universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Activation of secondary metabolites biosynthetic gene clusters from *Streptomyces* strains isolated in the Ethiopian desert for discovery of new biologically active compounds“

verfasst von / submitted by

Mag. Michael Maier

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

Magister der Pharmazie (Mag.pharm.)

Wien, 2022 / Vienna, 2022

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Univ.-Prof. Dr. Sergey Zotchev, PhD

Table of Contents

1. Zusammenfassung

Gesundheitsbehörden weltweit warnen vor der steigenden Gefahr von Antibiotika Resistenzen, was letztlich dazu führen könnte, dass antibiotische Arzneistoffe ihre Wirksamkeit verlieren. Im wesentlichen wurde beobachtet, dass alle Antibiotika die heute klinisch eingesetzt werden, von dieser Entwicklung betroffen sind und daher die Notwendigkeit besteht neue Antibiotika zu erforschen und zu entwickeln um so eine Gesundheitskrise abzuwenden.

Die Erfahrungen, die über ein Jahrhundert Arzneimittel Entwicklung gesammelt wurden, zeigen, dass Naturstoffe, insbesondere jene, die aus Bakterien und Pilzen isoliert werden konnten, einen vielversprechenden Ausgangspunkt für die Entdeckung und Entwicklung antibiotischer Arzneistoffe darstellen. *Actinobacteria*, insbesondere *Streptomyces* spp., haben in der Vergangenheit bewiesen, dass sie in der Lage sind eine große Anzahl an antibiotischen Substanzen zu produzieren.

Moderne DNA Sequenzierungstechniken und bioinformatische Methoden haben gezeigt, dass das genetische Potential von *Streptomyces* spp. Antibiotika zu produzieren noch nicht ausgeschöpft wurde. Eine große Anzahl an in biosynthetischen Genclustern (BGCs) organisierten Genen, die die enzymatische Maschinerie kodieren, die für die Produktion von antibiotisch wirksamen Sekundärmetaboliten notwendig ist, wird unter Laborbedingungen jedoch nicht exprimiert.

Das Ziel dieses Projekts war es biosynthetische Gencluster für Sekundärmetabolite aus *Streptomyces* sp. Ru-355 und *Streptomyces* sp. Go-475, die aus in der äthiopischen Wüste gewonnenen Bodenproben isoliert worden waren, durch Genome Mining zu aktivieren um neue biologisch aktive Verbindungen zu entdecken.

In einem ersten Schritt wurden die Bakterienstämme auf unterschiedlichen Nährmedien kultiviert um festzustellen welches Substrat am besten geeignet ist Sporenbildung zu induzieren. Danach wurden die Stämme verschiedenen Antibiotika ausgesetzt um sie auf mögliche Antibiotika Resistenzen zu untersuchen. Den Ergebnissen entsprechend konnte ein geeignetes Vektor Plasmid ausgewählt werden, das ein entsprechendes Resistenzgen enthielt. Dem folgte ein Pilotexperiment, im Zuge dessen das leere Vektor Plasmid in die *Streptomyces* Stämme konjugiert wurde.

Anschließend wurden die bakteriellen Genome mithilfe der Genome Mining Software antiSMASH analysiert um BGCs zu identifizieren, die Gene enthalten, die der Lage sein könnten interessante biologisch aktive Verbindungen zu produzieren. Im weiteren Ablauf

konnten regulatorische Gene innerhalb der BGCs lokalisiert werden, die möglicherweise die jeweiligen Cluster aktivieren. Anschließend wurden DNA Primer designt um diese regulatorischen Gene zu amplifizieren.

Genomische DNA wurde isoliert und die regulatorischen Gene mittels PCR amplifiziert. Sowohl die PCR Produkte als auch das isolierte Vektor Plasmid wurden mit Restriktions-Enzymen geschnitten und anschließend ligiert um ein rekombinantes Vektor Plasmid für die Konjugation in die *Streptomyces* Stämme zu generieren. Die Vektoren wurden mittels Restriktions-Analyse verifiziert und in *E.coli* ET12567 transformiert um anschließend in *Streptomyces* sp. Go-475 konjugiert zu werden und dort den jeweiligen BGC zu aktivieren.

Die rekombinanten Vektor Plasmide konnten nicht erfolgreich in *Streptomyces* sp. Go-475 konjugiert werden. Nach einer erfolgreichen Konjugation in *Streptomyces albus* J1074 wurden die rekombinanten Stämme in verschiedenen Medien fermentiert. Die methanolischen Extrakte aus diesen Medien wurden auf antibiotische Aktivität nach einer möglichen BGC Aktivierung untersucht.

Nachdem eine antibiotische Aktivität gegen *Bacillus subtilis* detektiert werden konnte, wurde die relevanten Extrakte weiteren Anaylsen mittels HPLC und MS unterzogen um die produzierten antibiotischen Substanzen weiter aufzuklären und zu charakterisieren. Dies führte zur Identifikation neuartiger Derivate von Deferoxamin und eines weiteren Peptids.

2. Abstract

World health authorities warn of the threat of emerging antibiotic resistance that could eventually render antibiotic drugs ineffective. Basically, all antibiotics used in the clinic today have been found to be affected by this development and there is urgent need to find and develop new antibiotics in order to avert a health crisis.

Experience from a century of drug development has shown that natural products, especially those isolated from bacteria and fungi, present a promising starting point for antibiotic drug discovery and development. Gram-positive *Actinobacteria,* in particular *Streptomyces* spp., have been found to produce a large number of antibiotic substances in the past.

Modern DNA sequencing techniques and bioinformatic tools have shown that the genomic potential for *Streptomyces* spp. to produce antibiotics has not been exhausted. A great number of genes encoding the enzymatic machinery to produce antibiotic secondary metabolites, that are organized in biosynthetic gene clusters (BGCs), are not expressed under lab conditions.

The aim of the project was to activate secondary metabolites biosynthetic gene clusters of *Streptomyces* sp. Ru-355 and *Streptomyces* sp. Go-475 that had been isolated from soil samples collected in the Ethiopian desert by genome mining in order to discover new biologically active compounds.

In a first step the bacterial strains were grown on different media in order to establish the substrate best suited to promote spore formation. Subsequently, the strains were subjected to different antibiotics in order to test for antibiotic resistances. According to the results a vector plasmid containing a suitable resistance gene could be selected. This was followed by a pilot experiment, in which the empty vector plasmid would be conjugated into the *Streptomyces* strains.

Then the bacterial genomes were analyzed using the genome mining software antiSMASH in order to identify BGCs which contained genes that might be able to produce interesting biologically active compounds. Moreover, regulatory genes could be located within these BGCs that might be able to activate the respective clusters. Subsequently, DNA primers were designed to amplify these regulatory genes.

Genomic DNA was isolated, and the regulatory genes were amplified using PCR. Both the PCR products and the isolated vector plasmid were cut using restriction enzymes and ligated to construct a recombinant plasmid vector for conjugation to *Streptomyces* strains. Subsequently, the vectors were verified by restriction analysis and transformed to

E.coli ET12567 to be conjugated to *Streptomyces* sp. Go-475 in order to activate the respective BGC.

The recombinant vector plasmids could not be successfully conjugated to *Streptomyces* sp. Go-475. After successful conjugation to *Streptomyces albus* J1074 the recombinant strains were fermented in different media. The methanolic media extracts were tested for antibiotic activity on a series of different test organisms after the possible activation of the BGCs.

After antibiotic activity against *Bacillus subtilis* could be detected the relevant extracts were eventually subjected to further analysis using HPLC and MS to further elucidate and characterize the biologically active compounds produced. This resulted in the identification of novel derivatives of deferoxamine and an additional peptide.

3. Introduction

Antibacterial drugs are one of the most important groups of pharmaceuticals used today. Throughout human history diseases caused by pathogenic microbes have been a serious problem for human health and societies and were responsible for many deaths and suffering. Pathogenic bacteria cause serious diseases including pneumonia, skin infections (*Staphylococcus aureus*), the plague (*Yersinia pestis*), tetanus (*Clostridium tetani*), gonorrhoea (*Neisseria gonorrhoeae*), syphilis (*Treponema pallidum ssp. pallidum*), chlamydia (*Chlamydia trachomatis*), leprosy (*Mycobacterium leprae*), tuberculosis (*Mycobacterium tuberculosis*), cholera (*Vibrio cholerae*) and pertussis (*Bordetella pertussis*) just to name a few.

Fortunately, the seminal discoveries of Paul Ehrlich, Gerhard Domagk, Alexander Flemming, Ernst Boris Chain, Howard Florey, Selman Waksman and many other scientists resulted in the availability of the first antibiotic drugs such as penicillins, sulfonamides and aminoglycosides in the first half of the 20th century. After further research and discovery of even more antibiotic drugs the world entered the golden age of antibiotics in the 1960-70s. It appeared that the fight against infectious disease had been won.

Unfortunately, the most basic process in the natural world has put an end to the golden age of antibiotics: evolution. Due to the relatively short time, minutes to hours, needed for bacteria to duplicate, several years have been sufficient time for bacteria to adapt to an environment flooded with antibiotic drugs. Over-prescriptions by doctors, the unwarranted use of broad-spectrum antibiotics, the extensive use in agriculture, low environmental standards in factories and poor patient compliance have accelerated the emergence of strains resistant to commonly used antibiotics.

By the second decade of the 21st century the world has come under the severe threat of entering a post-antibiotic age. After decades without great fear of infectious diseases “we now live in an era when people around the world […] are dying from untreatable infections because of the emergence and spread of antibiotic resistance.” (Centers for Disease Control and Prevention (U.S.), 2019)
The challenge of this century will not only be to use the antibiotic drugs still available to us with great care. Moreover, it will be necessary to raise public awareness of the problematic situation and to implement policies that incentivise research. (Dodds, 2017) It will be of utmost importance to identify and research new antibiotic substances with new mechanisms of action to keep up in the arms race with ever evolving pathogens around us.

3.1. Mechanisms of action and resistance

This section will briefly present different classes of antibiotic drugs derived from natural sources that are widely used to combat infectious disease today.

3.1.1. Mechanisms of action

Antibiotic drugs need to exert selective toxicity in bacteria. Thus, they need to bind to molecular targets that are essential for bacterial cells but absent in human cells in order not to harm the patient. There are several mechanisms of action that can achieve this goal to a certain extent.

3.1.1.1. Inhibition of cell wall synthesis

Beta-lactam antibiotics such as penicillins, cephalosporins, carbapenems and monobactams could be isolated from bacteria and fungi like *Penicillium notatum*, *Acremonium chrysogenum, Streptomyces cattleya, Pseudomonas acidophila* and *Gluconobacter sp..*

These substances target bacterial peptidoglycan synthesis by inhibition of penicillin binding proteins (PBPs) such as the enzyme D-alanine-transpeptidase, an enzyme essential for the cross-linking of peptides during cell wall synthesis. Without cell wall integrity bacterial cells burst due to osmotic pressure.

Glycopeptides like vancomycin and teicoplanin intervene in bacterial murein synthesis as well. Vancomycin was first isolated from *Amycolatopsis orientalis* in 1956. The fact that these large molecules do not target transpeptidases but their substrate, the D-Ala-D-Ala peptide C-terminus, makes them the drugs of choice against MRSA and other beta-lactam resistant gram-positive bacteria. (Aktories and Forth, 2013)

3.1.1.2. Inhibition of protein synthesis

Macrolide antibiotics such as erythromycin inhibit protein synthesis by binding to the 50S ribosomal subunit disrupting nascent chain elongation by blocking the ribosomal exit tunnel. (Wilson, 2009) Erythromycin could first be isolated from *Saccharopolyspora erythraea* and was introduced as a drug in the 1950s.

Tetracyclines interfere with protein synthesis by blocking the acceptor position on the 30S subunit, where aminoacyl-tRNAs bind to the ribosome. The first members of this class were isolated from *Streptomyces* strains in the 1940s e.g. chlortetracycline from *Streptomyces aureofaciens*.

Chloramphenicol inhibits protein synthesis by binding to the ribosomal peptidyl transferase center (PTC) on the 50S ribosomal subunit. It was first isolated in 1949 from *Streptomyces venezuelae.* It shows activity against a broad spectrum of pathogenic bacteria. (Wilson, 2009)

Lincosamides similarly inhibit translation by binding to the PTC site and lead to bacteriostasis. Lincomycin A was isolated from *Streptomyces lincolnensis* in the 1960s.

Aminoglycosides interfere in the protein synthesis of bacterial cells by binding to the 30S subunit of ribosomes. Streptomycin was the first member of this class described in 1944. The substance had been isolated from *Streptomyces griseus*.

3.1.1.3. Inhibition of RNA-polymerase

The naturally occurring antibiotic rifamycin could first be isolated from *Amycolatopsis mediterranei* and inhibits bacterial RNA-polymerase. Its semisynthetic derivative rifampicin was introduced in 1967 and is used in a combination therapy to treat tuberculosis.

3.1.1.4. Disruption of biomembrane integrity

The cyclic lipopeptide Daptomycin was discovered in 1986 and introduced in 2003. The substance is a secondary metabolite of *Streptomyces roseosporus*. By incorporation into the bacterial membrane the compound disrupts biomembrane integrity and thus exerts its bactericidal effect.

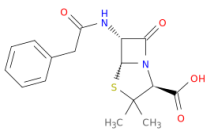
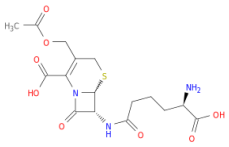
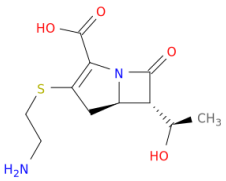
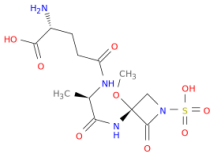
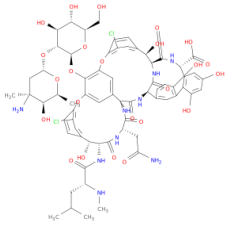
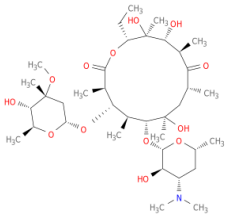
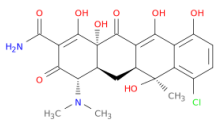
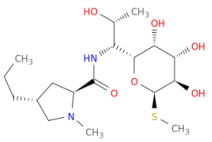
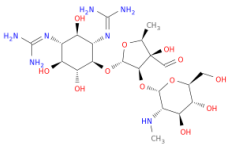
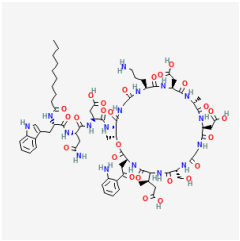
 Benzylpenicillin	 Cephalosporin C	 Thienamycin	 Sulfazecin	 Vancomycin
 Erythromycin	 Chlortetracycline	 Lincomycin	 Streptomycin	 Daptomycin

Fig. 1.: Antibiotic drugs isolated from fungal and bacterial sources. Structure information was obtained from https://pubchem.ncbi.nlm.nih.gov.

3.1.2. Modes of bacterial resistance

Antibiotics used in modern antibacterial therapy are subjected to mechanisms of resistance. Pathogenic bacteria have evolved over millions of years to survive exposure to antibiotic molecules by modification of the antibiotic, decreasing its cellular uptake, expressing efflux pumps and by modification of the target structure. The modes of bacterial resistances can be classified into two categories.

3.1.2.1 Intrinsic resistance

Certain bacteria are inherently not susceptible to certain antibiotics due to their biomolecular constitution. For example, gram-negative bacteria can resist cyclic lipopeptides, oxazolidinones and glycopeptides by not allowing diffusion of the compounds through their outer biomembrane.

3.1.2.2. Acquired resistance

Resistances can be acquired, either by spontaneous mutations in the genome that are favoured by selection pressure and passed on to the next generation via vertical gene transfer. It has also been found that the use of antibiotics in a low concentration can trigger cellular responses that promote the rate of spontaneous mutations. (Rodríguez-Rojas et al., 2013) This insight should have clear implications for the clinical practice so that antibiotics are not applied at a sub-therapeutic level which would further increase the incidence of bacterial resistances.

Another route of acquiring resistance is by means of horizontal gene transfer. (Blair et al., 2015) There are three mechanisms by which exchange of genetic information between bacteria can occur: transformation, transduction and conjugation. It has been observed that the main mechanism of dissemination of resistance genes among pathogenic bacteria of different species has been the conjugation of resistance plasmids. (Carattoli, 2013)

3.1.2.3. The resistome

All genetic information countering the effects of antibiotics is a product of evolution. As has been shown a great number of antibiotics or their precursors are produced by microorganisms. These secondary metabolites are produced to fend or kill off other microorganisms that are competitors for resources. It is however not surprising that counterstrategies have evolved a long time ago that render these chemical weapons ineffective. Consequently, many of these resistance genes will not only be found in pathogenic bacteria isolated from the tissue of patients but also in soil bacteria isolated from the environment. (Forsberg et al., 2012)

For every new antibacterial compound developed there is a distinct possibility that sooner or later a pre-existing gene encoding a mechanism of resistance will emerge and find its way to a pathogenic bacterial strain. Not only soil bacteria but also commensal bacteria can act as a

reservoir for resistance genes that can be transferred to pathogens. It is therefore essential to decrease the spreading of antibiotics and, consequently, resistant bacteria into the environment. Otherwise, commensal or facultative pathogenic bacteria will eventually share their resistance genes with pathogens.

Since antibiotic compounds have been used by humanity extensively and in large quantities over the last hundred years, these evolutionary mechanisms of adaptation have been accelerated. (Davies and Davies, 2010; Rodríguez-Rojas et al., 2013) It is time to take decisive measures in order not to lose the arms race against resistant pathogens.

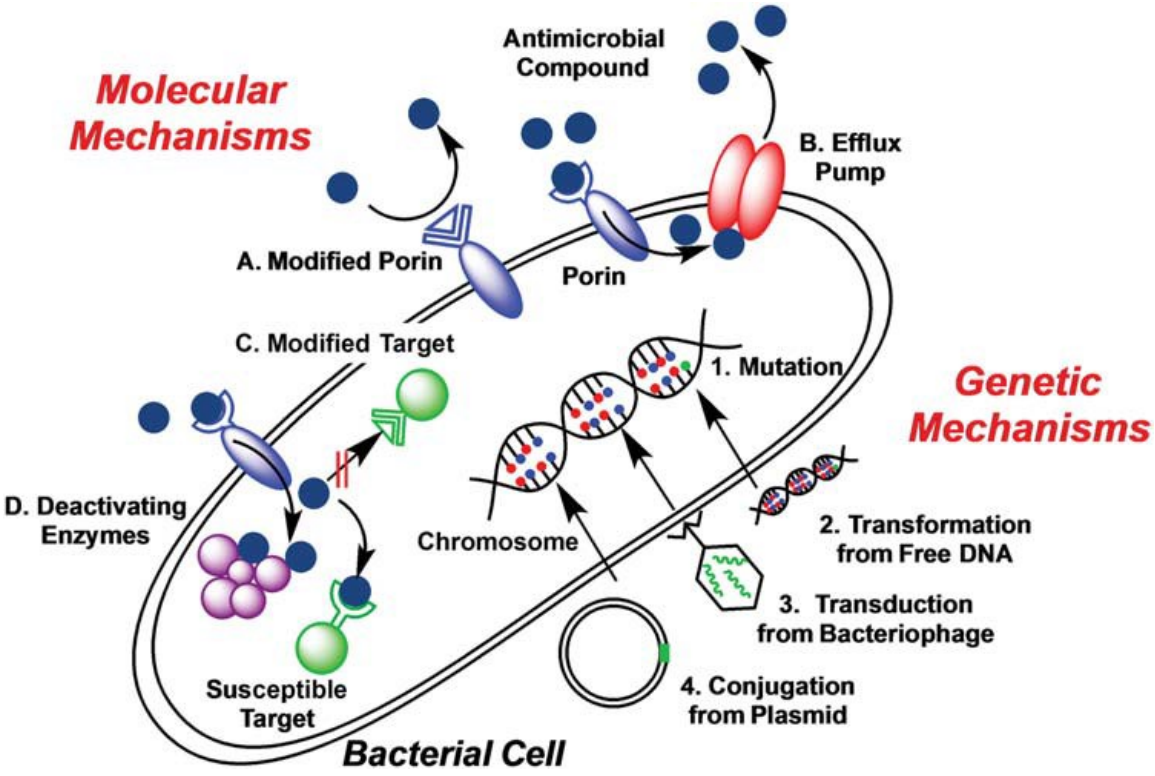


Fig. 2: Mechanisms of antibiotic resistance (Walsh, 2016)

3.1.3. Strategies and solutions

There are several pathways to tackle the problem of antibiotic resistance. The most important step to slow down the emergence of resistant pathogens would be to restrict and highly reduce the use of antibiotics both in agricultural and medical settings.

Moreover, antibiotics have been mostly used as stand-alone therapies. This may have caused the development of resistances, since the pathogens had only to adapt against one antibiotic agent. However, combination therapy is already used in treating tuberculosis or eradicating *Helicobacter pylori* infections. Thus, a possible solution to the development of new resistant strains would be to use several antibiotics with different modes of action simultaneously.

Previous successes and recent developments in the field of vaccinations illustrate that the most potent agent against pathogens is the human immune system. Therefore, the best prevention strategy against bacterial infection and disease would be the development of vaccines. Especially now, with the availability of novel technologies such as vector- and mRNA/DNA vaccines this may be a promising route to take. However, since bacterial surface antigens are changing rapidly very few vaccines against bacterial infections have been developed successfully so far.

Another strategy employed is the development of antibiotic adjuvants to complement established antibiotic therapies. These include beta-lactamase inhibitors, efflux pump inhibitors and compounds that increase cell membrane permeability for antibiotics. (Douafer et al., 2019) While it is a promising strategy to combine adjuvants with antibiotics, resistances against combinations of beta-lactams/beta-lactamase-inhibitors have already been described. (Ripoll et al., 2014) However, the combination of synergistic substances is an effective measure to keep a number of resistant bacterial strains under control a little longer.

Moreover, phage therapy, the possibilities of employing antimicrobial-peptides and anti-virulence compounds in antibacterial therapy have been investigated. (Pacios et al., 2020) Although these strategies provide some interesting approaches, issues of bioavailability, toxicity and immunogenicity are still to be dealt with. Yet, these approaches might indeed have an impact in special cases.

The fact remains that there is still an unmet need for novel antibiotics to treat infections. Research and development of new antibiotics in the past decades has predominantly relied on the molecular modification of chemical scaffolds of already established antibiotics. By this strategy the spectrum of effectiveness of certain classes could be increased and some resistance mechanisms circumvented. However, the number of possible chemical modifications is limited, and the occurrence of multi-drug-resistant bacterial strains is on the

rise. It is therefore necessary to obtain novel compounds with new scaffolds attacking new molecular targets. Experience of the past has shown that a promising place to look for them is nature. As illustrated in the previous section a large number of antibiotic compounds that have proven to be effective therapeutic drugs are natural products or derivatives thereof.

3.2. Drug discovery from nature

Nature has been providing human societies with remedies to diseases for thousands of years. A great number of drugs used in modern evidence-based medicine are compounds produced by living organisms or were inspired by them. (Cragg and Newman, 2013)
This section will provide a brief overview over natural products used in modern medicine.

The largest group of natural products that have been developed into pharmaceutical drugs originate from small molecules isolated from the kingdom of plants. Plant compounds like salicylic acid, morphine, scopolamine, ephedrine, paclitaxel, vinca alkaloids, colchicine and digoxin are still used today in their native form. Other active substances like acetylsalicylic acid, oxycodone, chloroquine, butylscopolamine and artemether are derivatives of naturally occurring compounds with improved pharmacological properties. In the case of small compounds these improvements can be achieved by minor chemical modifications like oxidations, acetylations or alkylations, while the compound scaffold remains intact.

A small number of pharmaceutical drugs were also inspired by peptides found in the animal kingdom, especially toxins. Ziconotide, a cyclic peptide found in the toxin produced by the marine organism conus magus, is used as a potent analgesic. The first ACE-inhibitor drug captopril was inspired by peptides found in the venom of the viper *Bothrops jararaca* while the GLP-1 analogue exenatide was discovered in the saliva of *Heloderma suspectum*, a lizard.

However, the organisms that provide the most abundant resource of pharmacologically active compounds are bacteria and fungi. Statins, immunosuppressants, anticancer drugs, antimycotics and the variety of antibacterial drugs mentioned earlier have been derived from compounds taken from bacterial and fungal sources. (Atanasov et al., 2021) While antibiotics derived from plants and animals are mostly peptides and terpenes, fungi and bacteria produce more chemically diverse compounds. (Zotchev, 2008) In comparison to plant natural products these compounds are larger, some of them peptides, and are more difficult to synthesize in the chemical lab. For this reason, many of them are biotechnologically produced, isolated and then chemically modified. Alternatively, if the biosynthesis pathways are understood, the producing microorganisms can be provided with modified precursor molecules that are then integrated into the secondary metabolite. Moreover, modern genomics enable scientists to even modify the enzymes responsible for the biosynthesis of the metabolites in order to optimize the properties of the product.

It can be argued that all these compounds have been optimized over millions of years by evolution through mutation and selection processes to serve their specific purpose. (Walsh and Fischbach, 2010) It has turned out that even though we might not fully understand the reason why organisms produce these compounds, they are of immense value to us, if these biologically active lead structures are optimized for our purposes using modern methods of chemistry, genetics and biotechnology. (Bérdy, 2012)

Natural products used for pharmaceutical purposes isolated from organisms are usually secondary metabolites. Their precursors are metabolites of primary metabolic pathways such as sugars, amino acids, shikimic acid, mevalonic acid or methylerythritol phosphate. In contrast to primary metabolites, secondary metabolites are not essential for cell growth and homeostasis, yet they have important functions regarding the interactions of the organism with its environment, most of them not yet entirely understood. These molecules are used for signalling throughout the ecosystem, communication among organisms and, as for antibiotics, chemical warfare between them.

3.2.1. *Streptomyces* spp.

Many microbial natural products with antibiotic activity have been isolated from *Streptomyces* spp. (Walsh, 2016) These are gram-positive aerobic filamentous soil dwelling *Actinobacteria* that have linear chromosomes with high GC-content. As resources become scarce the bacterial mycelium forms aerial filaments (hyphae) that eventually produce spores, called conidia. (Madigan et al., 2022) “[This] onset of morphological differentiation usually coincides with the production of secondary metabolites”. (Romero-Rodríguez et al., 2015) What makes them interesting for pharmaceutical research is the fact that *Streptomyces* spp. are well known producers of a large number of secondary metabolites that are used as immunosuppressants, anti-tumor, anti-fungal and anti-bacterial agents. (Paradkar et al., 2003; Zotchev, 2008) Moreover, it has been predicted that *Streptomyces* spp. might still yield plenty of yet undiscovered compounds. (Watve et al., 2001)

After the initial success of natural products isolated from *Streptomyces* strains, it became evident that no more novel bioactive compounds could be detected in strains that could be cultivated under laboratory conditions. Another problem faced by researchers was the high rate of rediscovery of already known compounds. However, advances in the field of genetics and biotechnology have opened new routes and possibilities. (Zarins-Tutt et al., 2016)

3.2.2. Silent/cryptic biosynthetic gene clusters

The genomic era has provided new methods of investigating the chemical potential of microorganisms. By 2021 hundreds of *Streptomyces* genomes have been sequenced. The availability of genome sequences now allows researchers to easily identify biosynthetic gene clusters using bioinformatics tools. (Blin et al., 2021) It has been shown that the genes encoding the information necessary to produce the enzymatic machinery, that is used to synthesize and export secondary metabolites, form clusters in the organism's genome. (van Wezel and McDowall, 2011) These biosynthetic gene clusters (BGCs) are composed of genes coding for proteins responsible for scaffold assembly, scaffold modification, transport of and resistance against the secondary metabolite. (Zotchev, 2008) It has been shown that *Streptomyces* spp. can produce a large number of pharmaceutically interesting secondary metabolites that are polyketides or non-ribosomal peptides that are encoded in these BGCs. These metabolites are the secret treasures modern drug discovery from nature tries to find.

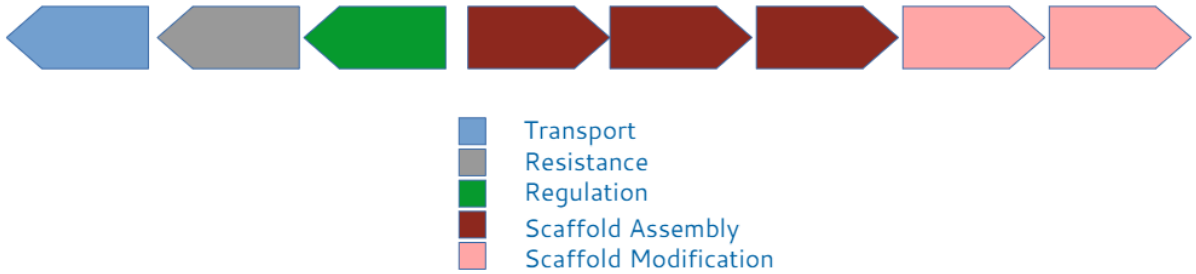


Fig. 3: Secondary metabolite biosynthetic gene cluster after Zotchev, 2008

Analyses have shown that up to 53 secondary metabolite biosynthetic gene clusters can be detected in *Streptomyces* genomes. (Ward and Allenby, 2018) However, it has become clear that many of these secondary metabolites are not produced, since their respective gene clusters are silent under the laboratory conditions. In the case of cryptic gene clusters, the secondary metabolites that could possibly be synthesized have not yet been identified. (Hoskisson and Seipke, 2020)

Since the production of these secondary metabolites and the expression of the enzymatic machinery responsible are energy-intensive processes, the expression of the encoding genes is tightly regulated responding to external signals from their natural environment. (Rutledge and Challis, 2015) These regulatory networks can be highly complex and are still not entirely studied and understood. However, several approaches have been developed to identify and to activate those silent BGCs in the lab and elicit the compounds of interest.

3.3. Genome mining

In the genomic era drug discovery from bacterial sources can be conducted by means of genome mining. One possible route of genome mining is to isolate new bacterial strains from environmental samples and to sequence their genomes. Subsequently, these genomes are analyzed using bioinformatics tools to identify secondary metabolite biosynthetic gene clusters of interest. The great challenge today is to activate these gene clusters in order to be able to characterize the secondary metabolites encoded by them. If the conditions are favourable these compounds are produced by bacterial cultures in the lab and subsequently extracted and tested for bioactivity. Bioactive extracts will then be chromatographically separated and fractionated to eventually isolate and characterize the natural product mediating bioactivity. Eventually these compounds might be developed further to become new pharmaceuticals.

3.3.1. BGC identification

Based on the information available on secondary metabolites biosynthetic pathways several bioinformatics tools, such as antiSMASH (Blin et al., 2021), have been developed to predict compounds produced by the enzymatic machinery encoded in their respective gene clusters.

BGCs are predicted from genomic and metagenomic sequence data, grouped into families, predicting the class and sometimes even a structure of a BGC's small molecule product, and gene cluster and molecular families are connected using networking approaches. (Medema and Fischbach, 2015)

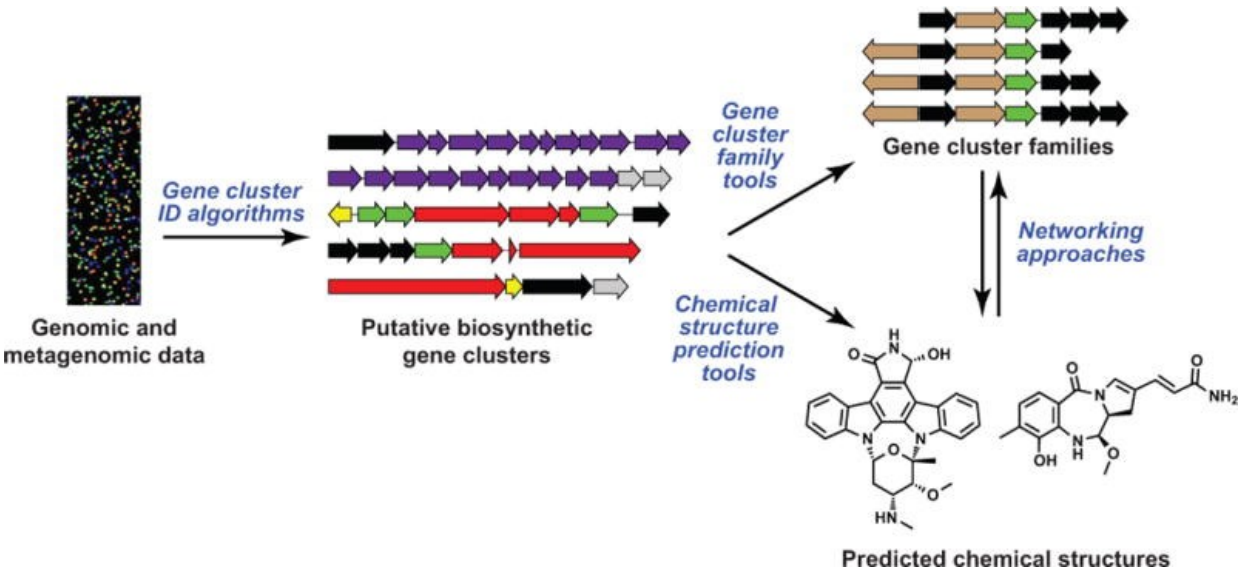


Fig. 4.: The role of computation in natural product discovery (Medema and Fischbach, 2015)

3.3.2. BGC activation

3.3.2.1 Classic pleiotropic approaches

The microbial metabolome can be influenced by the variation in growth conditions and signalling molecules. In its natural environment microorganisms respond and adapt to environmental triggers by activation or repression of expression of certain gene products (Zarins-Tutt et al., 2016).

Fermentation in different media is one approach to elicit the metabolic potential from *Streptomyces* bacteria. This strategy could already be employed in the pre-genomic era to elicit or optimize the yield of natural products produced by *Streptomyces* spp., since no prior knowledge of the BGCs was required. Although it is not clear which media compounds by their inclusion or exclusion trigger the production of secondary metabolites, the cultivation in different fermentation media may produce different extract compositions and is still a viable strategy in drug discovery. (Zarins-Tutt et al., 2016) However, it has been shown that variation of growth media alone has not been successful in yielding novel secondary metabolites in laboratory settings. Consequently, it is a reasonable approach that fermentation media variation be combined with other methods.

By now it is well understood that soil bacteria do not live in isolation. (Frey-Klett et al., 2011) They interact with a plethora of other bacteria, fungi or plants. They may even live in close symbiosis with their hosts as endophytes. Since secondary metabolites are a means to communicate with or react to these organisms in the natural ecosystem, it can be viable to emulate these interactions in the lab by co-cultivation of these species. (Nicault et al., 2021) Co-cultivation may not only trigger the activation of silent BGCs but also increase the yield of secondary metabolites. (Zarins-Tutt et al., 2016)

Another pleiotropic approach is to expose the microorganisms to a variety of specialised metabolites that can act as signalling molecules. These include antibiotics in sub-inhibitory concentrations, and auto-regulatory chemical compounds such as furans, gamma-butenolids and γ-butyrolactones. Such autoregulatory-signalling molecules might target regulators of the *TetR*-family and other repressors. (Niu et al., 2016) The exposure of bacterial strains to such compounds can also be used for the high-throughput elicitor screening approach (HiTES) which allows quick testing against large natural product libraries using reporter genes. (Kong et al., 2019)

Altering the transcriptional and translational machinery provides another approach to unlock the production of new secondary metabolites. This can be achieved by induction of point mutations in genes encoding RNA-polymerases and ribosomal RNA and proteins. The selection of mutant colonies is induced by exposing *Streptomyces* strains to high concentrations of antibiotics that target these enzymes. (Ochi and Hosaka, 2013)

Manipulation of global regulators and pleiotropic regulators that are situated outside BGCs presents the possibility to influence a great variety of genes situated throughout the genome. Global regulators influence various aspects of secondary metabolite synthesis, such as the supply of precursor molecules and the expression of biosynthetic enzymes. Pleiotropic regulators exert control over the expression of pathway-specific regulators such as PhoP, AfsR and AdpA. (Wang et al., 2012)

These methods predominantly rely on serendipity. Recent advances in the understanding of biosynthetic pathways and their regulatory networks have provided the possibility to complement and combine these classic methods with novel pathway-specific approaches.

3.3.2.2. Modern pathway-specific approaches

In principle, there are two novel modes of silent BGC activation that have been described in the genomic era. It has been shown that the regulation of gene expression can be overridden in the original bacterial host. Alternatively, the regulatory system can be bypassed entirely by genetically reengineering regulatory elements in heterologous bacterial hosts. (Ren et al., 2017)

3.3.2.2.1. Homologous expression

In order to activate a secondary metabolite BGC in the original host, cluster-situated regulators (CSRs) can be manipulated. Cluster-situated activator genes need to be overexpressed, while repressor genes need to be silenced.

3.3.2.2.1.1. Overexpression of positive regulators

Several regulatory gene families have been successfully overexpressed in *Streptomyces* strains to activate silent antibiotic BGCs. For example, *Streptomyces* antibiotic regulatory proteins (SARPs) are well known to trigger the production of secondary metabolites in *Streptomyces* spp. directly. (Wei et al., 2018)

Moreover, regulators of the LALs (Large ATP-binding regulators of the LuxR family) are often found to regulate BGCs and are thus an interesting target for overexpression.

A viable method to homologously overexpress such positive regulatory genes in the host is to put these pathway specific activator genes under the control of a constitutive promoter like *ermE*p* and to integrate them as a second, constitutively expressed, copy into the *Streptomyces* genome using an integrative vector. (van Wezel and McDowall, 2011)

It could be shown that the overexpression of *papR2*, a SARP regulatory gene, activated the transcription of a BGC in *Streptomyces* sp. SHP22-7, a strain isolated from a soil sample of the desert island Enggano in Indonesia. The activation resulted in the production of plicacetin , a nucleoside antibiotic showing both antibacterial and antiviral activity. (Krause et al., 2020)

The overexpression the positive regulator *samR0484,* a member of the LAL regulators group, the activation of the previously silent BGC in *Streptomyces ambofaciens* ATCC 23877 could be achieved. This resulted in the production of stambomycins, macrolide polyketides exerting cytotoxic effects. (Laureti et al., 2011)

Using a similar approach with the positive regulator *chal*, a silent angucycline-like biosynthetic gene cluster could successfully be activated in *Streptomyces chattanoogensis.* Thus, two angucycline polyketides, chattamycins A and B, could be elicited that have been demonstrated to possess antitumor and antibacterial activity. (Zhou et al., 2015)

3.3.2.2.1.2. Knockout of negative regulators

In contrast, transcriptional regulators can also act as repressors and need to be inactivated to trigger the expression of the BGCs controlled by them. Repressor genes can be deleted, disrupted and replaced by homologous recombination or cut from the genome employing CRISPR/Cas9. (Gust et al., 2004; Chen et al., 2019)

For example, by deletion of the negative regulatory gene encoding the γ-butyrolactone receptor homologue ScbR2 the production of a novel secondary metabolite with antibacterial activity could be induced in *Streptomyces coelicolor* A3(2). (Gottelt et al., 2010)

The *pimM* gene from the pimaricin cluster in *Streptomyces natalensis* that codes for a positive activator of the LuxR-family was expressed in *Streptomyces albus J1074* using an integrative vector under the control of the ermE*p promoter resulting in an increase of antimycins. (Olano et al., 2014)

3.3.2.2.1.3. Knock-in of constitutive promoters

In *Streptomyces albus* J1074 the constitutive promoter ermE*p was inserted upstream of key genes of a BGC coding for the pigment indigoidine leading to its expression bypassing its regulatory system. (Olano et al., 2014)

3.3.2.2.2. Heterologous expression

It has been observed that the production of natural products in native strains faces certain challenges. In many instances slow growth and low product yield pose a problem. In addition, difficulties in the genetic manipulation of BGCs have been encountered. Moreover, it has been found that in most cases secondary metabolite BGCs remain silent/cryptic in their native hosts. However, heterologous expression of entire BGCs or single regulatory genes in optimized and well-studied hosts offers a solution to these problems. (Nepal and Wang, 2019) In recent years, new techniques have been developed to introduce large

fragments of DNA into heterologous hosts that were specifically designed to express heterologous DNA. (Luo et al., 2016; Sekurova et al., 2019)

3.3.2.2.2.1. Expression of entire biosynthetic gene clusters

Such optimized expression hosts for heterologous BGCs like *Streptomyces albus* J1074 and *Streptomyces coelicolor* M1152, M1146 were constructed by deleting non-essential BGCs. Thus, the availability of precursor molecules could be increased.

For example, an entire kocurin BGC discovered in a *Kocuria rosea* strain could successfully be expressed in *Streptomyces coelicolor* M1146 by using the integrative shuttle vector pSET152 placing the BGC under the control of a constitutive promoter. (Linares-Otoya et al., 2017)

Metagenomic analyses have provided a plethora of microbial genomes harbouring secondary metabolite biosynthetic genes not yet investigated. The expression of such BGCs in specialized host systems is of great interest for metagenomic DNA of yet uncharacterized bacterial strains that cannot be cultivated in laboratory conditions. (Sekurova et al., 2019)

However, commonly used shuttle vector plasmids can only carry a limited size of DNA. Fortunately, it is now possible to assemble and clone larger segments of DNA in bacterial artificial chromosomes (BAC) and phage P1-derived artificial chromosome (PAC) vectors. (Baltz, 2016)

The insertion and heterologous expression of small genetic elements such as promoters and regulators also constitute a viable strategy to activate biosynthetic gene clusters already present in the host.

3.3.2.2.2.2. Synthetic biology approaches

With an increasing understanding of biosynthetic pathways and the regulatory networks controlling them it is now possible to combine several successful approaches. In order to bypass the complex regulatory networks controlling gene expression in *Streptomyces spp.*, entire synthetic BGCs have been designed to be expressed in specialized chassis-strains. The refactoring of targeted secondary metabolite BGCs aims to optimize the expression of the enzymatic machinery by introducing constitutive and inducible promoters, suitable terminator sequences, optimized UTRs and RBSs. Moreover, reporter genes can be inserted into BGCs of interest to study the expression of genes of interest. (Lee et al., 2019)

However, such synthetic biology approaches require an extensive knowledge of the relevant BGCs and entail a great amount of time and effort. Similarly, before an entire BGC is heterologously expressed it should be thoroughly studied and characterized. Thus, before undertaking projects of this magnitude, information concerning the regulatory networks and the secondary metabolites of interest should be gathered first to assess its feasibility.

In order to generate the knowledge necessary more basic and versatile methods can be applied.

For a first investigation of novel secondary metabolite biosynthetic gene clusters identified in recently discovered *Streptomyces* strains, a combination of overexpression of positive regulatory proteins and fermentation in different media is a viable approach to take.

4. Aim of this work

Antibiotic resistance has been emerging in pathogenic bacteria over the last decades. This development requires research and development of antibiotic drugs exerting novel mechanisms of action in order to maintain public health. Natural products, particularly obtained from fungal and bacterial sources, and their derivatives have proven to be effective drugs in the past. Genome mining using modern DNA sequencing techniques and bioinformatic tools has unveiled that there is an untapped potential of secondary metabolites encoded in the genomes of *Streptomyces* bacteria that could be a viable starting point for the development of new pharmaceutical drugs. It has been found that genes encoding the proteins responsible for the biosynthesis of these compounds are organised in clusters. The aim of this thesis was to identify and activate such secondary metabolite biosynthetic gene clusters of interest in *Streptomyces* strains isolated in the Ethiopian desert by genome mining in order to elicit and elucidate novel biologically active compounds.

5. Results

5.1. Cultivation of strains on agar medium

In order to establish the optimal growing conditions for sporulation in the lab *Streptomyces* sp. Ru-355 and *Streptomyces* sp. Go-475 were cultivated on three different agar media, ISP2, ISP4 and SFM, over a period of five days. Results of the cultivation are summarized in Tables 1 and 2.

***Streptomyces* sp. Ru-355**

Medium	Results
ISP2	light spores, plate transparent
ISP4	spores darker, higher density than on ISP2
SFM	spores dark/grey, high density, secondary colonies

Table 1: Growing results of *Streptomyces* sp. Ru-355 on different media

***Streptomyces* sp. Go-475**

Medium	Results
ISP2	Plate transparent, production of red pigment
ISP4	spores darker, higher density than on ISP2, little red pigment
SFM	lighter spores, high density, media amount of red pigment

Table 2: Growing results of *Streptomyces* sp. Go-475 on different media

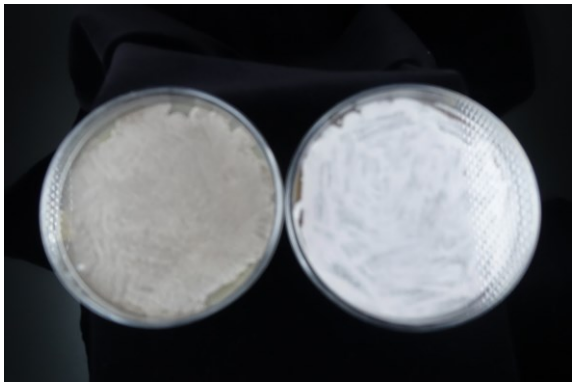


Fig. 5: *Streptomyces* sp. Ru-355 (left) and *Streptomyces* sp. Go-475 (right) on SFM agar

It was established that SFM agar produced the best sporulation results for both *Streptomyces* sp. Ru-355 and *Streptomyces* sp. Go-475 and would be used for the experiments.

5.2. Sensitivity to antibiotics

In order to be able to choose suitable conjugation vectors the sensitivity of *Streptomyces* sp. Ru-355 and *Streptomyces* sp. Go-475 to three antibiotics, thiostrepton, hygromycin and apramycin was investigated by growing the strains on SFM plates with antibiotics for three days. Results are presented in Tables 3 and 4.

***Streptomyces* sp. Go-457**

Antibiotic	**Result**
Apramycin 50µg/ml	No growth
Thiostrepton 30µg/ml	No growth
Hygromycin 100µg/ml	Isolated, scattered colonies

Table 3: Results of *Streptomyces* sp. Go-475 Antibiotic Resistance

***Streptomyces* sp. Ru-355**

Antibiotic	**Result**
Apramycin 50µg/ml	No growth
Thiostrepton 30µg/ml	Growth
Hygromycin 100µg/ml	Isolated, scattered colonies

Table 4: Results of *Streptomyces* sp. Ru-355 Antibiotic Resistance

It could be established that both *Streptomyces* sp. Ru-355 and *Streptomyces* sp. Go-475 were susceptible to the aminoglycoside antibiotic apramycin.
Thus, conjugation vectors containing the apramycin resistance gene (*ampR*) could be used in the following experiments.

5.3. Empty vector pilot experiment

In order to assess if the vectors available and the methods already established for other strains were suitable for the introduction of DNA into *Streptomyces* sp. Ru-355 and *Streptomyces* sp. Go-475, a pilot experiment using empty plasmid vectors was conducted.

The plasmid vectors pSOK806 and pSOK804 were isolated from *Escherichia coli* DH5-Alpha cells (Methods 9.11.). pSOK806 was then transformed to *E.coli* ET-12567 cells (Methods 9.17.).

Subsequently, pSOK806 was successfully conjugated to both *Streptomyces* sp. Ru-355 and *Streptomyces* sp. Go-475, exposing the spores to heat shock at 50°C for 10 minutes.

While conjugation efficiency was good for *Streptomyces* sp. Ru-355, it was rather poor for *Streptomyces* sp. Go-475. The efficiency could be slightly improved by reducing the heat shock temperature to 45°C. (Methods 9.18.).

By conducting conjugations to *Streptomyces* sp. Go-475, heat shock temperature at 45°C for 10 minutes, with plasmid vectors pSOK806, pSOK804 and pSET152 it could be shown that pSOK806 still showed the highest conjugation efficiency.

Thus, it could be established that pSOK806 was the most suitable plasmid vector for further experiments.

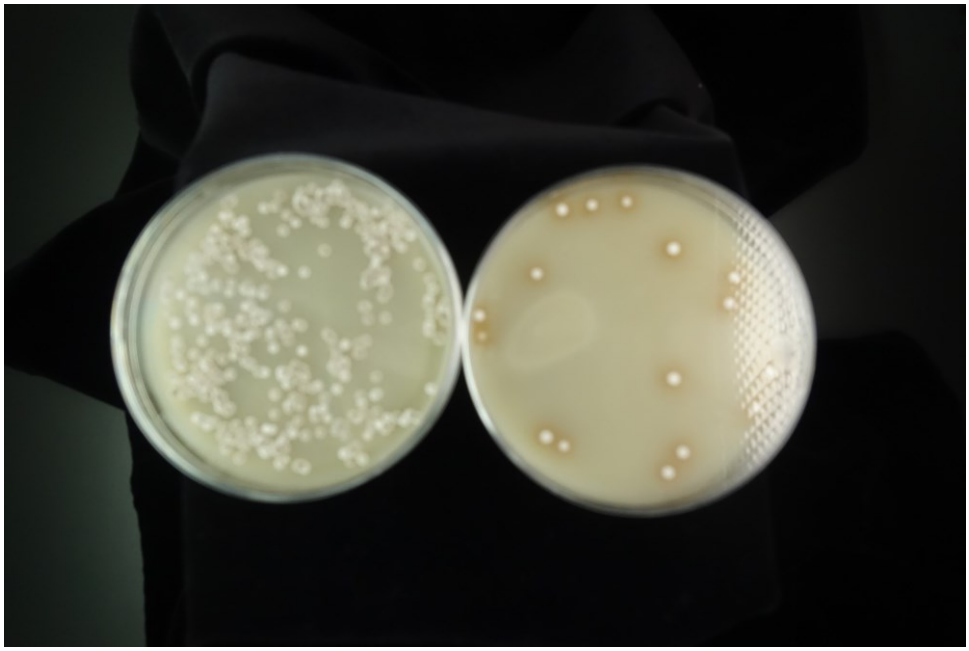
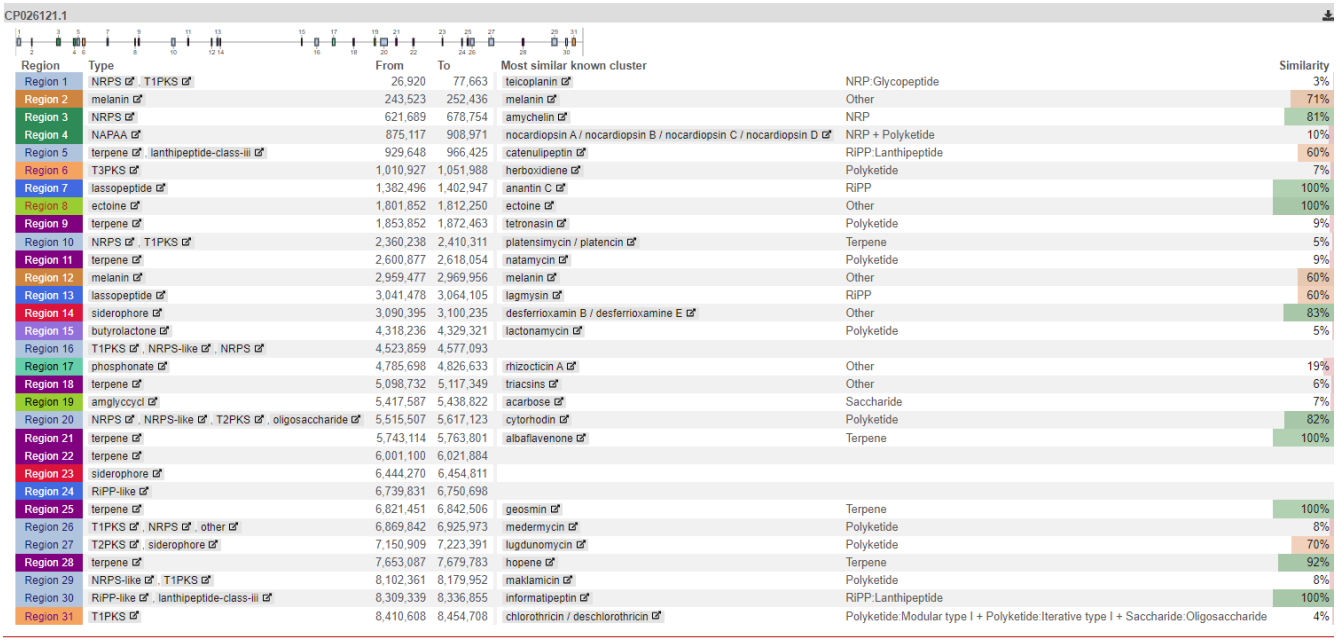


Fig. 6: Transconjugants *Streptomyces* sp. Ru-355_pSOK806 (left)
and *Streptomyces* sp. Go-475_pSOK806 (right)

5.4. BGC identification and analysis

The genomes of *Streptomyces* sp. Go-475 and *Streptomyces* sp. Ru-355 were analyzed using antiSMASH 5.0.0 which resulted in the identification of two biosynthetic gene clusters of interest in *Streptomyces* sp. Go-475.



CP026121.1

Region	Type	From	To	Most similar known cluster		Similarity
Region 1	NRPS , T1PKS	26,920	77,663	teicoplanin	NRP:Glycopeptide	3%
Region 2	melanin	243,523	252,436	melanin	Other	71%
Region 3	NRPS	621,689	678,754	amychelin	NRP	81%
Region 4	NAPAA	875,117	908,971	nocardiopsin A / nocardiopsin B / nocardiopsin C / nocardiopsin D	NRP + Polyketide	10%
Region 5	terpene , lanthipeptide-class-iii	929,648	966,425	catenulipeptin	RiPP:Lanthipeptide	60%
Region 6	T3PKS	1,010,927	1,051,988	herboxidiene	Polyketide	7%
Region 7	lassopeptide	1,382,496	1,402,947	anantin C	RiPP	100%
Region 8	ectoine	1,801,852	1,812,250	ectoine	Other	100%
Region 9	terpene	1,853,852	1,872,463	tetronasin	Polyketide	9%
Region 10	NRPS , T1PKS	2,360,238	2,410,311	platensimycin / platencin	Terpene	5%
Region 11	terpene	2,600,877	2,618,054	natamycin	Polyketide	9%
Region 12	melanin	2,959,477	2,969,956	melanin	Other	60%
Region 13	lassopeptide	3,041,478	3,064,105	lagmysin	RiPP	60%
Region 14	siderophore	3,090,395	3,100,235	desferrioxamin B / desferrioxamine E	Other	83%
Region 15	butyrolactone	4,318,236	4,329,321	lactonamycin	Polyketide	5%
Region 16	T1PKS , NRPS-like , NRPS	4,523,859	4,577,093			
Region 17	phosphonate	4,785,698	4,826,633	rhizocticin A	Other	19%
Region 18	terpene	5,098,732	5,117,349	triacsins	Other	6%
Region 19	amglyccycl	5,417,587	5,438,822	acarbose	Saccharide	7%
Region 20	NRPS , NRPS-like , T2PKS , oligosaccharide	5,515,507	5,617,123	cytorhodin	Polyketide	82%
Region 21	terpene	5,743,114	5,763,801	albaflavenone	Terpene	100%
Region 22	terpene	6,001,100	6,021,884			
Region 23	siderophore	6,444,270	6,454,811			
Region 24	RiPP-like	6,739,831	6,750,698			
Region 25	terpene	6,821,451	6,842,506	geosmin	Terpene	100%
Region 26	T1PKS , NRPS , other	6,869,842	6,925,973	medermycin	Polyketide	8%
Region 27	T2PKS , siderophore	7,150,909	7,223,391	lugdunomycin	Polyketide	70%
Region 28	terpene	7,653,087	7,679,783	hopene	Terpene	92%
Region 29	NRPS-like , T1PKS	8,102,361	8,179,952	maklamicin	Polyketide	8%
Region 30	RiPP-like , lanthipeptide-class-iii	8,309,339	8,336,855	informatipeptin	RiPP:Lanthipeptide	100%
Region 31	T1PKS	8,410,608	8,454,708	chlorothricin / deschlorothricin	Polyketide:Modular type I + Polyketide:Iterative type I + Saccharide:Oligosaccharide	4%

Fig. 7: antiSMAH analysis of biosynthetic gene clusters of *Streptomyces* sp. Go-475

Cluster 19 was predicted to produce an aminoglycoside/aminocyclitol-like compound and included *afsR*, a SARP regulatory gene:

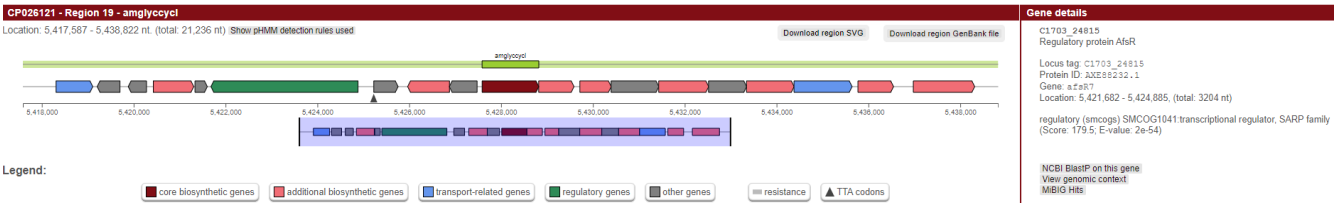


Fig. 8: antiSMAH analysis of BGC 19 in *Streptomyces* sp. Go-475

The analysis of Cluster 27 showed genes predicted to produce an aromatic polyketide. Among other regulatory genes, *yycF*, a regulator of the OmpR family, could be identified:

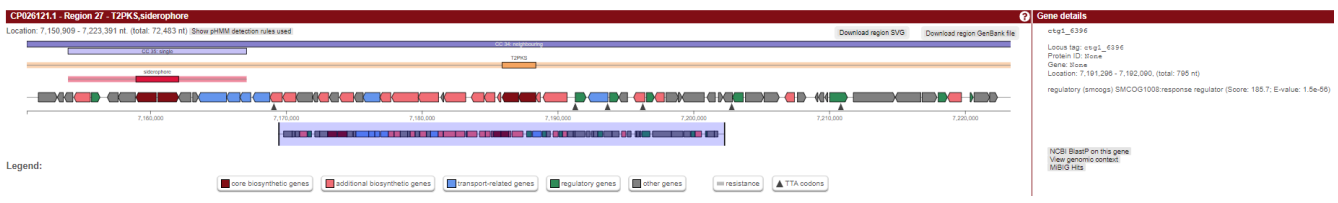


Fig. 9: antiSMAH analysis of BGC 17 in *Streptomyces* sp. Go-475

It was decided to clone the positive regulatory genes *afsR* and *yycF* into pSOK806, a plasmid containing the strong promoter ermE_up and the apramycin resistance gene *AmR*, and to introduce them into the *Streptomyces* sp. Go-475 wild type in order to activate the clusters controlled by them, cluster 19 and cluster 27.

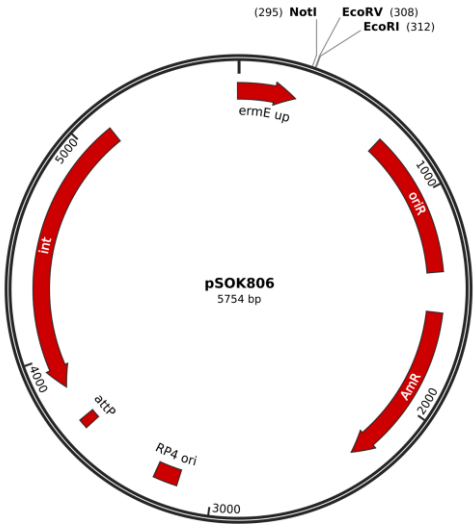


Fig. 10: Plasmid Map of pSOK806

5.5. Construction of the recombinant vectors

5.5.1. Isolation of genomic DNA

The genomic DNAs of *Streptomyces* sp. Ru-355 and *Streptomyces* sp. Go-475 were extracted (Methods 9.7.) and purified (Methods 9.12.). The results were verified by gel electrophoresis (Methods 9.8.).

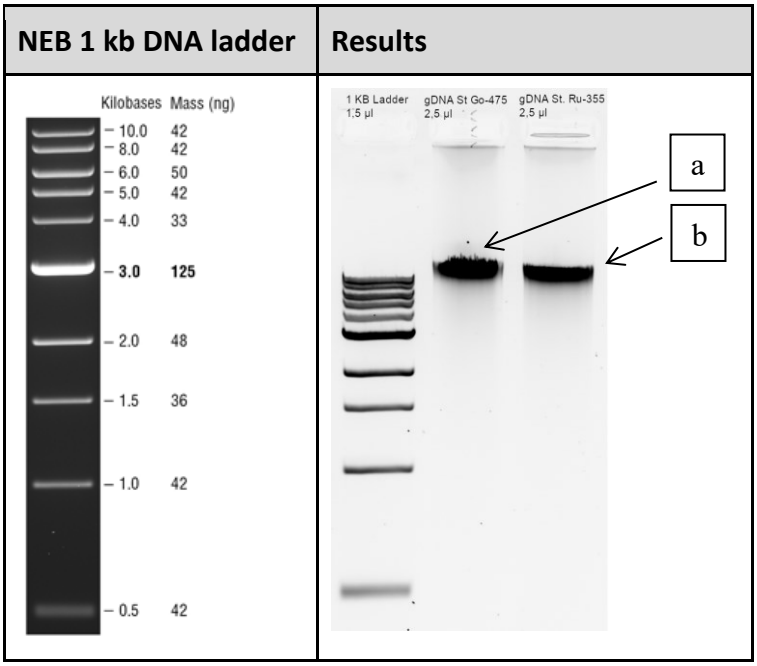


Fig. 11: Genomic DNA isolated from *Streptomyces* sp. Go-475 (a) and *Streptomyces* sp. Ru-355 (b)

5.5.2. Primer design

Forward and reverse primers for the genes *afsR* and *yycF* were designed using CloneManager introducing restriction sites for EcoRV and EcoRI in Cluster 19 and NotI and EcoRI in Cluster 27 (Methods 9.9.).

5.5.3. Amplification of regulatory genes

The regulatory genes *afsR*, situated in BGC 19, and *yycF*, situated in BGC 27, were amplified from the genomic DNA of *Streptomyces* sp. Go-475 using PCR with the respective forward and reverse RNA primers (Methods 9.10.).

5.5.4. Digestion of pSOK806 and PCR-products

pSOK806 was isolated (Materials 9.11.) from *E. coli* DH5-alpha cells and purified (Methods 9.12.). Two linear fragments of pSOK806 were created cutting he plasmid at its multiple cloning site using the enzymes EcoRV/EcoRI and NotI/EcoRI. The purified PCR product of cluster 19 was cut using EcoRV/EcoRI, while the purified PCR product of cluster 27 was cut using EcoRV/EcoRI (Methods 9.13.). The restriction results were validated by gel electrophoresis (Methods 9.8.).

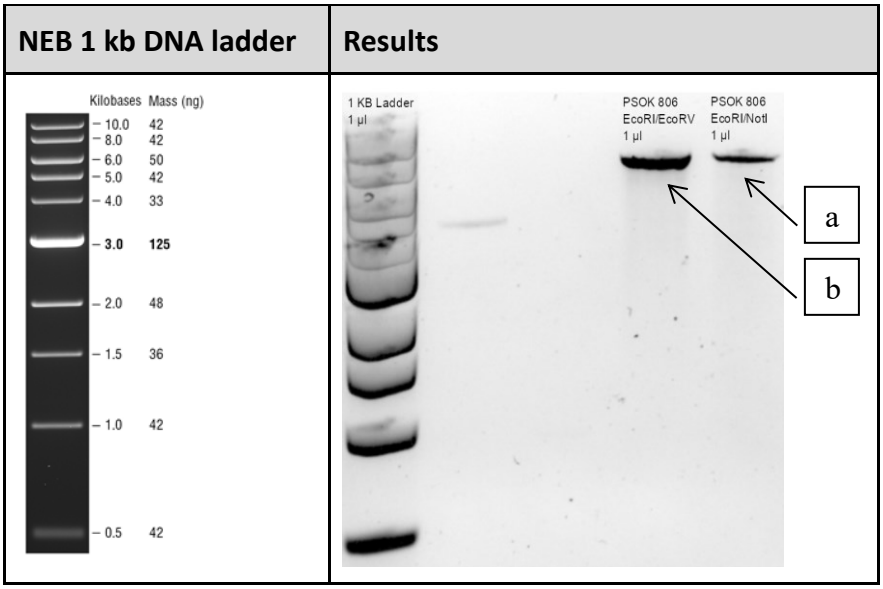


Fig. 12: pSOK806 cut with EcoRI/NotI (a) and pSOK806 cut with EcoRI/EcoRV (b)

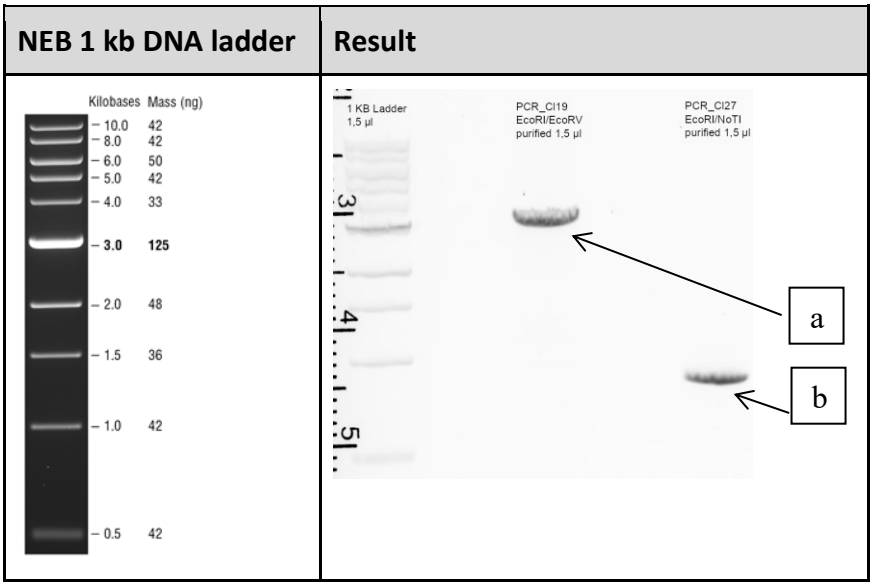


Fig. 13: PCR-product of cluster 19 cut with EcoRI/EcoRV (a) and PCR-product of cluster 27 cut with EcoRI/NotI (b)

5.5.5. DNA ligation

In the next step two new plasmid vectors were generated by ligation of the digested PCR products into the two plasmid vectors using T4-DNA-ligase (Methods 9.14.).

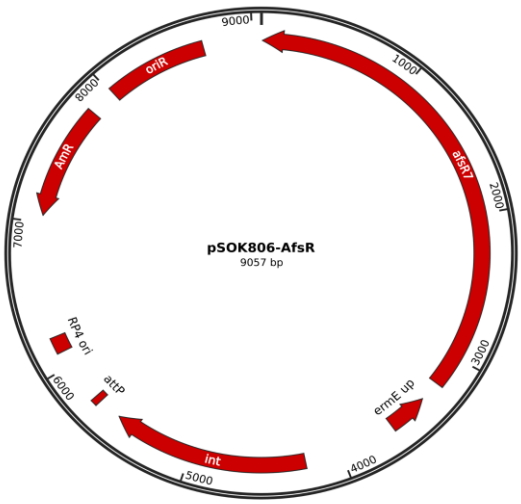


Fig. 14: Plasmid Map of pSOK806-AfsR

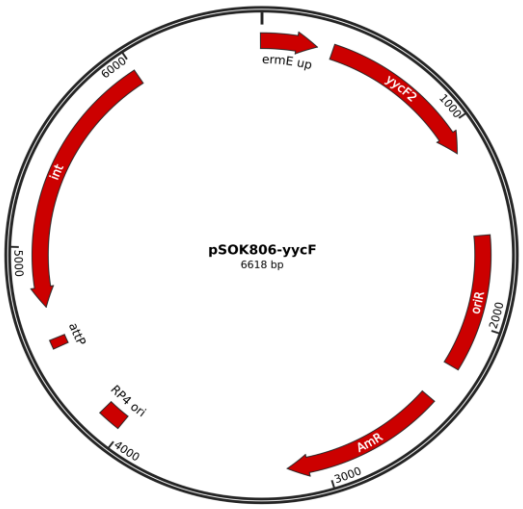


Fig. 15: Plasmid Map of pSOK806-yycF

5.5.6. Vector transformation

The vectors, containing an apramycin resistance gene, were transformed into *E. coli* XL1-Blue cells, which were then selected on LA agar plates containing apramycin 100µg/ml (Methods 9.15.).

The successfully transformed colonies were picked and liquid overnight cultures containing apramycin 100 µg/ml were prepared (Methods 9.5.4.). The following day the plasmids were isolated (Methods 9.11.) so that correct ligation could be confirmed by restriction analysis (Methods 9.16.).

5.5.7. Restriction analysis

The following fragments were expected in case of a successful ligation.

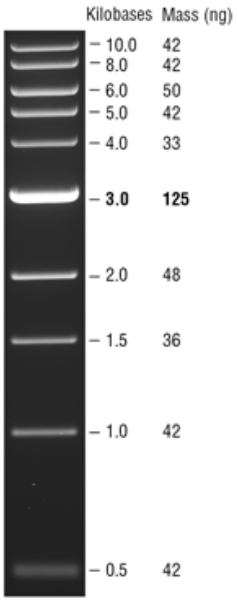
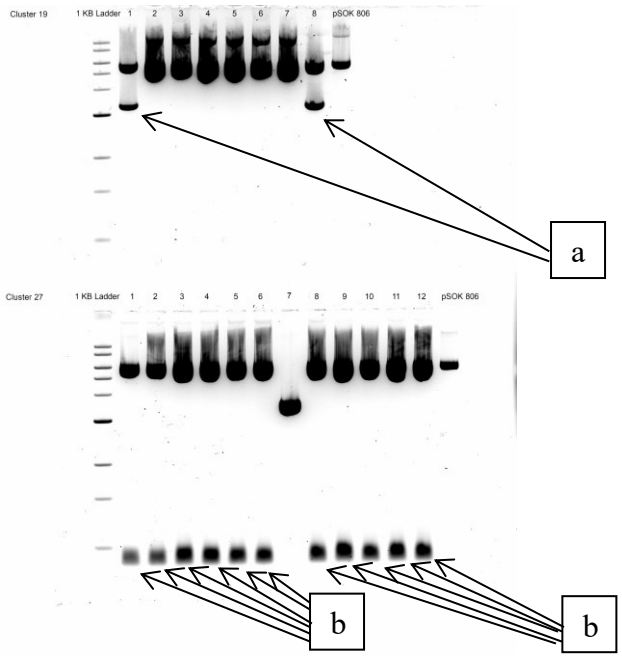
Fragments	NEB 1 kb DNA ladder	Result
1: pSOK806-AfsR EcoRI + EcoRV 1. 5746 bp 2. 3311 bp 1: pSOK806-yycF EcoRI + NotI 1. 5737 bp 2. 881 bp		

Fig. 16: Restriction Analysis with Restriction Enzymes EcoRI and EcorV/NotI. pSOK806-AfsR (a) could be isolated from XL1-Blue_Cl19 clones 1 and 8. pSOK806-yycF (b) could be isolated from XL1-Blue_Cl27 clones 1-6 and 8-12.

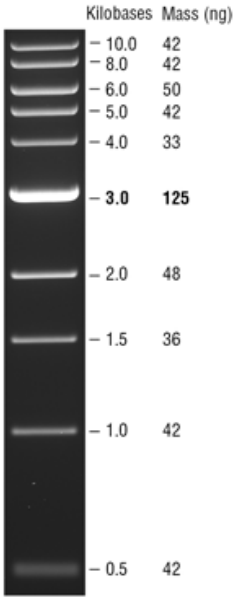
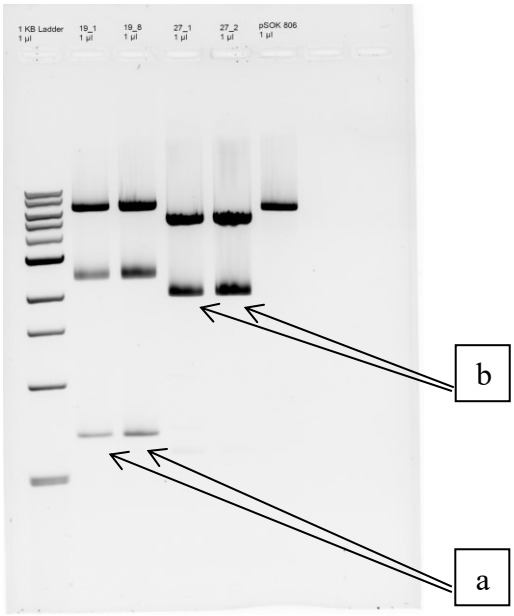
Fragments	NEB 1 kb DNA ladder	Result
1: pSOK806-AfsR AgeI 1. 6144 bp 2. 2246 bp 3. 667 bp 2: pSOK806-yycF AgeI 1. 4733 bp 2. 1885 bp		

Fig. 17: Restriction Analysis with Restriction Enzyme AgeI. pSOK806-AfsR (a) could be isolated from XL1-Blue_Cl19 clones 1 and 8. pSOK806-yycF (b) could be isolated from XL1-Blue_Cl27 clones 1 and 2.

The plasmids isolated from pSOK806-AfsR clone 1 and pSOK806-yycF clone 1 were selected to be introduced to *Streptomyces* sp. Go-475.

5.6. Conjugation to *Streptomyces*

For the conjugation to *Streptomyces* sp. Go-475 *E.coli* ET12567, a DNA methylation deficient strain, was used. The plasmid vectors were isolated from *E.coli* XL1-Blue (Methods 9.11.), transformed to ET12567 cells (Methods 9.17.) and conjugated to *Streptomyces* sp. Go-475 at a heat shock temperature of 45°C for 10 minutes (Methods 9.18.). While the conjugation of the control vector pSOK806 was successful at low efficiency, a conjugation of the vectors carrying the regulator genes could not be achieved after several attempts.

It was decided to conjugate the vectors to *Streptomyces albus* J1074 instead to investigate whether the heterologous expression of the regulators could induce the production of antibiotic compounds in this strain. The conjugation of the plasmid vectors pSOK806-AfsR, pSOK806-yycF and pSOK806 was successful at a heat shock temperature of 50°C for 10 minutes. (Methods 9.18.)

5.7. Fermentation

Overnight cultures of the recombinant strains *Streptomyces albus* J1074_pSOK, *Streptomyces albus* J1074_Cl19 and *Streptomyces albus* J1074_Cl27 were fermented in three different fermentation media, MYM, MP1, SM17 for 3 and 7 days (Methods 9.19.). The fermentation broths were lyophilized and extracted with methanol and stored at -20°C (Methods 9.20.).

5.8. Extract analysis

5.8.1. Disc diffusion assays

The methanolic extracts were tested for biological activity against several test organisms including gram-positive and gram-negative bacteria and yeast (Methods 9.21.1.). Results are presented in Tables 5 - 14.

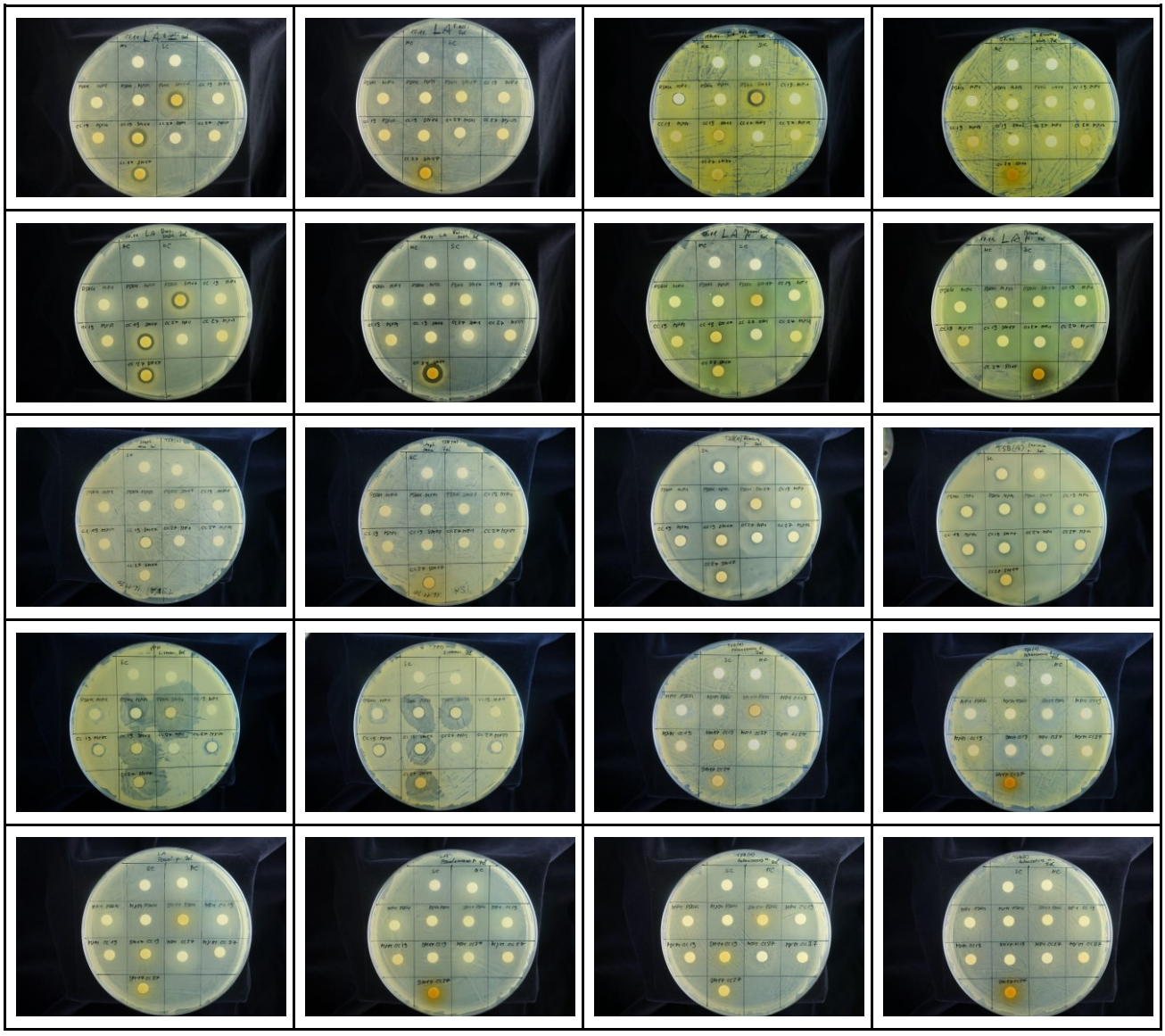


Fig. 18: Disc-diffusion Assays testing extract bioactivity on test organisms

Medium/Strain	*Streptomyces albus* J1074_pSOK	*Streptomyces albus* J1074_ Cl19	*Streptomyces albus* J1074_ Cl27
MP1 (3 days)	○	○	○
MP1 (7 days)	○	○	○
MYM (3 days)	○	○	○
MYM (7 days)	○	○	○
SM17 (3 days)	●	●	●
SM17 (7 days)	○	○	◌

Table 5: Activity of methanolic extracts of fermentation medium after 3 and 7 days of fermentation against *Escherichia coli* test strain.

Medium/Strain	*Streptomyces albus* J1074_pSOK	*Streptomyces albus* J1074_ Cl19	*Streptomyces albus* J1074_ Cl27
MP1 (3 days)	○	○	○
MP1 (7 days)	○	○	○
MYM (3 days)	○	○	○
MYM (7 days)	○	○	○
SM17 (3 days)	●	○	○
SM17 (7 days)	○	○	○

Table 6: Activity of methanolic extracts of fermentation medium after 3 and 7 days of fermentation against *Kocuria rhizophila* test strain.

Medium/Strain	*Streptomyces albus* J1074_pSOK	*Streptomyces albus* J1074_ Cl19	*Streptomyces albus* J1074_ Cl27
MP1 (3 days)	○	○	○
MP1 (7 days)	○	○	○
MYM (3 days)	○	○	○
MYM (7 days)	○	○	○
SM17 (3 days)	●	●	●
SM17 (7 days)	○	○	●

Table 7: Activity of methanolic extracts of fermentation medium after 3 and 7 days of fermentation against *Bacillus subtilis* test strain.

Medium/Strain	*Streptomyces albus* J1074_pSOK	*Streptomyces albus* J1074_ Cl19	*Streptomyces albus* J1074_ Cl27
MP1 (3 days)	◯	◯	◯
MP1 (7 days)	◯	◯	◯
MYM (3 days)	◯	◯	◯
MYM (7 days)	◯	◯	◯
SM17 (3 days)	◯	◯	◯
SM17 (7 days)	◯	◯	◯

Table 8: Activity of methanolic extracts of fermentation medium after 3 and 7 days of fermentation against *Pseudomonas floureszens* test strain.

Medium/Strain	*Streptomyces albus* J1074_pSOK	*Streptomyces albus* J1074_ Cl19	*Streptomyces albus* J1074_ Cl27
MP1 (3 days)	◯	◯	◯
MP1 (7 days)	◯	◯	◯
MYM (3 days)	◯	◯	◯
MYM (7 days)	◯	◯	◯
SM17 (3 days)	◯	◯	◯
SM17 (7 days)	◯	◯	◯

Table 9: Activity of methanolic extracts of fermentation medium after 3 and 7 days of fermentation against *Staphylococcus carnosus* test strain.

Medium/Strain	*Streptomyces albus* J1074_pSOK	*Streptomyces albus* J1074_ Cl19	*Streptomyces albus* J1074_ Cl27
MP1 (3 days)	◯	◯	◯
MP1 (7 days)	◯	◯	◯
MYM (3 days)	◯	◯	◯
MYM (7 days)	◯	◯	◯
SM17 (3 days)	◯	◯	◯
SM17 (7 days)	◯	◯	◯

Table 10: Activity of methanolic extracts of fermentation medium after 3 and 7 days of fermentation against *Erwinia persicina* test strain.

Medium/Strain	*Streptomyces albus* J1074_pSOK	*Streptomyces albus* J1074_ Cl19	*Streptomyces albus* J1074_ Cl27
MP1 (3 days)	○	○	○
MP1 (7 days)	○	○	○
MYM (3 days)	●	○	○
MYM (7 days)	●	○	○
SM17 (3 days)	●	●	●
SM17 (7 days)	●	●	●

Table 11: Activity of methanolic extracts of fermentation medium after 3 and 7 days of fermentation against *Saccharomyces cerevisiae* test strain.

Medium/Strain	*Streptomyces albus* J1074_pSOK	*Streptomyces albus* J1074_ Cl19	*Streptomyces albus* J1074_ Cl27
MP1 (3 days)	○	○	○
MP1 (7 days)	○	○	○
MYM (3 days)	○	○	○
MYM (7 days)	○	○	○
SM17 (3 days)	○	○	○
SM17 (7 days)	○	○	○

Table 12: Activity of methanolic extracts of fermentation medium after 3 and 7 days of fermentation against *Micrococcus luteus* test strain.

Medium/Strain	*Streptomyces albus* J1074_pSOK	*Streptomyces albus* J1074_ Cl19	*Streptomyces albus* J1074_ Cl27
MP1 (3 days)	○	○	○
MP1 (7 days)	○	○	○
MYM (3 days)	○	○	○
MYM (7 days)	○	○	○
SM17 (3 days)	○	○	○
SM17 (7 days)	○	○	○

Table 13: Activity of methanolic extracts of fermentation medium after 3 and 7 days of fermentation against *Pseudomonas putida* test strain.

Medium/Strain	*Streptomyces albus* J1074_pSOK	*Streptomyces albus* J1074_ Cl19	*Streptomyces albus* J1074_ Cl27
MP1 (3 days)	◯	◯	◯
MP1 (7 days)	◯	◯	◯
MYM (3 days)	◯	◯	◯
MYM (7 days)	◯	◯	◯
SM17 (3 days)	◯	◯	◯
SM17 (7 days)	◯	◯	◯

Table 14: Activity of methanolic extracts of fermentation medium after 3 and 7 days of fermentation against *Enterococcus mundtii* test strain.

◯ no activity ◯ moderate activity ● strong activity

The methanolic fermentation extracts showed antibiotic activity against *Escherichia coli, Kocuria rhizophila, Bacillus subtilis* and *Saccharomyces cerevisiae*. However, only the fermentation extract of *Streptomyces albus* J1074_Cl27 that had been fermented in SM17 for 7 days exerted strong antibiotic activity against *Bacillus subtilis*, while the control extract from *Streptomyces albus* J1074_pSOK did not. Thus, it could be concluded that the introduction of the plasmid Cl_27 (pSOK806-YycF) and the expression of the *yycF* gene in *Streptomyces albus* J1074_Cl27 was the determining factor for the antibiotic activity. In order to further characterize the bioactive compound an HPLC analysis of the extract was conducted.

5.8.2. HPLC-analysis

An analytical HPLC analysis showed a novel peak in the bioactive extract of *Streptomyces albus* J1074_Cl27 as compared to *Streptomyces albus* J1074_pSOK at 254 nm at RT: 8,7 mins. (Methods 9.21.2.)

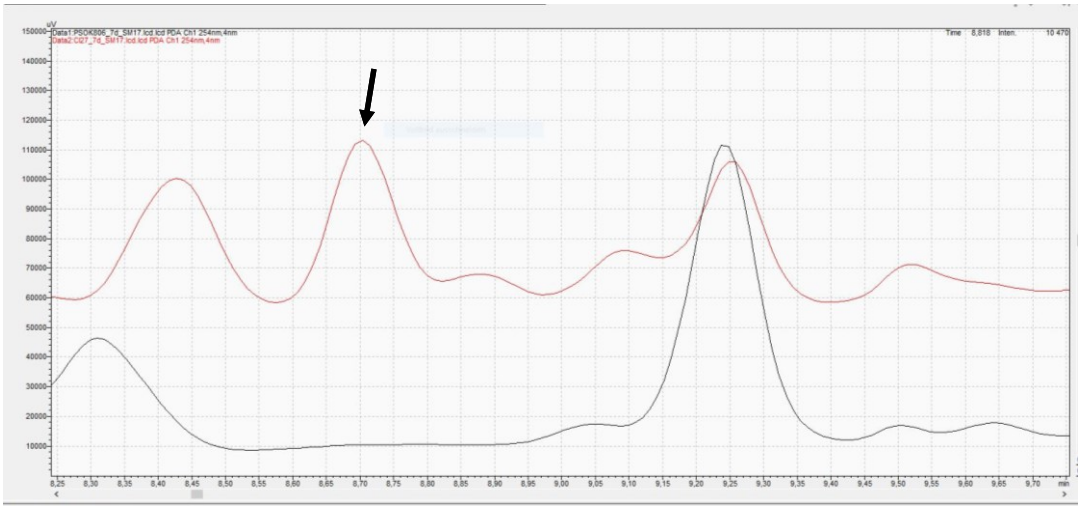


Fig. 19: Analytical HPLC: A novel peak (indicated by arrow) appears in the chromatogram of the methanolic extract of *Streptomyces albus* J1074_Cl27 fermented 7 days in SM17, as compared to *Streptomyces albus* J1074_pSOK control.

In order to further investigate the bioactive compounds a semi-preparative HPLC was conducted to separate several fractions. In fraction 4 (RT:15 -20 mins) several peaks could be identified. (Methods 9.21.2.)

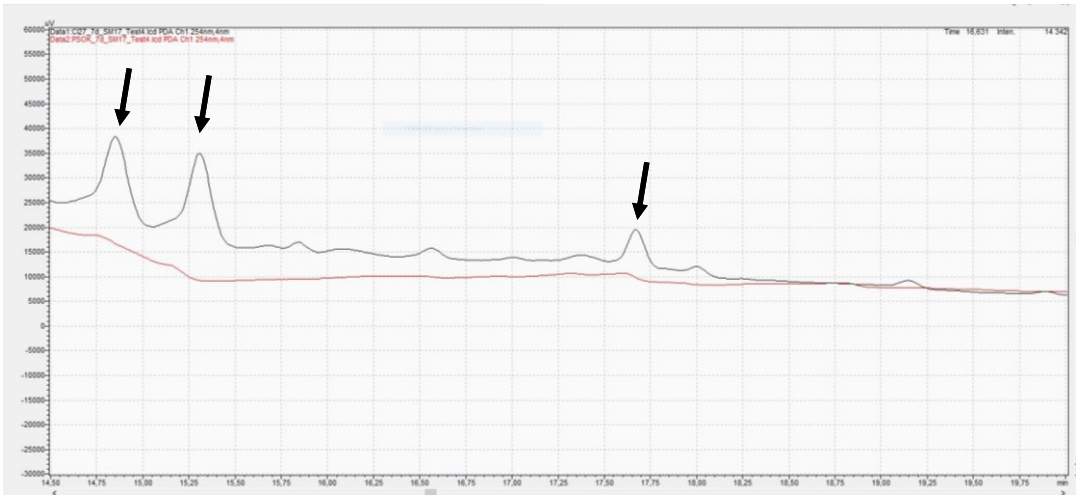


Fig. 20: Semi-preparative HPLC: Three novel peaks (indicated by arrows) appear in the chromatogram of the bioactive fraction 4 of the methanolic extract of *Streptomyces albus* J1074_Cl27 fermented 7 days in SM17, as compared to *Streptomyces albus* J1074_pSOK control.

To validate the bioactivity of the HPLC fractions they were once again tested against *Bacillus subtilis*.

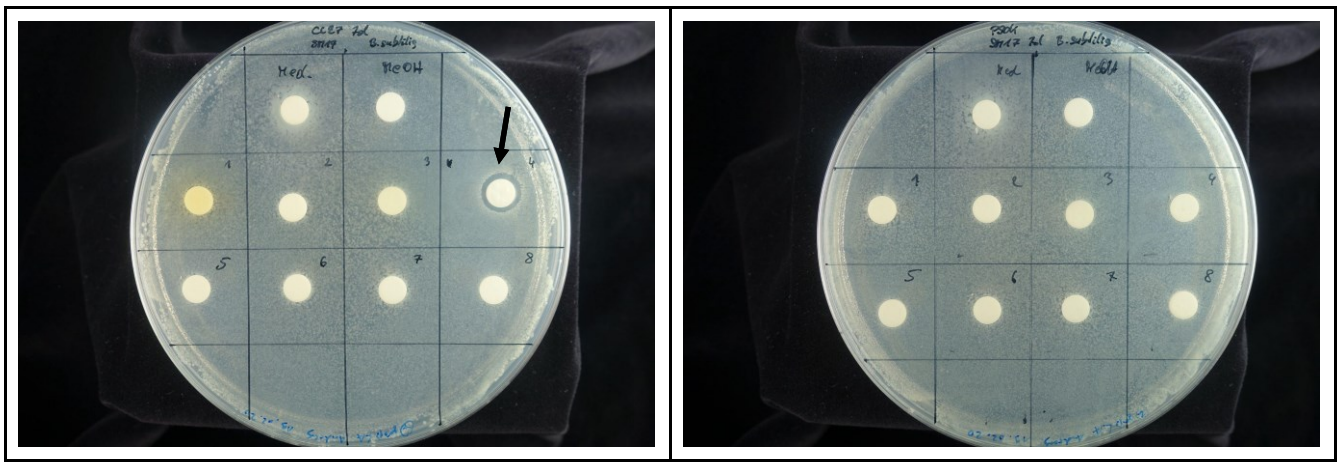


Fig. 21: Disc-diffusion assay against *Bacillus subtilis* of 8 fractions of the methanolic extract from fermentation in SM17 (7 days) of *Streptomyces albus* J1074_Cl27 as compared to *Streptomyces albus* J1074_pSOK. Fraction 4 (see arrow) showing antibiotic activity.

Since, out of 8 fractions, only fraction 4 of the methanolic extract of *Streptomyces albus* J1074_Cl27 fermented 7 days in SM17, as compared to *Streptomyces albus* J1074_pSOK control, exerted antibiotic activity against *Bacillus subtilis* it could be confirmed that fraction 4 contained the bioactive compound(s).

5.8.3. Mass spectrometry

In order to further investigate the compounds an MS-analysis was conducted by Dr. Martin Zehl at the Mass Spectrometry Centre of the University of Vienna. (Methods 9.21.3.)

Novel derivatives of deferoxamine appeared in the spectrum that could not be found in the dictionary of natural products (DNP) or SciFinder chemical databases. In addition, a putative peptide, most likely linear and not too heavily modified, could be identified in the bioactive fraction.

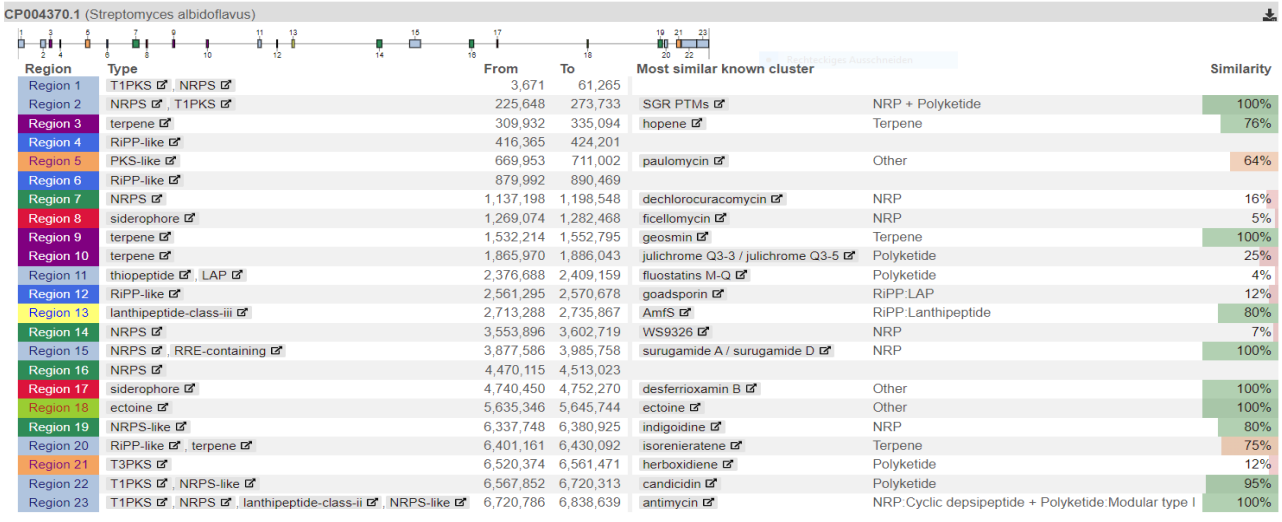
6. Discussion

As has been shown the final goal of this project to activate silent secondary metabolite biosynthetic gene clusters in *Streptomyces* sp. Ru-355 and *Streptomyces* sp. Go-475 could not be achieved with the methods employed.

However, it could be established that the growth media best suited to elicit spore formation in solid cultures of these novel strains is SFM. In addition, it could be shown that both *Streptomyces* sp. Ru-355 and *Streptomyces* sp. Go-475 are susceptible to the antibiotic apramycin.
Since the pilot experiment conjugating pSOK806 from *E.coli ET12567* to both strains was successful, it could be established that this vector system is potentially a viable method to introduce genetic information into these strains. These findings might prove to be useful in case further research on these strains should be conducted in the future.

The fact that neither recombinant vector containing a constitutively active promoter upstream of the regulatory genes *yycF* and *afSR* could be successfully conjugated to *Streptomyces* sp. Go-475 may suggest toxicity caused by the products of activated BGCs.

However, the conjugation to and subsequent heterologous expression of the regulatory genes in *Streptomyces albus* J1074 was successful. Interestingly, the expression of *yycF* in the culture grown in SMF17 medium over seven days produced secondary metabolites exerting antibiotic effect on *Bacillus subtilis*. Therefore, a previously silent genes could have been activated. Considering the identification of deferoxamine derivatives by MS-analysis, a possible explanation could be activation of genes encoding enzymes that can modify deferoxamine, and that these derivatives have antibiotic activity.



CP004370.1 (Streptomyces albidoflavus)

Region	Type	From	To	Most similar known cluster		Similarity
Region 1	T1PKS, NRPS	3,671	61,265			
Region 2	NRPS, T1PKS	225,648	273,733	SGR PTMs	NRP + Polyketide	100%
Region 3	terpene	309,932	335,094	hopene	Terpene	76%
Region 4	RiPP-like	416,365	424,201			
Region 5	PKS-like	669,953	711,002	paulomycin	Other	64%
Region 6	RiPP-like	879,992	890,469			
Region 7	NRPS	1,137,198	1,198,548	dechlorocuracomycin	NRP	16%
Region 8	siderophore	1,269,074	1,282,468	ficellomycin	NRP	5%
Region 9	terpene	1,532,214	1,552,795	geosmin	Terpene	100%
Region 10	terpene	1,865,970	1,886,043	julichrome Q3-3 / julichrome Q3-5	Polyketide	25%
Region 11	thiopeptide, LAP	2,376,688	2,409,159	fluostatins M-Q	Polyketide	4%
Region 12	RiPP-like	2,561,295	2,570,678	goadsporin	RiPP:LAP	12%
Region 13	lanthipeptide-class-iii	2,713,288	2,735,867	AmfS	RiPP:Lanthipeptide	80%
Region 14	NRPS	3,553,896	3,602,719	WS9326	NRP	7%
Region 15	NRPS, RRE-containing	3,877,586	3,985,758	surugamide A / surugamide D	NRP	100%
Region 16	NRPS	4,470,115	4,513,023			
Region 17	siderophore	4,740,450	4,752,270	desferrioxamin B	Other	100%
Region 18	ectoine	5,635,346	5,645,744	ectoine	Other	100%
Region 19	NRPS-like	6,337,748	6,380,925	indigoidine	NRP	80%
Region 20	RiPP-like, terpene	6,401,161	6,430,092	isorenieratene	Terpene	75%
Region 21	T3PKS	6,520,374	6,561,471	herboxidiene	Polyketide	12%
Region 22	T1PKS, NRPS-like	6,567,852	6,720,313	candicidin	Polyketide	95%
Region 23	T1PKS, NRPS, lanthipeptide-class-ii, NRPS-like	6,720,786	6,838,639	antimycin	NRP:Cyclic depsipeptide + Polyketide:Modular type I	100%

Fig. 22: Biosynthetic gene clusters of *Streptomyces albus* J1074. BGC17 is predicted to be responsible for the biosynthesis of deferoxamine.

In order to clarify this issue, more research beyond the scope of this project would have to be conducted. However, siderophores do not present a very promising class of compounds as regards to antibiotic drug discovery.

Alternatively, the peptide identified in the extract might still be an interesting subject of further investigation. In addition, the role of the YycF regulatory protein in the regulatory networks of *Streptomyces spp*. is still poorly investigated and might prove an interesting topic for future research.

7. Outlook

Emerging antimicrobial resistance presents an immense problem to the world. Besides flanking measures this challenge can only be met with an increased effort in drug discovery and development. The success stories of antibiotics throughout the 20th century show that most successful antibiotic compounds have been found or derived from natural sources, especially *Streptomyces* bacteria. The activation of previously silent BGCs will provide a viable strategy to further increase the understanding of the biochemical networks regulating the production of secondary metabolites.

While evolutionary pressure is responsible for the existence of resistance genes, it also led to the emergence of a plethora of bioactive secondary metabolites gene clusters. Compounds isolated from bacterial and fungal sources can provide great lead structures which can be chemically modified to eventually develop novel effective drugs to combat microbial infections. Bioprospecting in unusual habitats such as deserts or even the depths of the sea will unveil new organisms with yet unknown potential.
With the possibilities provided by modern sequencing and bioinformatics researchers will be able to uncover the treasures still hidden or locked away in genomes unknown.

8. Materials

8.1. Organisms

Name	Vector	Source
Streptomyces sp. Go-475	-	Provided by the lab
Streptomyces sp. Go-475_pSOK	Insertion of pSOK-806	Created in the project
Streptomyces sp. Ru-355	-	Provided by the lab
Streptomyces sp. Ru-355_pSOK	Insertion of pSOK-806	Created in the project
Streptomyces albus J1074	-	Provided by the lab
Streptomyces albus J1074_pSOK	Insertion of pSOK-806	Created in the project
Streptomyces albus J1074_Cl19	Insertion of pSOK-806-AfsR	Created in the project
Streptomyces albus J1074_Cl27	Insertion of pSOK-806-yycF	Created in the project

Table 15: *Streptomyces* strains, wild type and mutants, used in the project

Name	Vector	Source
DH5-alpha	pSOK-806	Provided by the lab
DH5-alpha	pSOK-804	Provided by the lab
DH5-alpha	PSET-152	Provided by the lab
XL1-Blue	-	Provided by the lab
XL1-Blue_pSOK	pSOK-806	Created in the project
XL1-Blue_Cl19	pSOK-806+AfsR	Created in the project
XL1-Blue_Cl27	pSOK-806+yycF	Created in the project
ET12567	-	Provided by the lab
ET12567_pSOK	pSOK-806	Created in the project
ET12567_804	pSOK-804	Created in the project
ET12567_152	pSET-152	Created in the project
ET12567_Cl19	pSOK-806-AfsR	Created in the project
ET12567_Cl27	pSOK-806-yycF	Created in the project

Table 16: *E.coli* strains, wild type and mutants, used in the project

Name	Type	Source
Bacillus subtilis	Gram-positive bacterium	Provided by the lab
Staphylococcus carnosus	Gram-positive bacterium	Provided by the lab
Kocuria rhizophila	Gram-positive bacterium	Provided by the lab
Enterococcus mundtii	Gram-positive bacterium	Provided by the lab
Micrococcus luteus	Gram-positive bacterium	Provided by the lab
Escherichia coli	Gram-negative bacterium	Provided by the lab
Erwinia persicina	Gram-negative bacterium	Provided by the lab
Pseudomonas putida	Gram-negative bacterium	Provided by the lab
Pseudomonas floureszens	Gram-negative bacterium	Provided by the lab
Saccharomyces cerevisiae	Yeast	Provided by the lab

Table 17: Test organisms used for bioassays

8.2. Plasmid vectors

Name	Source
pSOK806	Provided by the lab
Cl_19 (pSOK806-AfsR)	Created in the project
Cl_27 (pSOK806-YycF)	Created in the project
PSOK804	Provided by the lab
pSET152	Provided by the lab

Table 18: Vectors containing the apramycin resistance gene *AmR*

8.3. Agar cultivation media

(Kieser, 2000)

ISP Medium 2

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Agar	15 g
ddH_2O	Up to 1000 ml

ISP Medium 4

Soluble Starch	10 g
Dipotassium Phosphate	1 g
Magnesium Sulfate USP	1 g
Sodium Chloride	2 g
Ammonium Sulfate	2 g
Calcium Carbonate	20 g
Ferrous Sulfate	0.001 g
Manganous Chloride	0.001 g
Zinc Sulfate	0.001 g
ddH_2O	Up to 1000 ml

Mannitol Soya Flour Medium (SFM)

Agar	20 g
Mannitol	20 g
Soya flour	20 g
Trace Element Solution	1 ml
Tap Water	to 1000 ml

CP-6 Agar Medium

Com Steep Liquor (Sigma)	10 g
Starch Soluble	10 g
NaCl	3 g
$(NH_4)_2SO_4$	3 g
$CaCO_3$	3 g
Agar	20 g
Tap Water	to 1000 ml

L (Lennox) agar (LA)

Tryptone	10 g
NaCl	5 g
Agar	10 g
dH_2O	to 1000 ml

YPD Medium

Yeast extract	10 g
Peptone	20 g
Glucose	20 g
Agar	15 g
dH_2O	Up to 1000 ml

8.4. Liquid cultivation media

2 XYT

Tryptone	16 g
Yeast Extract	10 g
NaCl	5 g
dH_2O	to 1000 ml

Tryptone Soya Broth (TSB)

Tryptic Soy Broth	30 g
Agar	15 g
dH_2O	to 1000 ml

L (Lennox) Broth (LB)

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
dH_2O	to 1000 ml

8.5. Fermentation media

SM17

Glucose	2 g
Glycerol	40 g
Soluble Starch	2 g
Soy Flour	5 g
Peptone	5 g
Yeast Extract	5 g
NaCl	5 g
$CaCO_3$	2 g
Tap Water	up to 1000 ml

MYM pH 7.3

Maltose	4 g
Yeast Extract	4 g
Malt Extract	10 g
Tap Water	500 ml
dH_2O	Up to 500 ml
Trace elements as for R2YE	2 ml

MP1

Glucose	40 g
Yeast Extract	1.5 g
NH_4NO_3	2.5 g
$MgSO_4.7H_2O$	0.5 g
KH_2PO_4	0.5 g
$CaCO_3$	2 g
dH_2O	up to 1000 ml

8.6. Solutions

Antibiotics Stock Solutions

Apramycin	100 mg/ml in H_2O
Hygromycin	100 mg/ml in H_2O
Thiostrepton	30 mg/ml in DMSO
Kanamycin	25 mg/ml in H_2O
Chloramphenicol	25 mg/ml in EtOH
Nalidixic Acid	30 mg/ml in NaOH

MgCl2 Solution 1 M

$MgCl_2.6H_2O$	20.33 g
dH_2O	up to 100 ml

Glycerol Solution (20%)

Glycerol	20 ml
dH_2O	up to 100 ml

Trace Element Solution

$ZnCl_2$	40 mg
$FeCl_3.6H_2O$	200 mg
$CuCl_2.2H_2O$	10 mg
$MnCl_2.4H_2O$	10 mg
$Na_2BO_4.10H_2O$	10 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	10 mg
dH_2O	to 1000 ml

8.7. Agarose gel

Agarose	3.2 g
TBE Buffer	up to 400ml
GelRed Nucleic Acid Stain (10,000x)	20 µl

8.8. Buffers

TBE

Tris base	108 g
Boric acid	55 g
0.5M EDTA (pH 8.0)	51 ml
dH_2O	up to 1000 ml

TE

Tris-Hcl 2M (pH 8.0)	5 ml
0.5M EDTA (pH 8.0)	2 ml
dH_2O	up to 1000 ml

NEB CutSmart

T4 DNA Ligation Buffer

Gel Loading Dye

GelRed

8.9. Primers

DNA-primers were synthesised by Eurofins Genomics.

Regulator *afsR*

Forward Primer	5’-GATCGAATTCTGCCTCAGACCTGCTCCTT-3’
Reverse Primer	5’-GATCGATATCCGCAGCACACTTGACCAG-3’

Regulator *yycF*

Forward Primer	5’-GATCGCGGCCGCCTGTCGTTCAACACTTGATGA-3’
Reverse Primer	5’-GTCAGAATTCGAGGCGGCACCAGGAT-3’

8.10. Enzymes

NEB EcoRI-HF

NEB EcorV-HF

NEB NotI-HF

NEB AgeI-HF

T4 DNA Ligase

8.11. Kits

Zymoclean Gel DNA Recovery Kit

Promega Wizard ® Plus SV Minipreps DNA Purification System

NEB Monarch Genomic DNA Purification Kit

Q5 High-Fidelity 2X Master Mix

8.12. Software

Clone Manager
AntiSMASH v6.0.0
SnapGene

9. Methods

9.1. Preparation of media

Following the recipes listed in the materials section the media were prepared in Erlenmeyer flasks using a magnetic stirrer. Aliquots of 200 ml were then transferred into 500 ml flasks to be autoclaved for 20 minutes at 121°C. The sterile media were stored at room temperature and protected from light.

9.2. Preparation of spore suspensions

2 ml of glycerol solution were pipetted onto an SFM plate that had been sufficiently overgrown by the *Streptomyces* strain. The mycelium was carefully scratched off the plate using the tip of a plastic pipette to collect the spores. The spore suspension was then transferred into a syringe containing a cotton plug and filtered into 1.5 ml cryo-tubes. These were stored in the freezer at -80°C.

9.3. Preparation of antibiotic stock solutions

The antibiotics were weighed in and dissolved in the solvents appropriate. If applicable, the solutions were filtered using syringe filters with a pore size of 0.22 µm. Aliquots of 500 µl were filled into Eppendorf tubes and stored in the refrigerator at -20°C. The stock solutions were 1000 x concentrated.

9.4. Preparation of agar plates

The agar media were heated in the microwave until the agar was fully melted and cooled down in a water bath set to 50°C. If applicable, antibiotic stock solution was added to the bottle. The media were then transferred into sterile petri dishes with a pipette and cooled down to room temperature. The agar plates were stored at 4°C.

9.5. Cultivation

9.5.1 Cultivation of *Streptomyces* strains on solid growth media

100 µl of spore suspension were evenly distributed on a 94 mm x 16 mm agar plate, containing 30 ml of medium, using a sterile glass spreader or a metal loop. The dry plate was then placed into an incubator set to 28°C for several days until the formation of spores and air mycelium.

9.5.2. Cultivation of *Streptomyces* strains in liquid growth media

100 µl of spore suspension were transferred into a 12 ml cultivation tube containing 2 ml TSB. The tube was placed onto the shaker set to 28°C, 200 rpm and incubated overnight.

9.5.3 Cultivation of *E.coli* strains on solid growth media

100 µl of cell suspension were evenly distributed on a 94 mm x 16 mm agar plate, containing 20 ml of LA medium, using a sterile glass spreader or a metal loop. The dry plate was then placed into an incubator set to 37°C overnight.

9.5.4. Cultivation of *E.coli* in liquid growth media

E.coli were collected from the agar plate using a metal loop and transferred into a 12 ml cultivation tube containing 2 ml LB. If applicable, antibiotic stock solution was added. The tube was placed onto the shaker set to 37°C, 200 rpm and incubated overnight.

9.6. Preparation of *E.coli* cell suspensions

750 µl of a liquid overnight culture was transferred into an Eppendorf tube and centrifuged for 3 minutes at maximum speed. After the supernatant was discarded, the pellet was resuspended in 750 µl glycerol solution and transferred into 1.5 ml cryo-tubes. These were stored in the freezer at -80°C.

9.7. Genomic DNA isolation

2 ml of liquid Streptomyces overnight culture were prepared.

1 ml culture was transferred into an Eppendorf tube and 0.5 ml sterile water added, mixed and spun down at 13000 rpm for 3 min. Supernatant discarded, centrifuged briefly and the remaining liquid sucked out.

The pellet was resuspended by pipetting in 180 µl Lysis Buffer (20 mM Tris HCL, pH 8.0; 10 mM EDTA pH 8.0; 1.2% Triton X 100; 10-20 mg/ml lysozyme). Lysozyme was added just before the experiment. The suspension was incubated at 37°C for 15 min and mixed by gently tipping the end of the tube every 5 min.

10 µl Proteinase K and 6 µl RNase A were added to the resuspended pellet and mixed by vortexing briefly to ensure the enzymes are efficiently dispersed.

200 µl Cell Lysis Buffer was added and vortex immediately and thoroughly.
The suspension was incubated for 30 minutes at 56°C on a heating block and vortex occasionally.

600 µl gDNA Binding Buffer was added to the sample and mixed thoroughly by pulse-vortexing for 5-10 seconds.

The lysate/binding buffer mix (~600 µl) was transferred to a gDNA Purification Column pre-inserted into a collection tube, without touching the upper column area.

The cap was closed and the suspension centrifuged: first for 3 minutes at 1,000 x g (ca. 6000 rpm) to bind the gDNA

Then the suspension was centrifuged for 1 minute at maximum speed (>12,000 x g) to clear the membrane. The flow-through and the collection tube were discarded.

500 µl gDNA Wash Buffer was added. The cap was closed and the tube inverted a few times so that the wash buffer reached the cap. The suspension was centrifuged immediately for 1 minute at maximum speed, then the flow through was discarded.

The column was placed into the collection tube.
500 µl gDNA Wash Buffer was added and the cap closed. The tube was centrifuged immediately for 1 minute at maximum speed. The collection tube and flow through were discarded.

The gDNA Purification Column was placed in a DNase-free 1.5 ml microfuge tube (Eppendorf). 125 µl preheated (60°C) gDNA Elution Buffer was added, the cap was closed and the suspension incubated at room temperature for 15 minutes.

The tube was centrifuge for 1 minute at maximum speed (> 12,000 x g) to elute the gDNA.

The genomic DNA was stored in the refrigerator at -20°C and 2.5 µl of DNA was checked using gel electrophoresis.

9.8. Gel electrophoresis

For validation of DNA the sample was run on agarose gel (0,8%) & GelRed using flatbed electrophoresis. To each gel pocket 10 µl of sample were applied containing:

1 µl loading dye
10% of reaction volume DNA
up to 10 µl TBE

The gel was run at 100 volts for 45 minutes. Imaging was conducted using the Bio-Rad GelDoc Go Imaging System and Image Lab ™ software.

9.9. Primer design

Forward and reverse primers for the genes *afsR* and *yycF* were designed using the Clone Manager software. The sequence upstream of the start codon of the genes was to be between 20 and 50 nt long. While for *afsR* restriction sites for EcoRI and EcoRV were included, for *yycF* restriction sites for EcoRI and NotI were included. In addition 4 nt overhangs were added to the primers. Tm difference between the primers was set to be less than 5°C, and the highest Tm was set to be below 70°C.

9.10. Polymerase chain reaction

The primers were solved in the TE buffer and diluted to a concentration of 10 pm/ml using dH_2O. The reaction volume was set to 40 µl containing:

gDNA	1 µl
Q5-Reaction Mix	20 µl
forward primer	0.5 µl
reverse primer	0.5 µl
dsH_2O	18 µl

The PCR program for *afsR* was set to following parameters, steps 2-5 were repeated 25 times.

Phase	Time	Temperature
Initialization	1 minute	98°C
Denaturation	20 seconds	98°C
Annealing	1 minute	65 °C
Elongation	2, 5 minutes	72°C
Final Extension	5 minutes	72°C
Hold	∞	4°C

The PCR program for *yycF* was set to following parameters, steps 2-5 were repeated 25 times.

Phase	Time	Temperature
Initialization	1 minute	98°C
Denaturation	20 seconds	98°C
Annealing	1 minute	68 °C
Elongation	30 seconds	72°C
Final Extension	2 minutes	72°C
Hold	∞	4°C

9.11. Plasmid isolation

1 ml of overnight culture was pelleted at full speed for 5 minutes. The pellet was resuspended with 250µl of Cell Resuspension Solution. 250µl of Cell Lysis Solution was added to the sample and inverted 4 times to mix.

10µl of Alkaline Protease Solution was added; inverted 4 times to mix and Incubated 5 minutes at room temperature.

350µl of Neutralization Solution was added; inverted 4 times to mix.

The tube was centrifuge at top speed for 10 minutes at room temperature.

The Spin Column was inserted into the Collection Tube.

The cleared lysate was decanted into the Spin Column.

The tube was centrifuged at top speed for 1 minute at room temperature. The flowthrough was discarded, and the Column reinserted into the Collection Tube.

750µl of Wash Solution (ethanol added) was added and centrifuged at top speed for 1 minute. The flowthrough was discarded, and the column reinserted into the collection tube. The Step was repeated with 250µl of Wash Solution.

The tube was Centrifuged at top speed for 2 minutes at room temperature.

The Spin Column was transferred to a sterile 1.5ml microcentrifuge tube, being careful not to transfer any of the Column Wash Solution with the Spin Column.

60µl of Nuclease-Free Water was added to the Spin Column and centrifuged at top speed for 1 minute at room temperature.

The column was discarded, and the DNA stored at –20°C.

9.12. DNA purification

20 µl of DNA was run on an agarose gel, cut from the gel, recovered using the DNA Clean & Concentration Kit and eluted in 15 µl dH_2O.

9.13. Restriction

pSOK806 was isolated from *E.coli* DH5 alpha and incubated for 1,5 hours at 37°C using the following restriction enzymes. Following restriction, the sample was set to 65°C for 20 minutes to inactivate the enzymes.

pSOK I

pSOK806	4 µl
EcorV HF	0.5 µl
EcorI HF	0.5 µl
CutSmart	2 µl
dsH_2O	13 µl

pSOK II

pSOK806	4 µl
NotI HF	0.5 µl
EcorI HF	0.5 µl
CutSmart	2 µl
dsH_2O	13 µl

Subsequently the restricted DNA fragments of pSOK806, pSOK I and pSOK II, were purified.

The purified PCR products were incubated for 3 hours at 37°C using the following restriction enzymes. Following restriction, the sample was set to 65°C for 20 minutes to inactivate the enzymes.

Cl_19_cut

Cl_19_PCR	7 µl
EcorV HF	0.5 µl
EcorI HF	0.5 µl
CutSmart	2 µl
dsH_2O	10 µl

Cl_27_cut

Cl_27_PCR	7 µl
NotI HF	0.5 µl
EcorI HF	0.5 µl
CutSmart	2 µl
dsH_2O	10 µl

9.14. Ligation

The ligation reaction was conducted over ice overnight using the following components:

Cl_19

Cl_19_cut	10 µl
pSOK I	0.5 µl
T4 Ligase	1 µl
T4 Ligase Puffer	2 µl
dsH_2O	6.5 µl

Cl_27

Cl_27_cut	10 µl
pSOK II	0.5 µl
T4 Ligase	1 µl
T4 Ligase Puffer	2 µl
dsH_2O	6.5 µl

9.15. Transformation to XL-1 Blue cells

The plasmids formed in the ligation process were transformed to competent XL-1 Blue cells. 100 µl of cell suspension was taken from the -80°C storage and put on ice. After approximately 5 minutes the suspension had melted and 10 µl of the ligation mix was added to the tube, gently mixed with the pipette tip and put back on ice for approximately 20 minutes. The cells were exposed to heat shock at 42°C for 1 minute and put back on ice for 10 minutes. After 500 µl of LB had been added and the cells were transferred to an incubator set to 37°C for 80 minutes. 100 µl were spread on a LA plate containing Apramycin, 100 µg/ml. The remaining 500 µl of cell suspension were concentrated to 100 µl and transferred to another LA plate containing Apramycin, 100 µg/ml. The agar plates were incubated overnight at 37°C.
The next day the colonies were picked using a wooden toothpick and transferred to liquid overnight cultures containing Apramycin, 100 µg/ml. From these cell suspensions were prepared.

9.16. Restriction analysis

Restriction reaction was conducted in 1.5 ml Eppendorf tubes for 30 minutes in a water bath set to 37°C using the following components. Subsequently the results were checked by subjecting 2 µl of the sample to gel electrophoresis.

Plasmid DNA	1 µl
Restriction Enzyme HF	0.5 µl
CutSmart Buffer	2 µl
dsH_2O	up to 20 µl

9.17. Transformation to ET-12567 cells

The plasmids isolated from XL-1 blue cells were transformed to competent ET-12567 cells. 100 µl of cell suspension was taken from the -80°C storage and put on ice. After approximately 5 minutes the suspension had melted and 1 µl of the plasmids was added to the tube, gently mixed with the pipette tip and put back on ice for approximately 20 minutes. The cells were exposed to heat shock at 42°C for 1 minute and put back on ice for another 10 minutes. After 500 µl of LB had been added and the cells were transferred to the incubator set to 37°C for 80 minutes. 100 µl were spread on a LA plate containing Apramycin, 100 µg/ml. The remaining 500 µl of cell suspension were concentrated to 100 µl and transferred to another LA plate containing Apramycin, 100 µg/ml. The agar plates were incubated overnight at 37°C.

The following day three colonies were transferred to an LA plate containing three antibiotics: Apramycin 100 µg/ml, Chloramphenicol 25µg/ml and Kanamycin 25 µg/ml using a metal loop and incubated overnight at 37°C. The next day the bacteria were either used for conjugation or transferred to liquid overnight cultures containing Apramycin, 100 µg/ml Chloramphenicol 25µg/ml and Kanamycin 25 µg/ml to prepare cell suspensions.

9.18. Conjugation

50µl of *Streptomyces* stock spore suspension was added to an Eppendorf tube containing 350µl of 2XYT medium. The sample was subjected to 5 to 10 minutes of heat shock at 45°C-50°C and subsequently cooled down to room temperature.

A loop of ET12567 cells freshly cultivated on an LA plate containing apramycin 100 µg/ml, chloramphenicol 25µg/ml and kanamycin 25 µg/ml were transferred to and resuspended in an Eppendorf tube containing 500 µl of 2XYT medium.

50 µl of ET cells suspension was added to 400 µl *Streptomyces* spore suspension, gently tipped and centrifuged for 1 minute at 4000 rpms. The sample was concentrated to 250 µl which were applied to a dry SFM plate containing 1% 1M $MgCl_2$ solution and evenly distributed using a glass spatula. The dried plate was incubated at 28°C for 12 hours.

1 ml of a clear antibiotic solution containing 15 µl of apramycin and 30 µl of nalidixic acid stock solutions was added to the plate containing 30 ml SFM resulting in a concentration of approximately apramycin 50 µg/ml and nalidixic acid 30 µg/ml. The plate was incubated at 28°C for approximately three days.

Six transconjugant colonies were picked with wooden toothpicks and transferred to an SFM plate containing apramycin 50 µg/ml.

After the selection the recombinant *Streptomyces* strains were transferred with a metal loop and cultivated on SFM plates for approximately 3 days. Eventually, glycerol spore suspensions were prepared.

9.19. Fermentation

2.5 ml of a liquid *Streptomyces* overnight culture was added to 22.5 ml fermentation medium in a 250 ml fermentation flask. The culture was then incubated on a shaker set to 28°C for 3 and 7 days respectively.

9.20. Extraction

25 ml of fermentation culture were transferred to a 100 ml round-bottom flask and frozen in an ethanol bath. The flask was attached to a freeze dryer for approximately 24 hours until all the ice had been sublimated. The dried extract was then extracted at room temperature with 25 ml of methanol for one hour on a shaker set to 120 rpms. The extract was eventually concentrated to 1 ml using a rotavapor at 40°C.

9.21. Analytics

9.21.1. Bio-assays

The antibiotic activity of the extracts was tested using disc diffusion bioassays. A volume of 1.5 ml of test organism overnight culture was concentrated to 500 µl and applied to 15 cm diameter plates containing the respective growth medium using a glass spatula and dried for approximately 30 minutes. Then 6 mm antibiotic assay discs to which 25 µl of the extracts and controls (methanol and fermentation medium) had been applied, were placed on the plates using a sterile metal tweezer. The plates were incubated overnight at suitable growth temperatures. The next day the plates were assessed in regard to inhibition zones.

9.21.2. High pressure liquid chromatography

HPLC analysis of the fermentation broth extracts was conducted using the Shimadzu UHPLC system.
For qualitative analysis a volume of 5 µl a 1:25 dilution of the extract was prepared with methanol. The stationary phase was Phenomenex Luna 5 µm C18(2) 100Â LC Column 250 x 4.6 mm. Mobile phase was Acetonitrile / dH_2O formic acid 0.1%. The flow rate was set to 0.5 ml/min. A concentration gradient was used. The concentration of acetonitrile was increased by from 5% to 95% over 45 minutes.

For Semi Preparative HPLC a volume of 3 ml of a 1:62.5 dilution of the extract was applied to a Phenomnex Luna 5 µm C18(2) 100 A LC Column 250 x 10 mm. 1ml/min The fractions were collected and concentrated to 1 ml using a vacuum centrifuge.

9.21.3. Mass spectrometry

MS analysis was conducted in the lab of Dr. Martin Zehl at the Mass Spectrometry Centre (MSC), a Core Facility at the Faculty of Chemistry of the University of Vienna. For further details please refer to Dr. Martin Zehl.

10. Bibliography

Aktories, K., Forth, W. (Eds.), 2013. Allgemeine und spezielle Pharmakologie und Toxikologie: für Studenten der Medizin, Veterinärmedizin, Pharmazie, Chemie und Biologie sowie für Ärzte, Tierärzte und Apotheker; mit 305 Tabellen; [Plus im Web, mediscript], 11., überarb. Aufl. ed. Elsevier, Urban & Fischer, München.

Atanasov, A.G., Zotchev, S.B., Dirsch, V.M., Supuran, C.T., 2021. Natural products in drug discovery: advances and opportunities. Nat. Rev. Drug Discov. 20, 200–216. https://doi.org/10.1038/s41573-020-00114-z

Baltz, R.H., 2016. Genetic manipulation of secondary metabolite biosynthesis for improved production in Streptomyces and other actinomycetes. J. Ind. Microbiol. Biotechnol. 43, 343–370. https://doi.org/10.1007/s10295-015-1682-x

Bérdy, J., 2012. Thoughts and facts about antibiotics: Where we are now and where we are heading. J. Antibiot. (Tokyo) 65, 385–395. https://doi.org/10.1038/ja.2012.27

Blair, J.M.A., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock, L.J.V., 2015. Molecular mechanisms of antibiotic resistance. Nat. Rev. Microbiol. 13, 42–51. https://doi.org/10.1038/nrmicro3380

Blin, K., Shaw, S., Kloosterman, A.M., Charlop-Powers, Z., van Wezel, G.P., Medema, M.H., Weber, T., 2021. antiSMASH 6.0: improving cluster detection and comparison capabilities. Nucleic Acids Res. 49, W29–W35. https://doi.org/10.1093/nar/gkab335

Carattoli, A., 2013. Plasmids and the spread of resistance. Int. J. Med. Microbiol. 303, 298–304. https://doi.org/10.1016/j.ijmm.2013.02.001

Centers for Disease Control and Prevention (U.S.), 2019. Antibiotic resistance threats in the United States, 2019. Centers for Disease Control and Prevention (U.S.). https://doi.org/10.15620/cdc:82532

Chen, Y.-W., X.-C. Liu, F.-X. Lv, and P. Li. 'Characterization of Three Regulatory Genes Involved in Enduracidin Biosynthesis and Improvement of Enduracidin Production in *Streptomyces Fungicidicus*'. *Journal of Applied Microbiology* 127, no. 6 (December 2019): 1698–1705. https://doi.org/10.1111/jam.14417

Cragg, G.M., Newman, D.J., 2013. Natural products: A continuing source of novel drug leads. Biochim. Biophys. Acta BBA - Gen. Subj. 1830, 3670–3695. https://doi.org/10.1016/j.bbagen.2013.02.008

Davies, J., Davies, D., 2010. Origins and Evolution of Antibiotic Resistance. Microbiol. Mol. Biol. Rev. 74, 417–433. https://doi.org/10.1128/MMBR.00016-10

Dodds, D.R., 2017. Antibiotic resistance: A current epilogue. Biochem. Pharmacol. 134, 139–146. https://doi.org/10.1016/j.bcp.2016.12.005

Douafer, H., Andrieu, V., Phanstiel, O., Brunel, J.M., 2019. Antibiotic Adjuvants: Make Antibiotics Great Again! J. Med. Chem. 62, 8665–8681. https://doi.org/10.1021/acs.jmedchem.8b01781

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., Dantas, G., 2012. The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. Science 337, 1107–1111. https://doi.org/10.1126/science.1220761

Frey-Klett, P., Burlinson, P., Deveau, A., Barret, M., Tarkka, M., Sarniguet, A., 2011. Bacterial-Fungal Interactions: Hyphens between Agricultural, Clinical, Environmental, and Food Microbiologists. Microbiol. Mol. Biol. Rev. 75, 583–609. https://doi.org/10.1128/MMBR.00020-11

Gottelt, M., Kol, S., Gomez-Escribano, J.P., Bibb, M., Takano, E., 2010. Deletion of a regulatory gene within the cpk gene cluster reveals novel antibacterial activity in Streptomyces coelicolor A3(2). Microbiology 156, 2343–2353. https://doi.org/10.1099/mic.0.038281-0

Gust, B., Chandra, G., Jakimowicz, D., Yuqing, T., Bruton, C.J., Chater, K.F., 2004. Red-Mediated Genetic Manipulation of Antibiotic-Producing Streptomyces 22.

Hoskisson, P.A., Seipke, R.F., 2020. Cryptic or Silent? The Known Unknowns, Unknown Knowns, and Unknown Unknowns of Secondary Metabolism. mBio 11. https://doi.org/10.1128/mBio.02642-20

Kieser, T. (Ed.), 2000. Practical Streptomyces genetics. Innes, Norwich.

Kong, D., Wang, X., Nie, J., Niu, G., 2019. Regulation of Antibiotic Production by Signaling Molecules in Streptomyces. Front. Microbiol. 10, 2927. https://doi.org/10.3389/fmicb.2019.02927

Krause, J., Handayani, I., Blin, K., Kulik, A., Mast, Y., 2020. Disclosing the Potential of the SARP-Type Regulator PapR2 for the Activation of Antibiotic Gene Clusters in Streptomycetes. Front. Microbiol. 11, 225. https://doi.org/10.3389/fmicb.2020.00225

Laureti, L., Song, L., Huang, S., Corre, C., Leblond, P., Challis, G.L., Aigle, B., 2011. Identification of a bioactive 51-membered macrolide complex by activation of a silent polyketide synthase in Streptomyces ambofaciens. Proc. Natl. Acad. Sci. 108, 6258–6263. https://doi.org/10.1073/pnas.1019077108

Lee, N., Hwang, S., Lee, Y., Cho, S., Palsson, B., Cho, B.-K., 2019. Synthetic Biology Tools for Novel Secondary Metabolite Discovery in Streptomyces. J. Microbiol. Biotechnol. 29, 667–686. https://doi.org/10.4014/jmb.1904.04015

Linares-Otoya, L., Linares-Otoya, V., Armas-Mantilla, L., Blanco-Olano, C., Crüsemann, M., Ganoza-Yupanqui, M.L., Campos-Florian, J., König, G.M., Schäberle, T.F., 2017. Identification

and heterologous expression of the kocurin biosynthetic gene cluster. Microbiology 163, 1409–1414. https://doi.org/10.1099/mic.0.000538

Luo, Y., Enghiad, B., Zhao, H., 2016. New tools for reconstruction and heterologous expression of natural product biosynthetic gene clusters. Nat. Prod. Rep. 33, 174–182. https://doi.org/10.1039/C5NP00085H

Madigan, M.T., Bender, K.S., Buckley, D.H., Sattley, W.M., Stahl, D.A., Brock, T.D., 2022. Brock Biology of Microorganisms, Sixteenth edition, global edition. ed. Pearson Education Limited, Harlow.

Medema, M.H., Fischbach, M.A., 2015. Computational approaches to natural product discovery. Nat. Chem. Biol. 11, 639–648. https://doi.org/10.1038/nchembio.1884

Nepal, K.K., Wang, G., 2019. Streptomycetes: Surrogate hosts for the genetic manipulation of biosynthetic gene clusters and production of natural products. Biotechnol. Adv. 37, 1–20. https://doi.org/10.1016/j.biotechadv.2018.10.003

Nicault, M., Zaiter, A., Dumarcay, S., Chaimbault, P., Gelhaye, E., Leblond, P., Bontemps, C., 2021. Elicitation of Antimicrobial Active Compounds by Streptomyces-Fungus Co-Cultures. Microorganisms 9, 178. https://doi.org/10.3390/microorganisms9010178

Niu, G., Chater, K.F., Tian, Y., Zhang, J., Tan, H., 2016. Specialised metabolites regulating antibiotic biosynthesis in Streptomyces spp. FEMS Microbiol. Rev. 40, 554–573. https://doi.org/10.1093/femsre/fuw012

Ochi, K., Hosaka, T., 2013. New strategies for drug discovery: activation of silent or weakly expressed microbial gene clusters. Appl. Microbiol. Biotechnol. 97, 87–98. https://doi.org/10.1007/s00253-012-4551-9

Olano, C., García, I., González, A., Rodriguez, M., Rozas, D., Rubio, J., Sánchez-Hidalgo, M., Braña, A.F., Méndez, C., Salas, J.A., 2014. Activation and identification of five clusters for secondary metabolites in S treptomyces albus J 1074. Microb. Biotechnol. 7, 242–256. https://doi.org/10.1111/1751-7915.12116

Pacios, O., Blasco, L., Bleriot, I., Fernandez-Garcia, L., González Bardanca, M., Ambroa, A., López, M., Bou, G., Tomás, M., 2020. Strategies to Combat Multidrug-Resistant and Persistent Infectious Diseases. Antibiotics 9, 65. https://doi.org/10.3390/antibiotics9020065

Paradkar, A., Trefzer, A., Chakraburtty, R., Stassi, D., 2003. Streptomyces Genetics: A Genomic Perspective. Crit. Rev. Biotechnol. 23, 1–27. https://doi.org/10.1080/713609296

Ren, H., Wang, B., Zhao, H., 2017. Breaking the silence: new strategies for discovering novel natural products. Curr. Opin. Biotechnol. 48, 21–27. https://doi.org/10.1016/j.copbio.2017.02.008

Ripoll, A., Galan, J.-C., Rodriguez, C., Tormo, N., Gimeno, C., Baquero, F., Martinez-Martinez, L., Canton, R., SEIMC Quality Control Study Group, 2014. Detection of Resistance to Beta-Lactamase Inhibitors in Strains with CTX-M Beta-Lactamases: a Multicenter External

Proficiency Study Using a Well-Defined Collection of Escherichia coli Strains. J. Clin. Microbiol. 52, 122–129. https://doi.org/10.1128/JCM.02340-13

Rodríguez-Rojas, A., Rodríguez-Beltrán, J., Couce, A., Blázquez, J., 2013. Antibiotics and antibiotic resistance: A bitter fight against evolution. Int. J. Med. Microbiol. 303, 293–297. https://doi.org/10.1016/j.ijmm.2013.02.004

Romero-Rodríguez, A., Robledo-Casados, I., Sánchez, S., 2015. An overview on transcriptional regulators in Streptomyces. Biochim. Biophys. Acta BBA - Gene Regul. Mech. 1849, 1017–1039. https://doi.org/10.1016/j.bbagrm.2015.06.007

Rutledge, P.J., Challis, G.L., 2015. Discovery of microbial natural products by activation of silent biosynthetic gene clusters. Nat. Rev. Microbiol. 13, 509–523. https://doi.org/10.1038/nrmicro3496

Sekurova, O.N., Schneider, O., Zotchev, S.B., 2019. Novel bioactive natural products from bacteria via bioprospecting, genome mining and metabolic engineering. Microb. Biotechnol. 12, 828–844. https://doi.org/10.1111/1751-7915.13398

van Wezel, G.P., McDowall, K.J., 2011. The regulation of the secondary metabolism of Streptomyces: new links and experimental advances. Nat. Prod. Rep. 28, 1311. https://doi.org/10.1039/c1np00003a

Walsh, C., 2016. Antibiotics: Challenges, Mechanisms, Opportunities. ASM Press, Washington, DC, USA. https://doi.org/10.1128/9781555819316

Walsh, C.T., Fischbach, M.A., 2010. Natural Products Version 2.0: Connecting Genes to Molecules. J. Am. Chem. Soc. 132, 2469–2493. https://doi.org/10.1021/ja909118a

Wang, T., Bai, L., Zhu, D., Lei, X., Liu, G., Deng, Z., You, D., 2012. Enhancing macrolide production in Streptomyces by coexpressing three heterologous genes. Enzyme Microb. Technol. 50, 5–9. https://doi.org/10.1016/j.enzmictec.2011.09.014

Ward, A., Allenby, N., 2018. Genome mining for the search and discovery of bioactive compounds: The Streptomyces paradigm. FEMS Microbiol. Lett. https://doi.org/10.1093/femsle/fny240

Watve, M., Tickoo, R., Jog, M., Bhole, B., 2001. How many antibiotics are produced by the genus Streptomyces? Arch. Microbiol. 176, 386–390. https://doi.org/10.1007/s002030100345

Wei, J., He, L., Niu, G., 2018. Regulation of antibiotic biosynthesis in actinomycetes: Perspectives and challenges. Synth. Syst. Biotechnol. 3, 229–235. https://doi.org/10.1016/j.synbio.2018.10.005

Wilson, D.N., 2009. The A–Z of bacterial translation inhibitors. Crit. Rev. Biochem. Mol. Biol. 44, 393–433. https://doi.org/10.3109/10409230903307311

Zarins-Tutt, J.S., Barberi, T.T., Gao, H., Mearns-Spragg, A., Zhang, L., Newman, D.J., Goss, R.J.M., 2016. Prospecting for new bacterial metabolites: a glossary of approaches for

inducing, activating and upregulating the biosynthesis of bacterial cryptic or silent natural products. Nat. Prod. Rep. 33, 54–72. https://doi.org/10.1039/C5NP00111K

Zhou, Z., Xu, Q., Bu, Q., Guo, Y., Liu, S., Liu, Y., Du, Y., Li, Y., 2015. Genome Mining-Directed Activation of a Silent Angucycline Biosynthetic Gene Cluster in Streptomyces chattanoogensis. ChemBioChem 16, 496–502. https://doi.org/10.1002/cbic.201402577

Zotchev, S.B., 2008. Antibiotics: Biosynthesis, in: Wiley Encyclopedia of Chemical Biology. John Wiley & Sons, Inc., Hoboken, NJ, USA, p. wecb014. https://doi.org/10.1002/9780470048672.wecb014

11. Acknowledgments

I would like to extend my gratitude to my supervisor Prof. Sergey Zotchev who provided me with a most interesting research topic and the opportunity to conduct this diploma project in his work group and kindly offered his support in matters of bioinformatics and in composing this thesis.

Moreover, I would like to thank Dr. Olga Sekurova who helped me plan and conduct my project and who kindly offered her guidance and counsel during my practical work at the lab and emotional support, when experiments failed.

I am also very grateful to Dr. Olha Schneider whose assistance in conducting the HPLC analysis was greatly appreciated and to Dr. Martin Zehl who conducted the MS analysis of my samples.

In addition, I deeply appreciate the help and support of the entire pharmaceutical biotechnology work group, especially Mag. Fabian Malfent and Mag. Sophie Graffius who were always ready to share their experience and answer my many questions.

Most of all I would like to thank my friends and family who supported me throughout the many years of my studies, kept believing in me and made it all possible.