* BL21 for protein expression. 6x His…Histidin-tag essential for Ni-NTA-affinity chromatography.

The efficiency of the cloning strategy was confirmed by restriction analysis. (Figure 16)

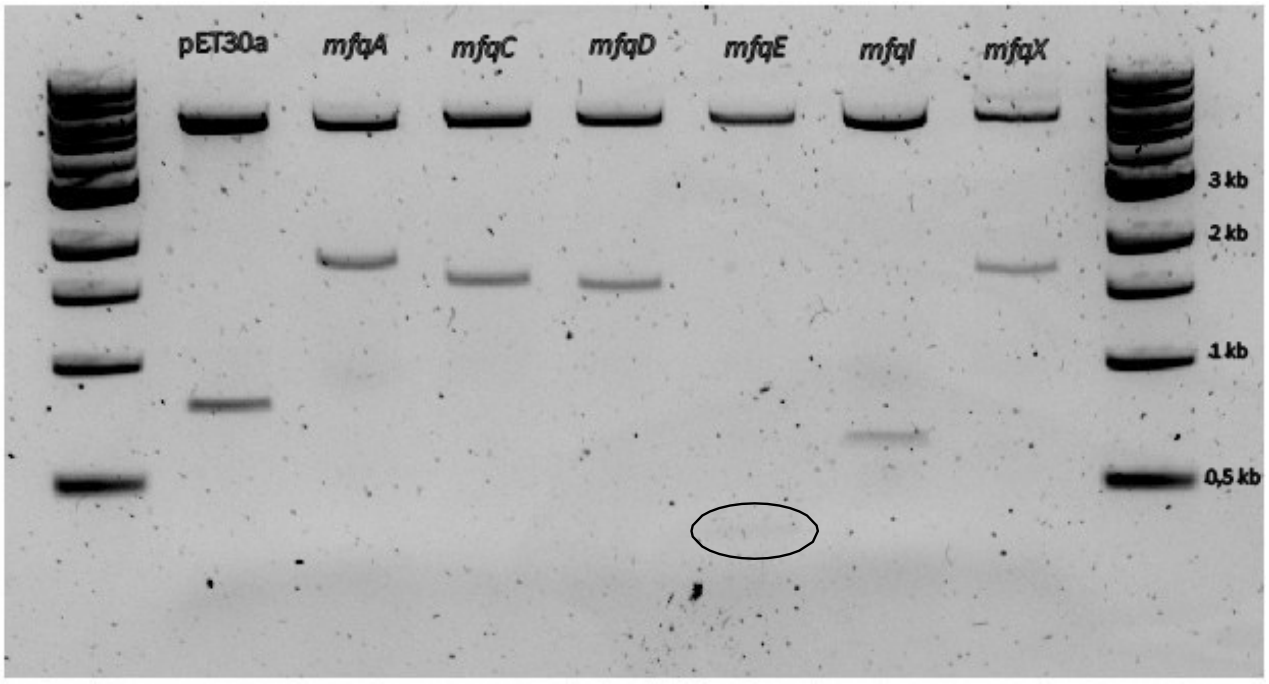

Figure 16: Gel electrophoresis to check the correct DNA expression constructs in *E. coli* BL21 pET-30a clones. The isolated plasmids were cut with *Bam*HI and *Nde*I. The fragment sizes are listed in Table 4.

The expected sizes of the His-tagged *mfq* genes in base pairs are listed in Table 4. The fragment sizes were all correct and in proper intensity, except for *mfqE,* which is therefore marked with a circle in Figure 16. The weak intensity of *mfqE* band seen in the gel, may result from a small size of the insert, but had no negative impact on the efficiency of protein expression.

Table 4: Selected his-*mfq*-genes sizes in bp

Gene	**Sizes [bp]**	**Expected fragment sizes after restriction digest with *Bam*HI /*Nde*I**
His-*mfqA*	1806	5276;1799
His-*mfqC*	1593	5276;1586
His-*mfqD*	1545	5276;1538
His-*mfqE*	330	5276;323
His-*mfqI*	639	5276;632
His-*mfqX*	1677	5276;1670

6.1.2. Extraction and purification of Mfq proteins from *E. coli* BL21

After the heterologous expression in *E. coli* BL21 the His-Mfq proteins were isolated from the cell lysates and purified with Ni-NTA affinity chromatography. To determine the purity of extracted proteins they were separated in SDS-PAGE and compared to different steps of the purification process. Table 5 lists the protein molecular weights in kDA calculated with Clone Manager.

Table 5: Protein molecular weights calculated with Clone Manager and predicted enzyme function

Protein	**Molecular weight of proteins with 6x His-tag [kDA]**	**Predicted function**
MfqA	64,58	FAD-binding monoxygenase
MfqC	57,77	Acyl-CoA ligase
MfqD	54,67	FAD-binding oxidoreductase
MfqE	12,04	Monoxygenase
MfqI	22,04	NADPH-dependent oxidoreductase
MfqX	58,53	Terpene cyclase

Figures 17a and 17b show His-tagged proteins MfqA and MfqE after SDS-PAGE stained with Coomassie-Blue, compared to different purification steps (Materials and methods). The dark clear bands of the final eluates align with the expected molecular weights of each protein.

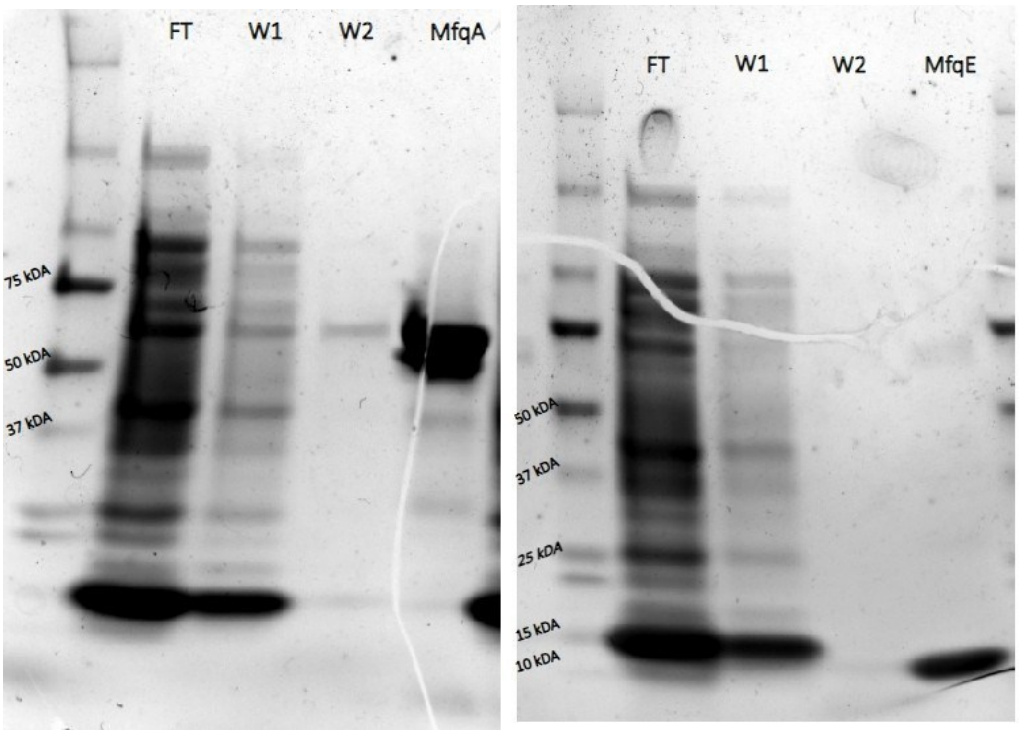

Figure 17a (left): His-MfqA (64,58 kDA) compared to different purification steps in SDS-PAGE after Coomassie-Blue staining. FT: flowthrough of lysate after incubation with Ni-NTA (10 mM imidazol). W1 and W2: wash steps 1 and 2 with washing buffer (20 mM imidazol). His-MfqA: final eluat with 4 mL elution buffer (250 mM imidazol) and dialysis.

Figure 17b (right): SDS-PAGE analysis for His-MfqE (12,04 kDa) coloured with Coomassie-Blue. FT: flowthrough of lysate after incubation with Ni-NTA (10 mM imidazol). W1 and W2: wash steps with washing buffer (20 mM imidazol). His-MfqE: final eluat with 4 mL elution buffer (250 mM imidazol) after dialysis.

Figure 18 displays the final eluates of His-tagged MfqC, MfqI and MfqX extraction in SDS-PA-gel with Coomassie-Blue stain compared to the cell lysates. The strongest bands of the eluates matched with the expected protein molecular weight. The weaker bands may indicate the presence of by-products in the extracts or partial degradation of the proteins.

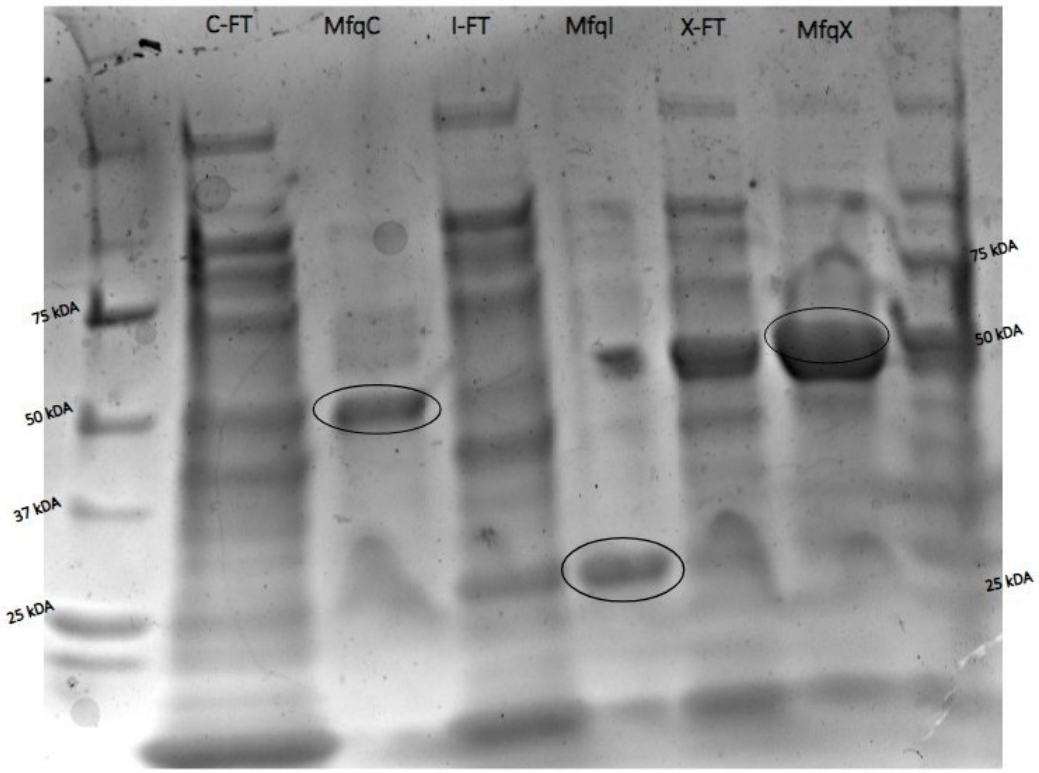

Figure 18: SDS-PAGE analysis of His-MfqC (57,77 kDa), His-MfqI (22,04 kDa) and His-MfqX (58,53 kDa) coloured with Coomassie-Blue. C-FT/I-FT/X-FT: lysate-flowthrough of corresponding protein before mixing with Ni-NTA (10 mM imidazol). MfqC/MfqI/MfqX: final eluat of His-tagged proteins with 4 mL of elution buffer (250 mM imidazol)

The successful heterologous expression and purification of the targeted proteins enables further studies, to determine the function and the enzymatic kinetics of the selected Mfq proteins in the marfuraquinocins biosynthesis.

6.2. CRISPR/Cas9 mediated gene knockout

For the investigation of certain marfuraquinocin biosynthesis steps, several *mfq*-genes were chosen for gene knock-out using CRISPR/Cas-9 assisted in-frame deletions. *Saccharomyces cerevisiae* TC3 was used for gene deletion in the *mfq* BGC, as it harbours the plasmid for Cas9- expression. The primers for sgRNA in vector pTJOS, expressing the sgRNA, were designed with CRISPy-web (Blin et al. 2016; Jakočiūnas et al. 2015). The sgRNA should guide the Cas9-endonuclease to the target sequences in the pSH plasmid carrying *mfq*-BGC. After the double strand break introduced by Cas9, the homology directed repair will occurr and due to the introduction of a knockout cassette consisting of double stranded oligonucleotides replacing original genes in the *mfq*-BGC (Figure 19).

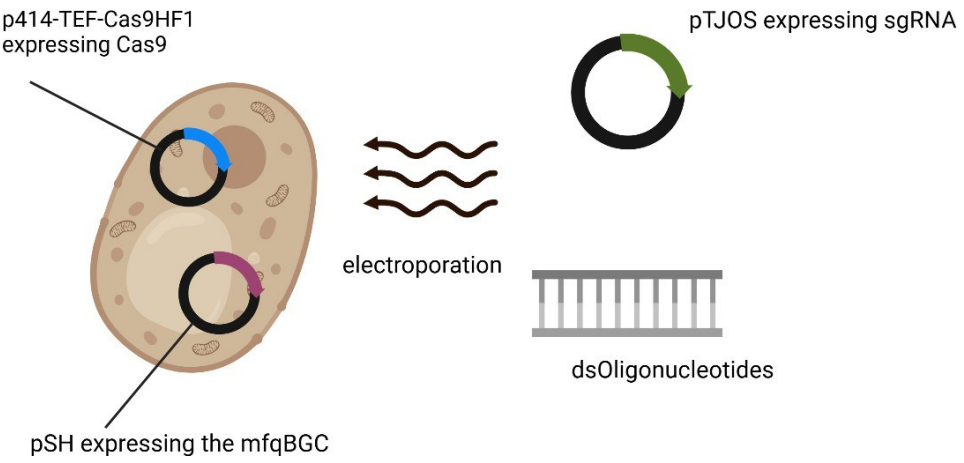

Figure 19: Schematic illustration of electroporation of sgRNA-expressing pTJOS vector and dsOligonucleotides into *S. cerevisiae* TC3 to facilitate CRISPR/Cas9-assisted gene deletion.

The knockout cassette was created beforehand using SOEing PCR (Figure 20). All sgRNAs, flanks and SOEing products were analysed via gel electrophoresis, extracted from the gel and sent for sequencing (by Microsynth). In case of *mfqD* the company BioCat synthesized the final SOEing product which was then used for the CRISPR/Cas9-assisted gene deletion.

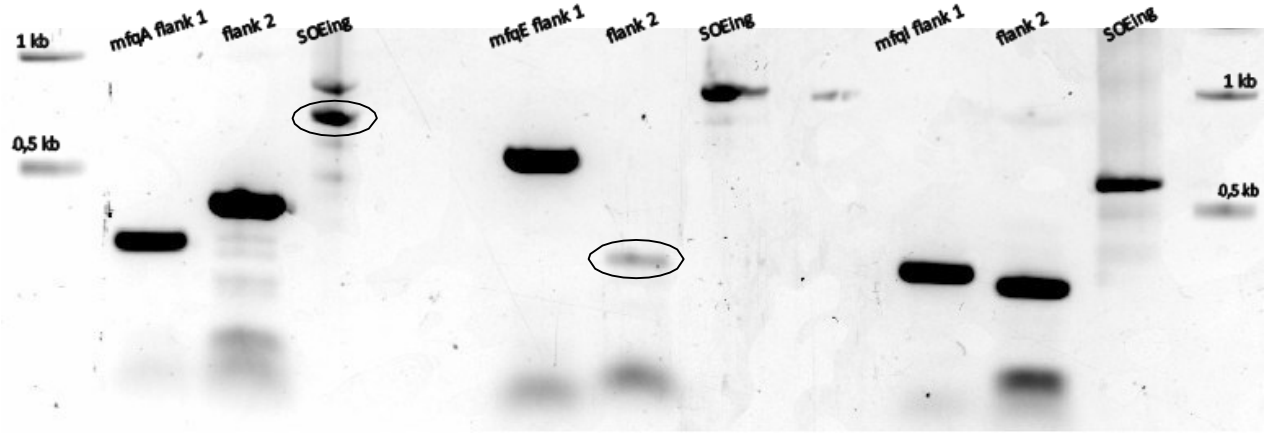

Figure 20: Gelelectrophoresis after PCR of flanks and spliced overlap extension (SOE) products for *mfqA, mfqE* and *mfqI*. lane 1: mfqA flank_1 (308 bp), lane 2: *mfqA* flank_2 (425 bp), lane 3: *mfqA*_SOEing product (713 bp), lane 5: *mfqE* flank_1 (598 bp), lane 6: *mfqE* flank_2 (303 bp), lane 7: *mfqE*_SOEing product (882 bp), lane 9: *mfqI* flank_1 (321 bp), lane 10: *mfqI* flank_2 (302 bp, lane 11: *mfqI*_SOEing product (604 bp)

6.2.1. Gene knockout validation in yeast

Knockout cassettes and pTJOS-vectors expressing the sgRNAs were introduced into *Sac. cerevisiae* TC3 via electroporation transformation and grown on Y1771 solid medium without leucin, tryptophan and uracil for selection. For each construct a control sample was prepared without the addition of DNA and growth rate was compared to validate the success of the Cas9

reaction. The further validation included plasmid isolation and restriction analysis or colony PCR. The sequencing confirmed correct clones for about 50% of every gene targeted.

6.3. Conjugation

After the successful knock-outs, the verified mutant pSH plasmids carrying specific *mfq* gene deletions were transformed into *Escherichia coli* ET12567/pUZ8002 and therefrom conjugated into *Streptomyces coelicolor* M1154. The obtained *S. coelicolor* M1154 strains were then subjected to a second conjugation, which introduced either empty pUWLoriT (control) or its variant harbouring Streptomyces Antibiotic Regulator Protein (SARP) MfqF to activate expression of the *mfq* BGC.

The modified pSH-vectors were transformed into *E. coli* ET12567/pUZ8002 (described in Materials and methods) and selected on solid medium with Apra, Cml and Kan. Restriction analysis with *Nde*I and *Hind*III was done to check the quality of DNA. Table 6 lists the plasmids which were introduced into *S. coelicolor* M1154 -strains during the first and second conjugation.

Table 6: Plasmids introduced via conjugation from *E. coli* ET12567/pUZ8002 into *S. coelicolor* M1154.

Gene	First conjugation	Second conjugation
mfqA	pSH-*ΔmfqA*	pSH-*ΔmfqA*-pUWLoriT+ SARP
		pSH-*ΔmfqA*-pUWLoriT
mfqC	pSH-*ΔmfqC*	
mfqE	pSH-*ΔmfqE*	pSH-*ΔmfqE*-pUWLoriT+ SARP
		pSH-*ΔmfqE*-pUWLoriT
mfqI	pSH-*ΔmfqI*	pSH-*ΔmfqI*-pUWLoriT+ SARP
		pSH-*ΔmfqI*-pUWLoriT
mfqX	pSH-*ΔmfqX*	

6.4. Fermentation

Fermentation of bacterial strains is done to stimulate the biosynthesis of secondary metabolites. Table 7 lists the modified strains of *S. coelicolor* M1154 carrying *mfq* BGC with deleted *mfqA* and *mfqI* used for the fermentation in this project. The media and conditions are listed in Materials and methods. In theory, the biosynthesis would terminate at the step

catalysed by the enzyme which gene was deleted, resulting in accumulation of the preceding intermediate.

Table 7: Plasmid genotypes in recombinant *S. coelicolor* M1154 used for fermentation.

Gene	***Streptomyces* - Strain**	**Remarks**
mfqA	pSH-Δ*mfqA*	*mfq*-BGC with deleted *mfqA* in pSH vector
	pUWLoriT-Δ*mfqA*	Deleted *mfqA* without SARP regulator
	pUWLoriT-Δ*mfqA* + pSARP	Deleted *mfqA* with overexpressed SARP regulator
mfqI	pSH-Δ*mfqI*	*mfq*-BGC with deleted *mfqI* in pSH vector
	pUWLoriT-Δ*mfqI*	Deleted *mfqI* without SARP regulator
	pUWLoriT-Δ*mfqI* + pSARP	Deleted *mfqI* with overexpressed SARP regulator

6.5. Preparation of culture extracts and their LC-MS analysis

After fermentation the compounds were extracted from the cultures as described in Materials and methods. All methanolic extracts were analysed with LC-MS by Dr. Martin Zehl from the Department of Analytical Chemistry of the University of Vienna. This analysis aimed to determine whether deletion of the gene resulted in any changes in metabolite accumulation, thereby providing evidence for the gene's function in the biosynthesis of marfuraquinocins.

6.5.1. Deletion of *mfqA* encoding a putative FAD-binding monoxygenase

Figures 22 and 24 show the chromatograms of extracts from the supernatants of engineered *S. coelicolor* M1154 strains carrying either pSH-Δ*mfqA*_SARP (line 1) or unmodified *mfq*-BGC with SARP (line 2). Deletion of *mfqA* resulted in higher marfuraquinocin production in both pellet and supernatant extracts. In MYM medium, production levels remained high, with marfuraquinocin E (Figure 22) representing one of the major peaks in the pellet extract (Figure 23). Additionally, accumulation of flaviolin and 8-aminoflaviolin was detected in the supernatant samples. (Figure 21, line 1)

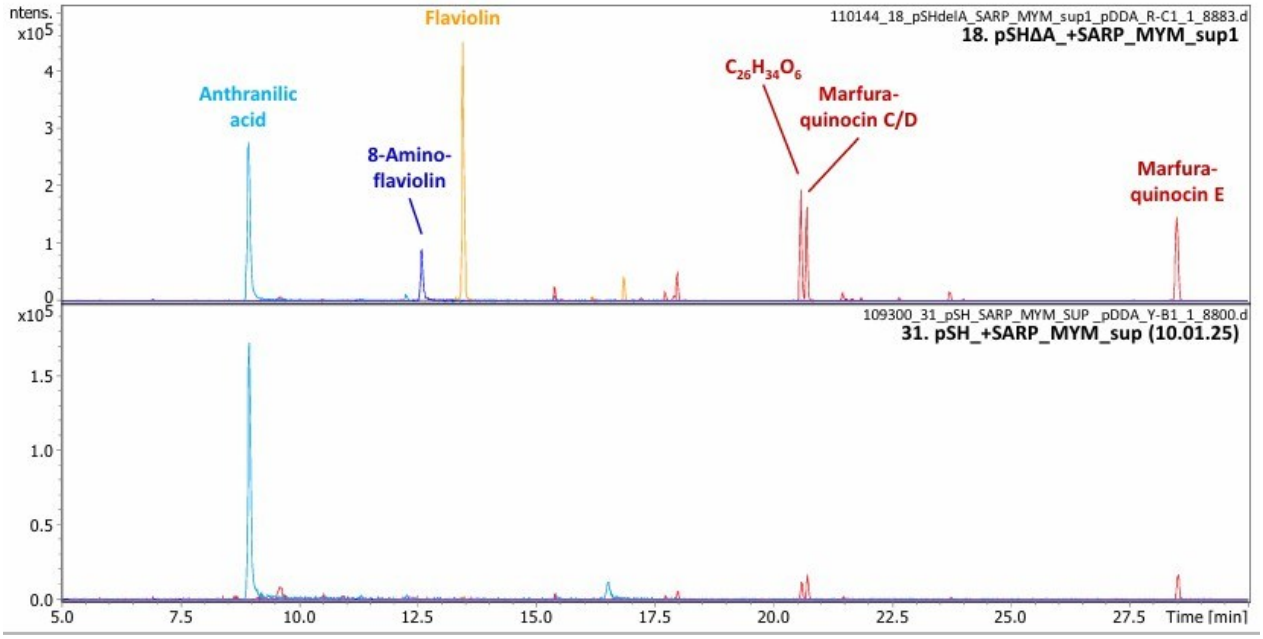

Figure 21: timsTOF flex LC-MS comparison of two engineered *S. coelicolor* M1154 strains cultivated in MYM. line 1: pSH-Δ*mfqA*_SARP_MYM_supernatant1; line 2: pSH_SARP _MYM_supernatant. Higher production levels of 8-aminoflaviolin, flaviolin and marfuraquinocins C/D and E occurred in the strain with *mfqA* -deletion.

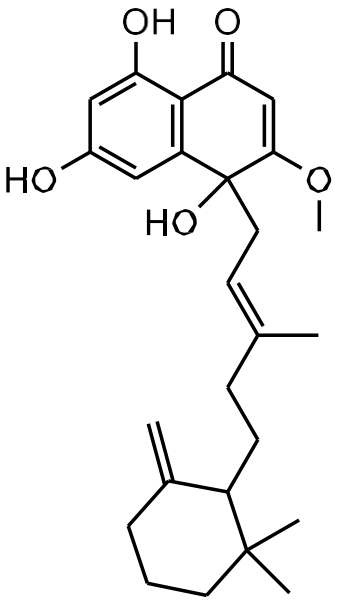

Figure 22: Marfuraquinocin E

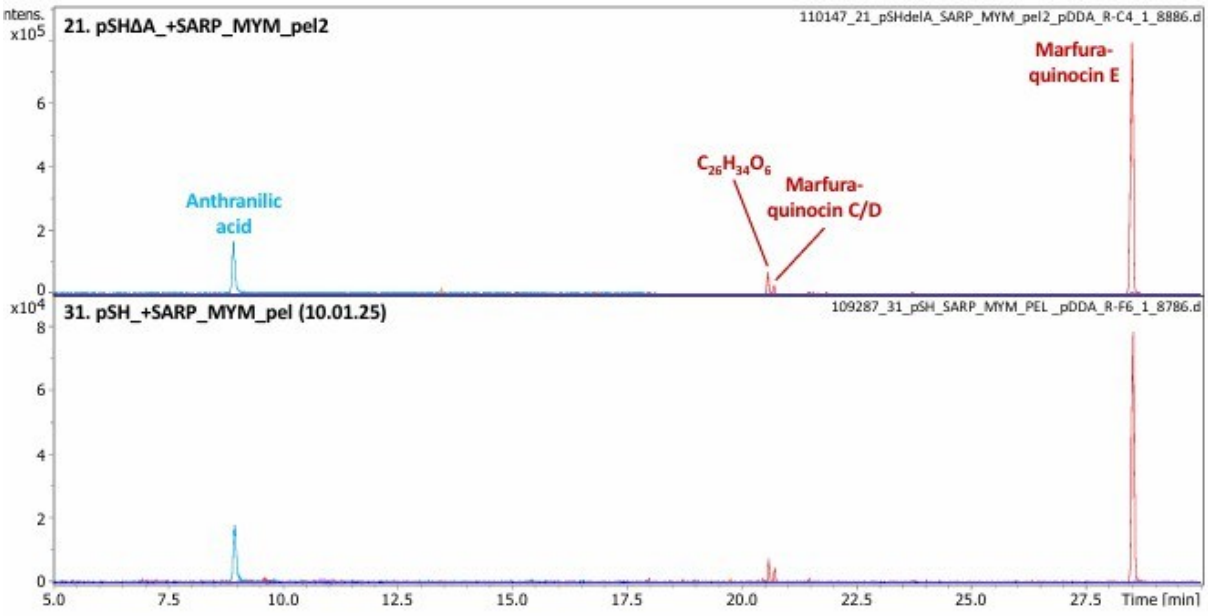

Figure 23: timsTOF flex LC-MS comparison of engineered *S. coelicolor* M1154 strains cultivated in MYM. line 1: pSH-Δ*mfqA*_SARP_MYM_pellet2; line 2: pSH_SARP _MYM_pellet. Production levels of marfuraquinocin E are 10 times higher in *mfqA* mutant than in wild type.

For marfuraquinocin E, the production levels in the pellet extract of the *mfqA* mutant cultivated in MYM, were about 10 times higher compared to the wild type (Figure 23). The oxidized products, marfuraquinocin C or D were also observed in their typical ratios. These findings indicate that *mfqA* is not essential marfuraquinocin biosynthesis under the tested laboratory conditions. Notably, MYM medium supported the highest levels of production. In contrast, in SFM medium, marfuraquinocin production was very low overall, and no significant differences were observed between the control strain and the *mfqA*-deleted strain. (see Appendix)

6.5.2. Deletion of *mfqI* encoding a putative NADPH- dependent oxidoreductase

In both fermentation media (MYM and SFM), the pSH-*mfqI*_SARP mutant exhibited a significantly altered metabolite profile compared to control strains (data in Appendix). The mutant consumed fewer medium-derived compounds and produced reduced levels of host-associated metabolites, although some host metabolites remained detectable. This suggests a broad impact of *mfqI* deletion on the metabolic processes of the strain, possibly reflecting disruptions in both primary and secondary metabolism.

Additionally, the mutant displayed noticeably slower growth, which may be associated with impaired metabolic activity or the accumulation of toxic intermediates resulting from the biosynthetic block.

7. Discussion

The successful heterologous expression and purification of selected Mfq proteins in *E. coli* BL21 demonstrates the feasibility of using this host system for functional characterisation of the enzymes encoded by the *mfq* -BGC. The Ni-NTA affinity chromatography yielded proteins of the expected molecular weights, with clear bands observed in the eluates, confirming that the His-tag facilitated efficient purification. Especially the eluates of His-MfqA and His-MfqE showed very low contamination, suggesting that the purification protocol was largely effective for these two proteins. The presence of by-products in samples His-tagged MfqC, MfqI and MfqX may indicate partial degradation or co-purification of host proteins. This could be addressed in future optimizations of the purification process by including additional steps or protease inhibitors to prevent degradation.

The CRISPR/Cas9-mediated gene knock-outs in *Sac. cerevisiae* TC3 further validated the functional tools for manipulating the *mfq*-BGC. The successful generation of knockout mutants for *mfqA, mfqC, mfqE, mfqI* and *mfqX* shows the applicability of CRISPR/Cas9 system for targeted genome editing using this system (Rainha et al. 2020).

The conjugation of modified pSH vectors into *S. coelicolor* M1154 enabled the generation of engineered strains with targeted gene deletions in the *mfq*-BGC. The subsequent second conjugation with the pUWLoriT vector carrying an overexpressed SARP gene aimed to activate marfuraquinocin biosynthesis pathway. The establishment of stable transconjugants in both conjugation steps confirms the suitability of the employed vectors and selection conditions for genetic manipulation in *S. coelicolor*.

Fermentation of the Δ*mfqA* and Δ*mfqI* strains in different media revealed that MYM medium supported higher levels of marfuraquinocin production compared to SFM, which highlights the importance of culture medium for production of secondary metabolites in *Streptomyces* bacteria.

LC-MS analysis of methanolic extracts showed that deletion of *mfqA* provided significant changes in the qualitative metabolite profile. Typical marfuraquinocin derivatives including marfuraquinocin E, C and D, were detected in higher ratios in the mutant strain compared to the wild type in both supernatant and pellet extracts. In particular, the *mfqA* mutant cultivated in MYM medium exhibited approximately tenfold higher levels for marfuraquinocin E compared to the wild type. These findings suggest that *mfqA* may suppress the biosynthesis of marfuraquinocins under the tested conditions. Further studies should be done to identify the function of *mfqA*.

The reduced growth rate with deletion of *mfqI* indicates that *mfqI* might play a critical role in detoxification of an intermediate that may accumulate during the marfuraquinocin biosynthesis and thus inhibits the growth of recombinant *S. coelicolor*.

The results obtained during this Master project provide a solid foundation for further analysis of the marfuraquinocin biosynthetic pathway. Future work should focus on the functional characterisation of the Mfq- proteins via in vitro enzyme assays, substrate feeding experiments and kinetic studies using the purified proteins. With these findings it could become possible to rationally modify the *mfq* BGC and design novel bioactive derivatives with pharmaceutical relevance.

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9. Appendix

9.1.1. LC-MS analysis of fermented extracts

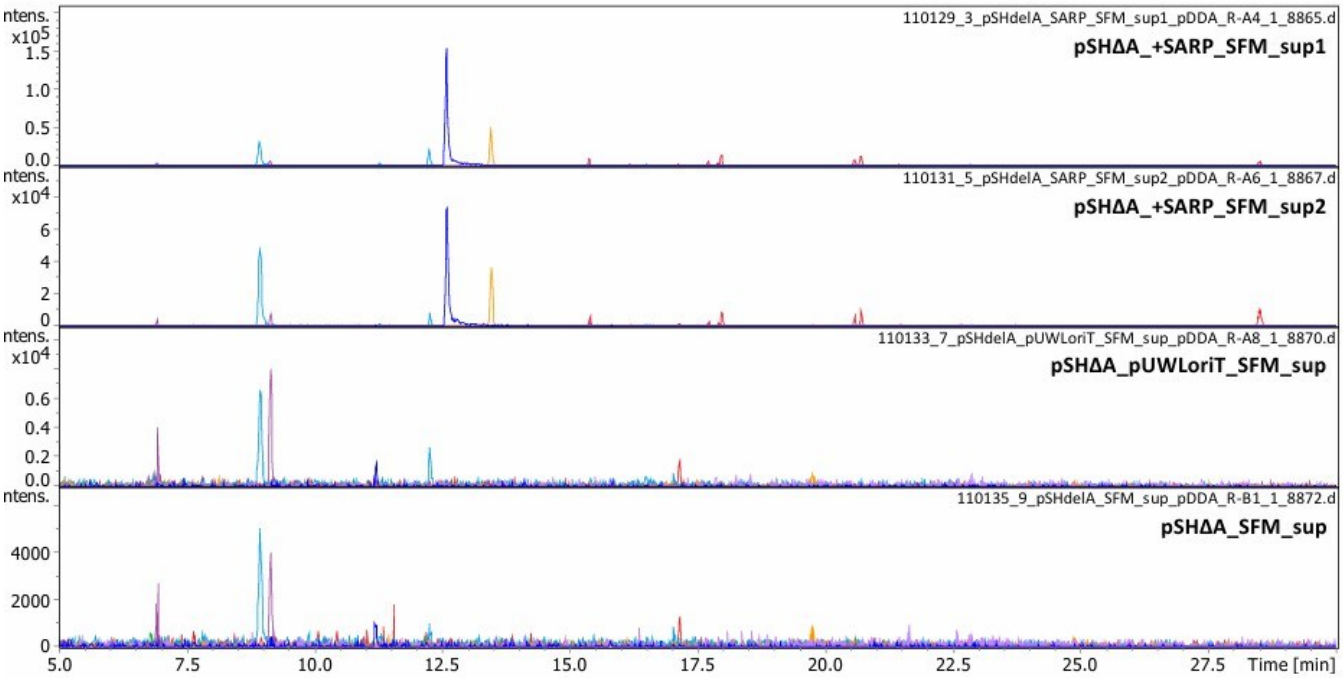

Figure 25: LC-MS comparison of four engineered S. coelicolor M1154 cultivated in SFM

line 1: pSH-ΔmfqA_SARP_SFM_supernatant1 – exhibits the highest metabolite production with the corresponding signal intensity (up to 10^5)

line 2: pSH-ΔmfqA_SARP_SFM_supernatant2

line 3: pSH-ΔmfqA_pUWLoriT_SFM_supernatant

line 4: pSH-ΔmfqA_SFM_supernatant

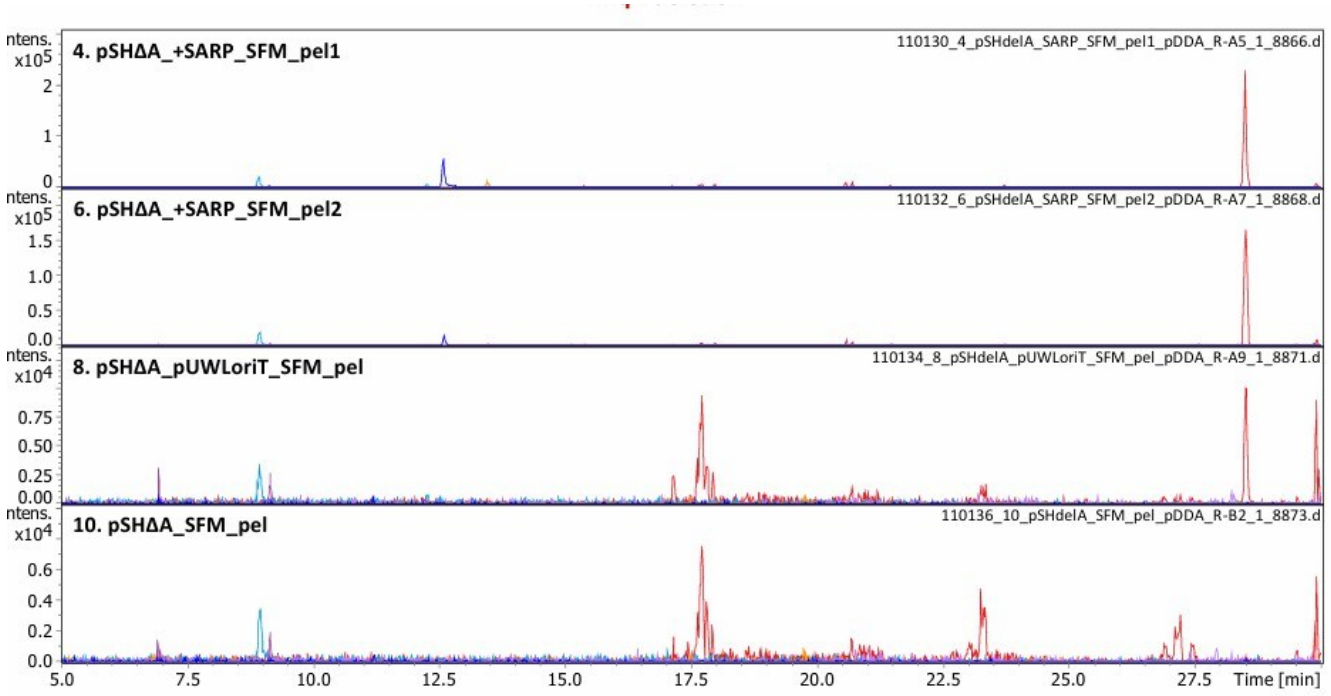

Figure 26: LC-MS analysis of four samples from genetically modified S. coelicolor M1154 cultivated in SFM. Highest metabolite signals are observed in the samples with overexpressed SARP (line 1 and 2).

line 1: pSH-ΔmfqA_SARP_SFM_pellet1

line 2: pSH-ΔmfqA_SARP_SFM_pellet2

line 3: pSH-ΔmfqA_pUWLoriT_SFM_pellet2

line 4: pSH-ΔmfqA_SFM_pellet

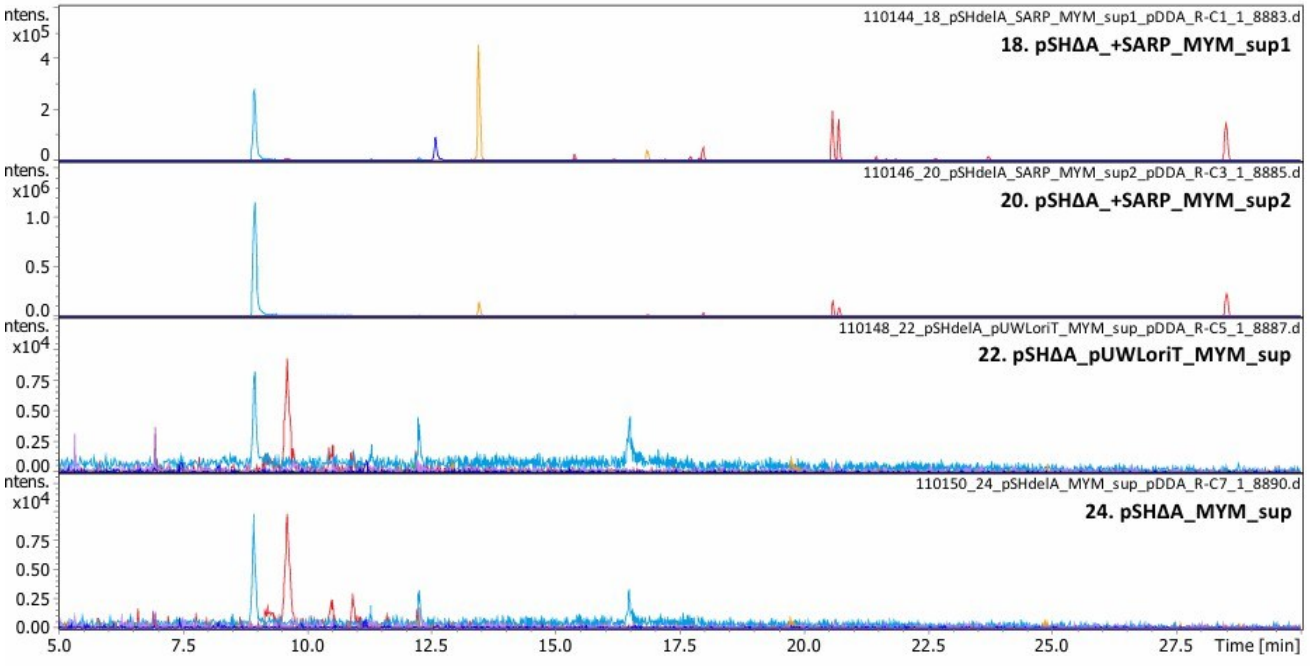

Figure 27: LC-MS comparison of four samples from engineered S. coelicolor M1154 cultivated in MYM. Strains with overexpressed SARP show higher metabolite production profiles, while pSH-ΔmfqA

control shows lowest signal intensity.

line 1: pSH-ΔmfqA_SARP_MYM_supernatant1

line 2: pSH-ΔmfqA_SARP_MYM_supernatant2

line 3: pSH-ΔmfqA_pUWLoriT_MYM_supernatant

line 4: pSH-ΔmfqA_MYM_supernatant.

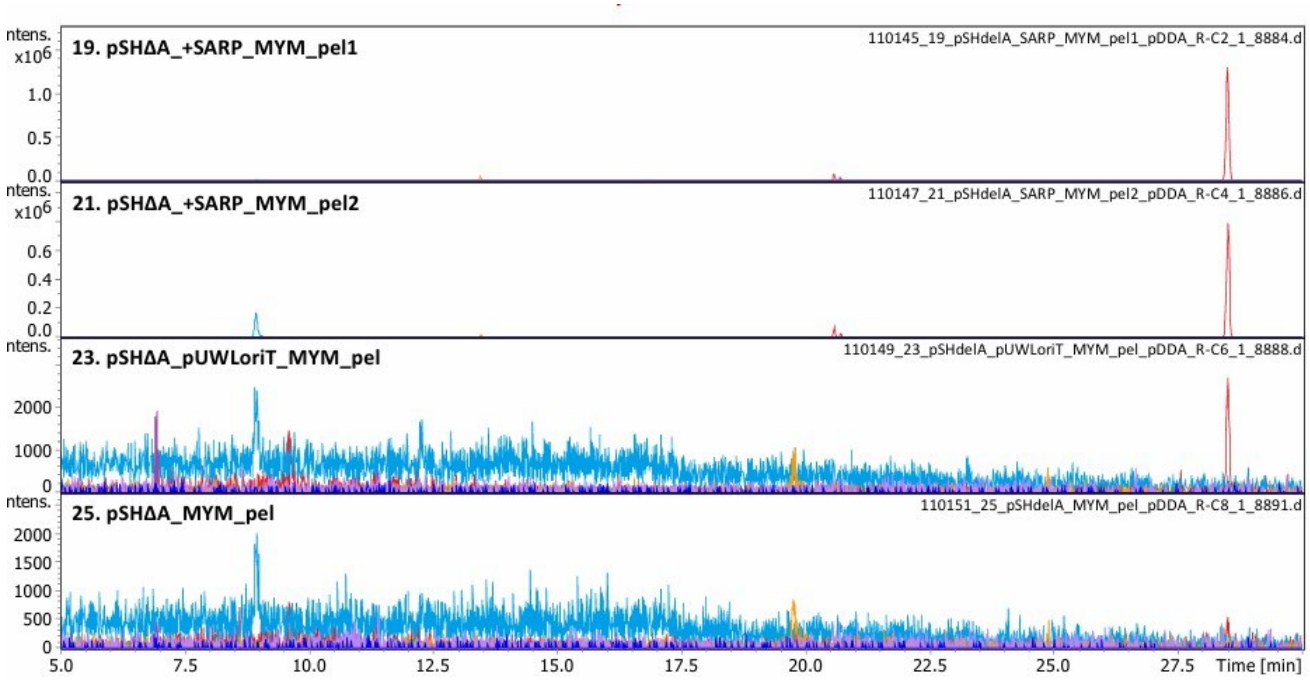

Figure 28: Comparison of four LC-MS chromatograms of samples from engineered S. coelicolor M1154 strains cultivated in MYM.

line 1: pSH-ΔmfqA_SARP_MYM_pellet1

line 2: pSH-ΔmfqA_SARP_MYM_pellet2

line 3: pSH-ΔmfqA_pUWLoriT_MYM_pellet

line 4: pSH-ΔmfqA_MYM_pellet

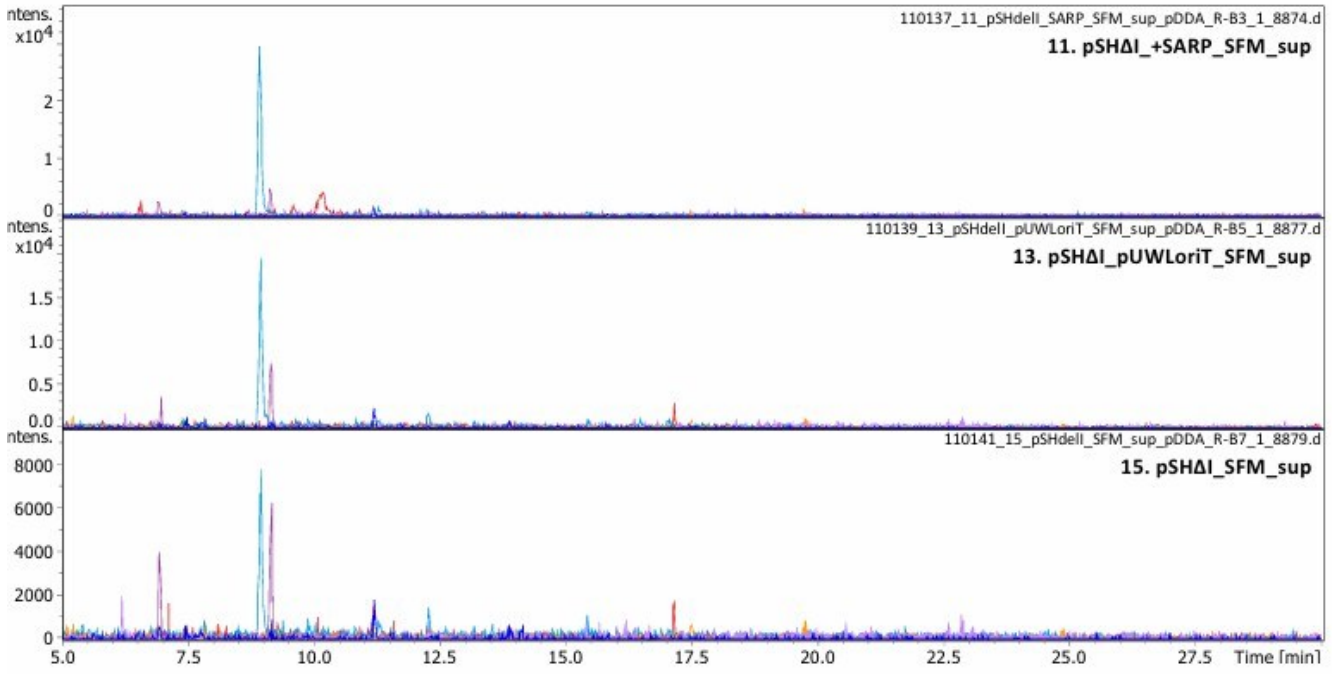

Figure 29: LC-MS comparison of three genetically modified S. coelicolor M1154 strains cultivated in SFM.

line 1: pSH-ΔmfqI_SARP_SFM_supernatant

line 2: pSH-ΔmfqI_pUWLoriT_SFM_supernatant

line 3: pSH-ΔmfqI_SFM_supernatant

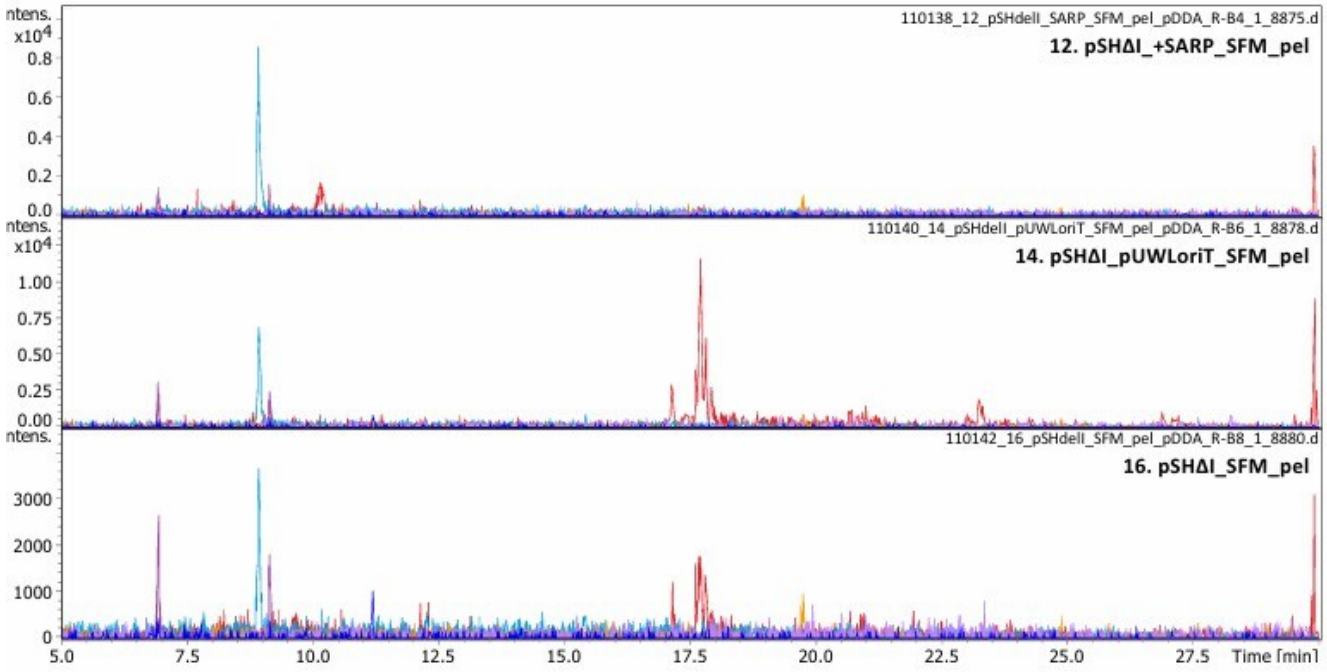

Figure 30: LC-MS comparison of three modified S. coelicolor M1154 strains cultivated in SFM.

line 1: pSH-ΔmfqI_SARP_SFM_pellet

line 2: pSH-ΔmfqI_pUWLoriT_SFM_pellet

line 3: pSH-ΔmfqI_SFM_pellet.

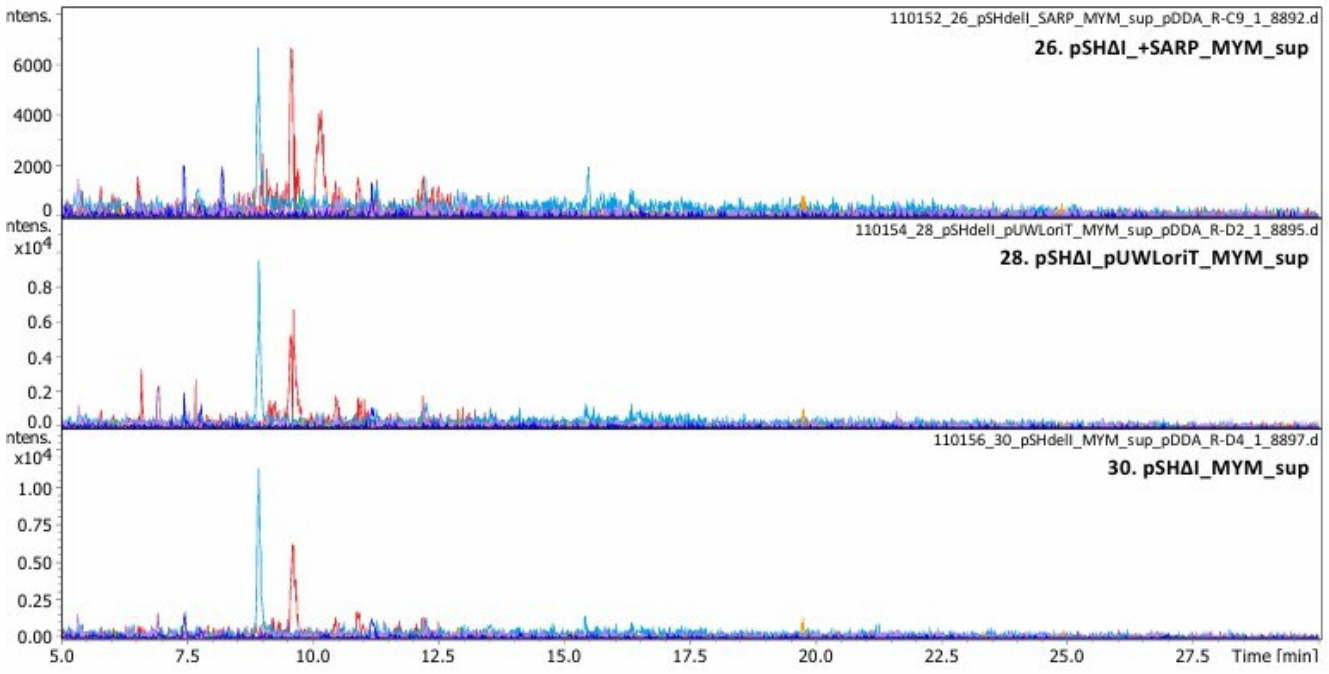

Figure 31: LC MS compraison of three engineered S. coelicolor M1154 strains cultivated in MYM.

line 1: pSH-ΔmfqI_SARP_MYM_supernatant

line 2: pSH-ΔmfqI_pUWLoriT_MYM_supernatant

line 3: pSH-ΔmfqI_MYM_supernatant

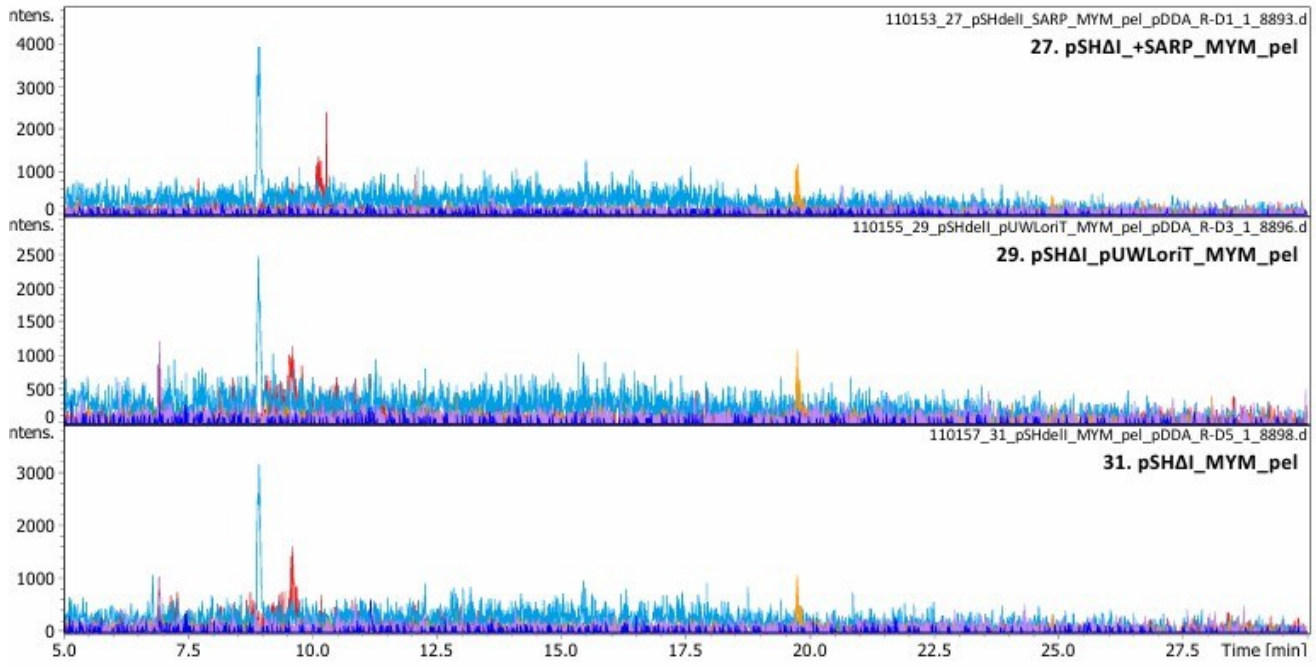

Figure 32: LC-MS comparison of three engineered S. coelicolor M1154 strains cultivated in MYM.

line 1: pSH-ΔmfqI_SARP_MYM_pellet

line 2: pSH-ΔmfqI_pUWLoriT_MYM_pellet

line 3: pSH-ΔmfqI_MYM_pellet