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Titel | Title

Construction of biosensors detecting secondary metabolites in Streptomyces

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1. Zusammenfassung

Actinomyceten und insbesondere *Streptomyces*, produzieren eine Vielzahl von Naturstoffen, welche vor allem im Bereich der Medizin und Landwirtschaft genutzt werden. Globale Probleme wie der Anstieg der Krebsinzidenz, die zunehmende bakterielle Resistenz gegenüber antimikrobiellen Wirkstoffen und die steigende Resistenz von Organismen gegenüber Pestiziden machen die Entdeckung neuer Wirkstoffe erforderlich. Die Gene, welche für die Produktion eines Sekundärmetaboliten verantwortlich sind, liegen im Genom von *Streptomyces* als Cluster vor. Diese Sequenzen werden als Biosynthese Gencluster (BGC) bezeichnet. Mit Hilfe von genome mining können BGCs identifiziert werden. Viele dieser BGCs produzieren ihren zugehörigen Metaboliten nur unter bestimmten Bedingungen in ihrer natürlichen Umgebung und bleiben unter Laborbedingungen stumm. Es gibt verschiedene Ansätze, um einen stillen BGC zu aktivieren; eine davon ist die Überexpression regulatorischer Gene. Biosensoren können helfen, den analytischen Aufwand nach Aktivierungsexperimenten zu reduzieren.

Für dieses Projekt wurden fünf TetR-ähnliche Repressoren aus Region 2.34 von Streptomyces RLA012, isoliert aus der Rhizosphäre von Leontopodium nivale subsp. alpinum, ausgewählt, um rekombinante Biosensorstämme zu erzeugen, um die Aktivität dieses BGC zu untersuchen. Diese basieren auf dem Reportergen bpsA, welches zur Produktion des blauen Pigments Indigoidin führt. Ein Biosensorstamm zeigte die Produktion von Indigoidin. Um zu untersuchen, ob dies auf die Aktivität des BGC zurückzuführen ist, wurde ein Knockout-Vektor konstruiert. Außerdem wurden Überexpressionsvektoren basierend auf fünf regulatorischen Sequenzen erstellt. Die Einführung des Knockout-Vektors und der meisten Überexpressionsvektoren, mit Hilfe von Conjugation, schlug fehl. Die erzeugten rekombinanten Stämme einer Fermentation unterzogen. Die daraus hergestellten Extrakte wurden mittels Hochleistungsflüssigchromatographie (HPLC) analysiert. Eine Farbveränderung konnte in einem Stamm beobachtet werden. Ein Extrakt zeigte eine leichte Bioaktivität gegenüber zwei Testorganismen in einem Scheibendiffusionstest. Um zu identifizieren, welche Verbindung für die Bioaktivität verantwortlich ist, ob sie mit Region 2.34 oder einem anderen zuvor stillen BGC assoziiert werden kann und ob es sich möglicherweise um eine unbekannte Verbindung handelt, wären weitere Untersuchungen erforderlich.

1. Abstract

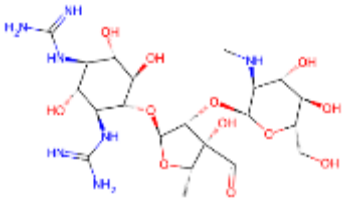
Actinomycetes and in particular *Streptomyces* are prolific bacterial producers of secondary metabolites with widespread usages in medicine and agriculture. Global problems such as the rise of cancer incidence, growing bacterial antimicrobial resistance and increasing resistance of organisms to pesticides call for the discovery of new active ingredients. In *Streptomyces* the genes responsible for the production of a secondary metabolite are clustered together on the genome. These assemblies of neighbouring genes are called biosynthetic gene clusters (BGCs), which can be identified using advanced bioinformatics. Many of these BGCs only generate their corresponding metabolites under certain circumstances in their natural environments and remain silent under laboratory conditions. There are different approaches to activate a silent BGC, one such method being the overexpression of regulatory genes. Biosensors, which can sense the production of BGC-specific metabolite can help to reduce the analytical workload after activation experiments.

For this project 5 TetR like repressors, from region 2.34 of *Streptomyces* sp. RLA012 isolated from the rhizosphere of *Leontopodium nivale* subsp. *alpinum*, were selected to create recombinant biosensor strains based on the reporter gene *bpsA* responsible for production of the blue pigment indigoidine, to examine the activity of a selected BGC. 1 Biosensor strain showed production of indigoidine, and to investigate whether this was due to the activity of the BGC a knockout vector was constructed. Furthermore, overexpression vectors, based on 5 regulatory sequences, were constructed. The introduction of the knockout vector and most overexpression vectors into the biosensor strain, however, failed for unknown reasons. The recombinant strains created through this process were fermented nonetheless and extracts were analysed with high performance liquid chromatography. A change in colour could be observed in 1 strain and one extract showed some bioactivity against 2 test organisms in a disc diffusion assay. To identify which compound is responsible for the bioactivity, and whether it can be linked to a particular BGC in *Streptomyces* sp. RLA012, further investigations are needed.

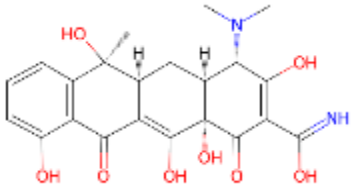
2. Introduction

2.1. Natural products

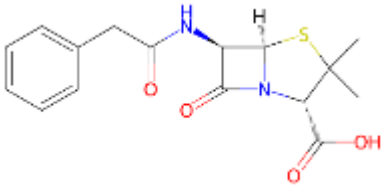
Natural products are a large group of chemically diverse compounds produced by living organisms such as animals, fungi, plants and microorganisms via secondary metabolic pathways. Many of them have found different uses, most notably in human or veterinary medicine and agriculture (Katz & Baltz, 2016; Nahar & Sarker, 2020). Biomolecules synthesized by the metabolic pathways of those organisms can be divided into two groups: primary metabolites and secondary metabolites. Primary metabolites are the compounds which play a role in growth and reproduction of the organism which means they are essential in its survival. Typically, they are polysaccharides, proteins, nucleic and fatty acids present in all living organisms (Elshafie, Camele & Mohamed, 2023). On the contrary, secondary metabolites are not essential for the survival of the organism but facilitate its communication with its environment, providing survival benefits (Seyedsayamdost, 2019). They are structurally extremely diverse, typically of a low molecular weight and can have a wide array of different functions (Bérdy, 2005). In bacteria, for example, they can function as agents of chemical warfare against other microbes, fungi, animals or plants, as metal transporting agents, as factors which facilitate symbiosis between them and other organisms or differentiation effectors (Demain & Fang, 2000). In plants secondary metabolites can function as repellents for microbes and herbivores, mediate their communication with other organisms or as protective factors against abiotic stress (Zaynab et al., 2018). Animals are known to produce fewer secondary metabolites because many of those complex molecules are taken in with food or produced by their microbiomes. Nonetheless, some are produced in humans, for example steroids, prostaglandins, lipids, melanin and neurotransmitters with an array of different functions (Torres & Schmidt, 2019). In fungi they can serve in providing protection from environmental stressors, aid in acquiring nutrients, facilitate virulence against their host or mediate competition (Keller, 2019).



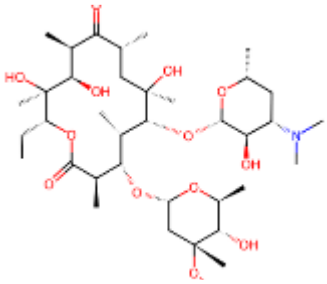
Strepromycin (aminoglycoside, antibiotic) *Streptomyces griseus*



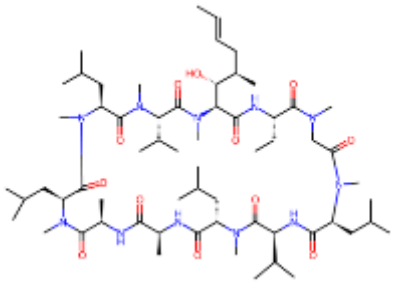
Tetracycline (tetracycline, antibiotic) *Streptomyces griseus*



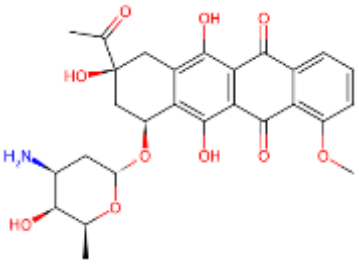
Penicillin G (β-lactam antibiotic) *Penicillium notatum*



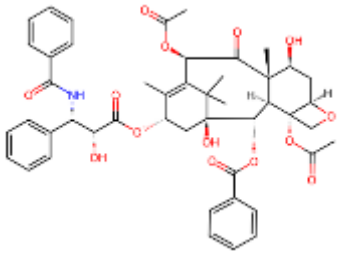
Erythromycin (macrolide, antibiotic) *Saccharopolyspora erythraea*



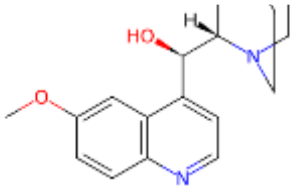
Cyclosporin A (calcineurin inhibitor, immunosuppressant) *Beauveria nivea*



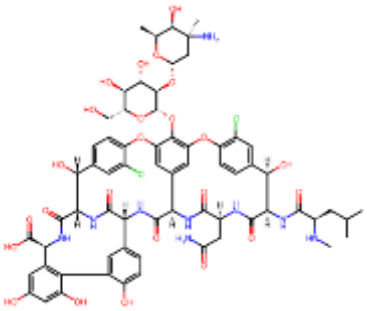
Daunorubicin (Anthracycline, cytostatic) *Streptomyces peucetius*



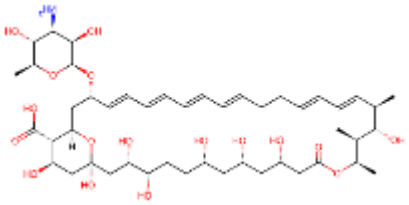
Paclitaxel (Mitotic inhibitor, cytostatic) *Taxus brevifolia*



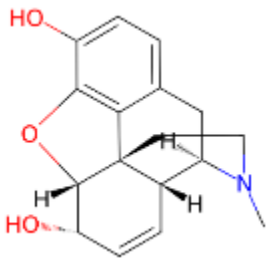
Quinine (antimalarial agent) *Chinchona officinalis*



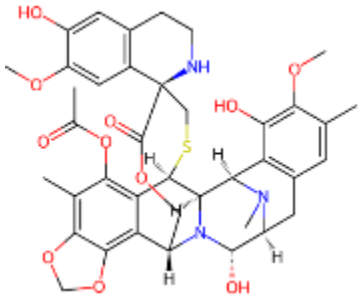
Vancomycin (Glycopeptide, antibiotic) *Streptomyces orientalis*



Nystatin (polyene, antimycotic) *Streptomyces noursei*

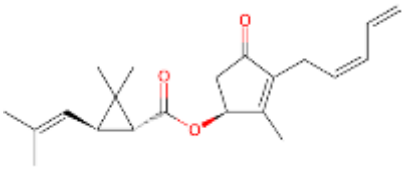


Morphin (opioid agonist, analgesic) *Papaver somniferum*

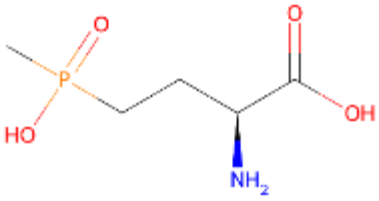


Trabectedin (cytostatic) *Ecteinascidia turbinata*

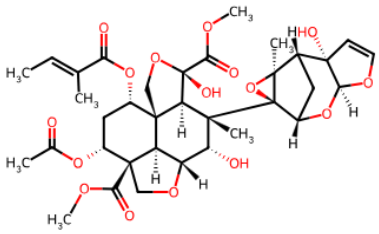
Figure 1: Examples of natural products as clinically used drugs (ChemSpider CSID 18508, n.d.; ChemSpider CSID 10257122, n.d.; ChemSpider CSID 5693, n.d.; ChemSpider CSID 12041, n.d.; ChemSpider CSID 4447449, n.d.; ChemSpider CSID 28163, n.d.; ChemSpider CSID 10368587, n.d.; ChemSpider CSID 84989, n.d.; ChemSpider CSID 14253, n.d.; ChemSpider CSID 23078586, n.d.; ChemSpider CSID 4450907, n.d.; ChemSpider CSID 97236, n.d.)



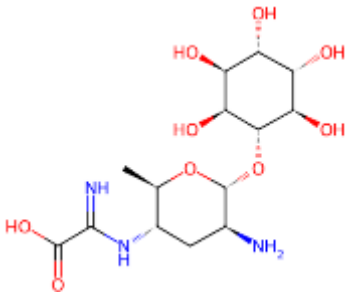
Pyrethrin 1 (insecticide)
Chrysanthemum cinerariaefolium



Phosphinothricin (herbicide)
Streptomyces viridichromogenes



Azadirachtin (Insect growth regulator) *Azadirachta indica*



Kasugamycin (fungicide)
Streptomyces kasugaensis

Figure 2: Examples of natural products as agrochemicals (ChemSpider CSID 4444510, n.d.; ChemSpider CSID 4630, n.d.; ChemSpider CSID 4444685, n.d.; ChemSpider CSID 16736502, n.d.)

2.2. The need for the discovery of new natural products

More than 50% of all pharmaceutical drugs which are currently on the market are either natural products, derived from them or mimic them in some way. Areas of disease in which natural products play the most prominent role are the fields of anticancer and antibacterial therapies (Newman & Cragg, 2019). A similar picture emerges when looking at agriculture, where 17% off all crop-protecting compounds, i.e. fungicides, herbicides, insecticides, acaricides, nematicides and bactericides are natural products and this number rises to 50% when synthetic equivalents of natural products, that have a natural product origin are included (Sparks & Bryant, 2021).

According to the last report of the WHO on cancer from 2020, one in six deaths occurs due to cancer. In 2018 there were an estimated 18 million new cases of cancer reported, and this number predicted to rise to 29-37 million new cases of cancer in the year 2040 (World Health Organization, 2020). Causes for this are the increasing age of the population, progressing environmental pollution, ionizing radiation, chemical pollution to list just a few. A variety of

treatments are already available including chemotherapy, this however often comes with a multitude of severe side effects and the potential of the cancer cells developing resistance (Naeem et al., 2022).

Another severe threat to human health, which calls for new active substances, is the rapid rise of resistance to established antibiotics in different pathogenic bacteria (Review on Antimicrobial Resistance, 2016). In 2019 there have been an estimated 4,95 million deaths associated with bacterial antimicrobial resistance, including 1,27 million deaths directly attributed to bacterial antimicrobial resistance. (Antimicrobial Resistance Collaborators, 2019) According to a review from 2016 the number of deaths caused by bacterial antimicrobial resistance could increase to 10 million deaths per year in 2050 (Review on Antimicrobial Resistance, 2016). Main reason for increasing occurrence of these resistant pathogens is the widespread use and misuse of antibiotics, for example unnecessary prescription of antibiotics by physicians and the intense use of antibiotics as prophylactic or growth promoters in animal farming and agriculture (Venter, Henningsen & Begg, 2017; Harbarth et al., 2015). According to a report from 2022 by 2050 the global food production will have to increase by 50% to face the needs of the growing world population which by then will have reached around 10 billion. 80% of this additional demand will need to come from plant products. Each year 20-40% of global crop production is lost to pests and diseases (Food and Agriculture Organization of the United Nations, Plant Production and Protection Division, 2022). Like the rise of bacterial antimicrobial resistance in human pathogens, there is a severe rise in resistances to pesticides in plant pathogens, insects and weeds. The wide use of agrochemicals puts these organisms under selective pressure which leads to the development of resistances (Beckie et al., 2021). The rise of cancer incidence, growing bacterial antimicrobial resistance and increasing resistance of organisms to pesticides are three examples of problems which call for the discovery of new active ingredients. Previously undiscovered natural products can be a source for those urgently needed new compounds (Newman & Cragg, 2019; Sparks & Bryant, 2021).

2.3. *Streptomyces* species

Streptomyces are a genus of bacteria of the phylum *Actinomycetes.* They are gram-positive and aerobic, and form one of the largest bacterial genera. Numbers of how many species of *Streptomyces* are known vary from source to source but get as high as 1100 and are only rising because new species are discovered and isolated frequently (Lorková et al., 2025; Schlimpert & Elliot, 2023). *Streptomyces* are ubiquitous bacteria primarily known for being found in the

soil. There they play an ecological key role because they can colonize the organic remains of organisms such as fungi and plants, which they then are able to break down and use as nutrients (Chater et al., 2010). *Streptomyces* can also be found in variety of ecological niches, from extreme terrestrial and marine environments to being symbionts of animals and endophytes in plants (Alam et al., 2022).

The genome of *Streptomyces* consists of one linear chromosome in contrast to most bacteria chromosomes which are circular. It typically ranges from 6-12Mbp in size and has a high GC content which usually ranges around 70-74%. The chromosome follows a specific organisation where the so called "core" genes shared by different species of *Streptomyces* are found in the centre of the chromosome, whilst more variable genes are located in the arms of the chromosome. Some *Streptomyces* spp-. also carry one or more linear or circular plasmids (Schlimpert & Elliot, 2023; Lorenzi et al., 2021) Typical *Streptomyces* chromosome contains between 7000 and 10000 genes, of which ca 12% are regulatory genes (Romero-Rodríguez, Robledo-Casados & Sánchez, 2015).

More than half of all natural products with applications in medicine or agriculture originate from *Streptomyces* (Alam et al., 2022), with examples being streptomycin from *Streptomyces griseus* (Schatz, Bugie & Waksman, 1944), tetracycline from *Streptomyces aureofaciens* (Duggar, 1948), nystatin from *Streptomyces noursei* (Hazen & Brown, 1951), doxorubicin from *Streptomyces peucetius var. caesius* (Arcamone, 1969) and phosphinothricin from *Streptomyces viridichromogenes* (Bayer et al., 1972).

Streptomyces have a rather unusual life cycle for a bacterium, as they have a filamentous lifestyle which reminds of fungi, although it is not the same. It starts with the germination of a spore from which one or two germ tubes start growing and developing into hyphae. The hyphae grow by extending at the tip and branching. Those branches then interconnect to form the so called vegetative mycelium or substrate mycelium. If exposed to environmental stressors, such as nutrient depletion, a part of the mycelium undergoes programmed cell death. The dead cells release nutrients which then are used to form aerial hyphae and spores. The initiation of sporulation is linked to the production of secondary metabolites that probably aid the organisms in competing with other microbes (van der Meij et al., 2017) (Figure 3).

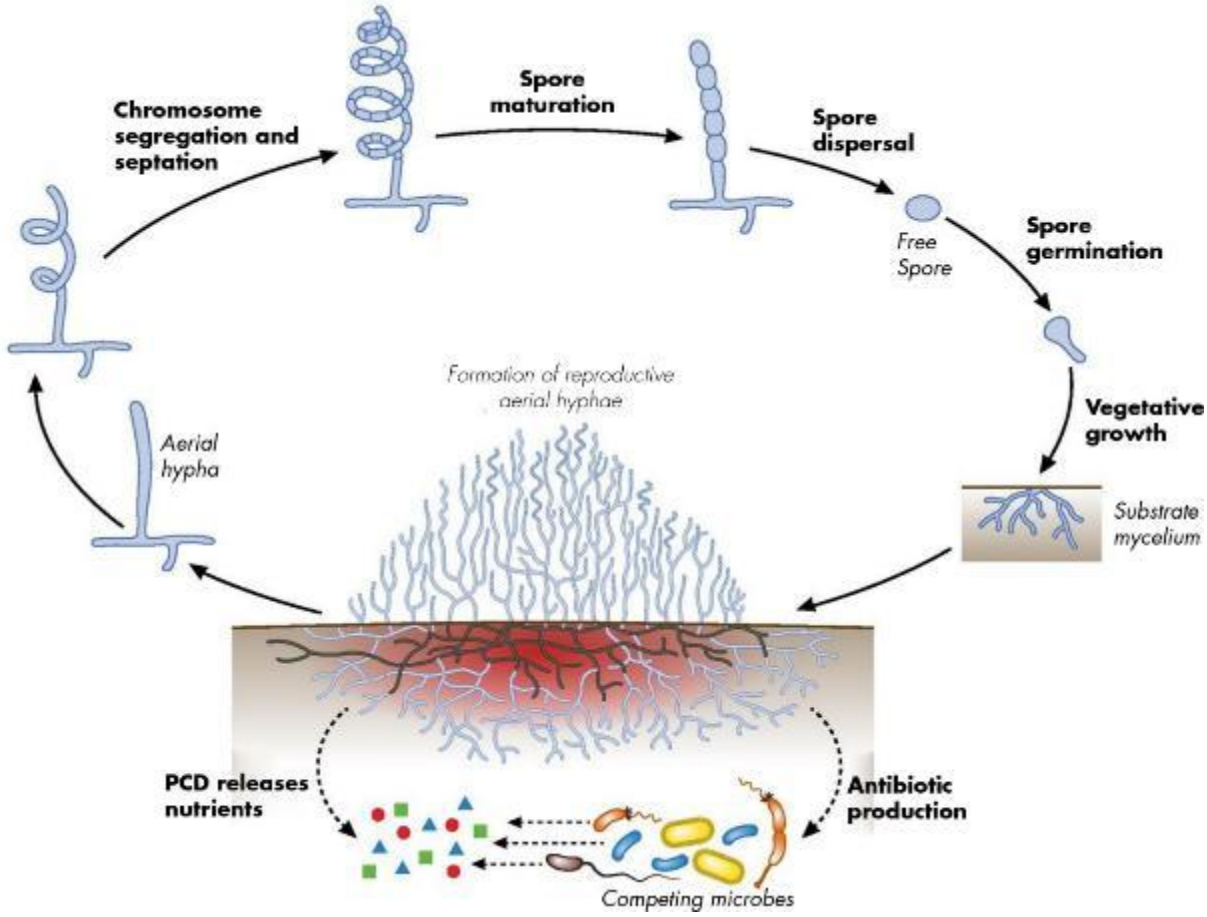


Figure 3: Streptomyces life cycle (van der Meij et al., 2017)

2.4. Biosynthetic Gene Clusters

The biosynthesis of a secondary metabolite in *Streptomyces* is typically controlled by 10-30 genes with different functions, that are located next to each other on the genome. Together, these genes form a so-called biosynthetic gene cluster (BGC) (Zotchev, 2014). Medema et al. (2015) define a BGC as follows: "A BGC can be defined as a physically clustered group of two or more genes in a particular genome that together encode a biosynthetic pathway for the production of a specialized metabolite". A BGC typically contains genes for scaffold synthesis, which are responsible for forming the skeleton of the produced compound, scaffold modification genes such as genes for oxidases, hydrolases, methyltransferases and regulatory genes. Scaffold modification enzymes are often crucial for the bioactivity of produced compound. Often, the product is toxic to the producing cell, so the BGCs may also contains genes for resistance. There are two types known: genes for efflux pumps which transport the

compound out of the cell or genes for a resistance protein that can render the compound inactive (Zotchev, 2014) (Figure 4).

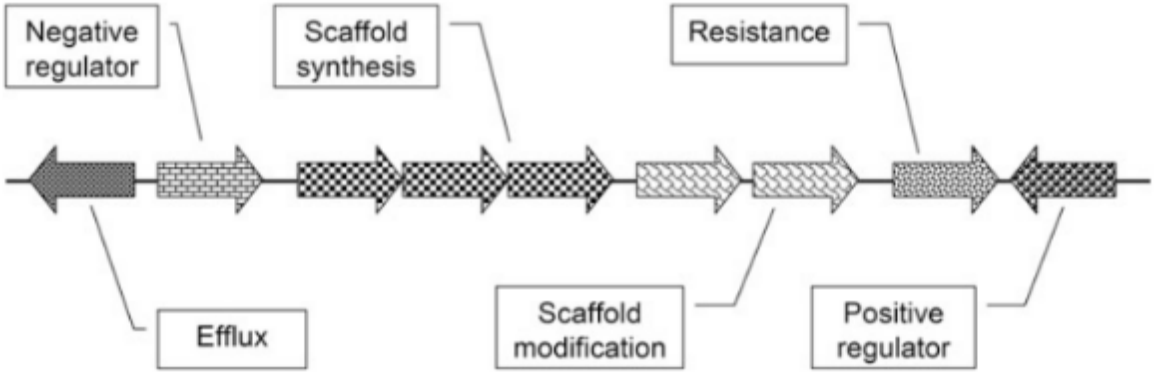


Figure 4: Setup of a BGC containing genes for scaffold synthesis, scaffold modification, regulators and resistance (Zotchev, 2014)

A variety of different molecules can be produced by *Streptomyces* spp., including polyketides (PK), non ribosomal peptides (NRP), terpenoids, lactams and alkaloids (Lacey & Rutledge, 2022). PKs and NRPs are the most commonly produced compounds, assembled by the enzymes called polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS), respectively. (Albarano et al., 2020) The building blocks for the scaffolds of these products are simpler molecules derived from primary metabolism, such as amino acids, acyl-CoAs, sugars, nucleotides etc (Doroghazi & Metcalf, 2013) (Figure 5).

In 2002 the genome of the first *Streptomyces* bacterium, *Streptomyces coelicolor* was sequenced. For decades *Streptomyces coelicolor* was used as a model organism to study genetics etc. and was known to produce 4 secondary metabolites. This genome sequencing, however, revealed 18 more BGCs whose products were not produced under laboratory conditions (Bentley et al., 2002). Typically, a *Streptomyces* genome contains 20-50 BGC of which around 90% are not expressed in laboratory conditions. These BGCs are called silent or cryptic (Liu et al. 2021).

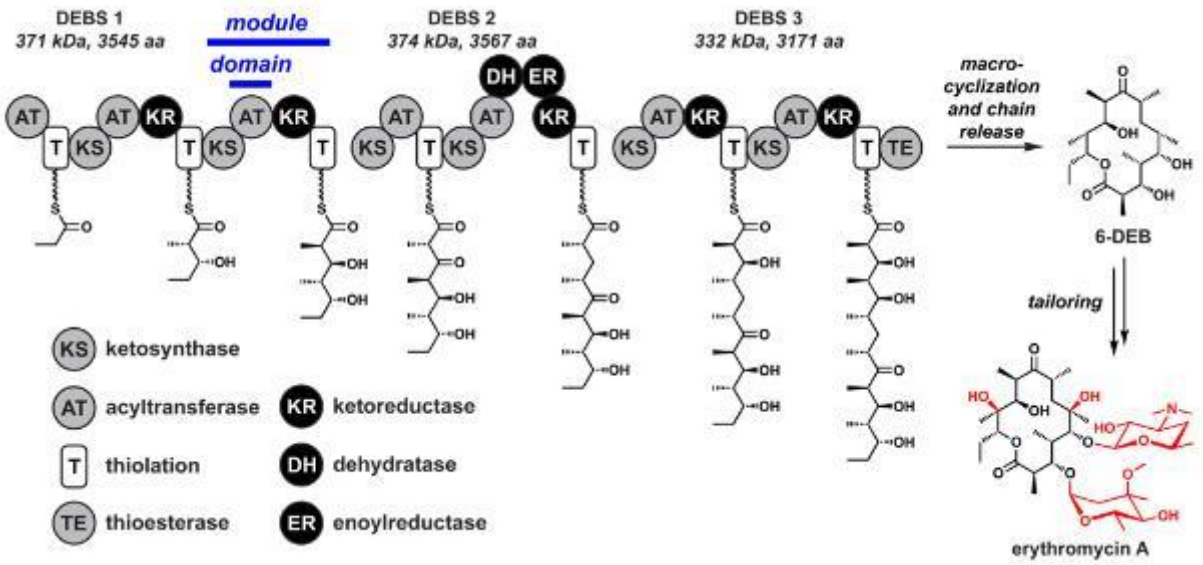


Figure 5: Biosynthesis of a secondary metabolite shown on the example of erythromycin from Saccharopolyspora erythraea. The erythromycin synthase is composed 28 domains organized in 7 modules. Each module is responsible for incorporating one building block into the elongating chain (Walsh & Fischbach, 2010)

2.5. Genome mining

Since the discovery of the first antibiotic penicillin from *Penicillium* by Fleming in 1928/29 (Fleming, 1929) and the discovery of the first effective agent against tuberculosis, streptomycin from *Streptomyces griseus* in 1944 (Schatz et al., 1944) it was clear that microorganisms, most notably the class of *Actinomycetes*, are a rich source for the discovery of potential drugs (Reen et al., 2015). Following these discoveries the antifungal nystatin, the antibacterials erythromycin and vancomycin and the anticancer agent doxorubicin were found in the 1950-1970s in actinomycetes. Those compounds however were relatively easy to discover and represent so called “low hanging fruits”. (Sekurova et.al., 2019). In that time the common approach to the discovery of natural products was a bioactivity-guided screening. This method represents the traditional screening for new compounds and starts with isolation and cultivation of the microorganism, followed by fermentation and extract preparation and bioactivity screening. After that, a stepwise fractionation is done until the pure compound is obtained and, as last step, structure elucidation follows. This approach, however, led to the constant rediscovery of already known compounds (Gaudêncio et al., 2023). The inability to find metabolites encoded by silent BGCs and the high cost of this classical approach gave rise to the practice of genome mining (Bachmann, Van Lanen & Baltz, 2015). Genome mining is a term used to describe bioinformatics, genetic engineering and analytical

chemistry-assisted approach aimed at detecting of previously uncharacterized natural products specified by BGCs within the genome of sequenced organism (Albarano et al., 2020). With sequencing techniques getting more advanced and cheaper, there is now many genomes sequenced and a large amount of data accessible (Shendure & Ji, 2008). A widely used tool for the bioinformatics-guided identification of uncharacterized BGCs is antiSMASH (antibiotics and secondary metabolites analyses shell). This software compares gene products of identified regions with all previously characterized BGCs which are connected to specific molecules. Through this process the software can identify BGCs as well as the genes within and connect them to classes of secondary metabolites like PKs and NRPs and the function of the genes. Sometimes a prediction of the chemical core structure is possible (Medema et al., 2011). Other tools that can be used to automatically identify BGCs are DeepBGC that implements an artificial intelligence approach (Hannigan et al., 2019), CLUSEAN (Weber et al., 2009), SMURF which is a software specialised on fungi (Khaldi et al., 2010) to name just a few examples (Zhu et al., 2025). There are different ways in which the strategy of genome mining can be applied: by the phenotype of the producing organism, by targeting a specific chemical class of product and by the potential uniqueness of the bacterial isolate (Sekurova etl., 2019) (Figure 6).

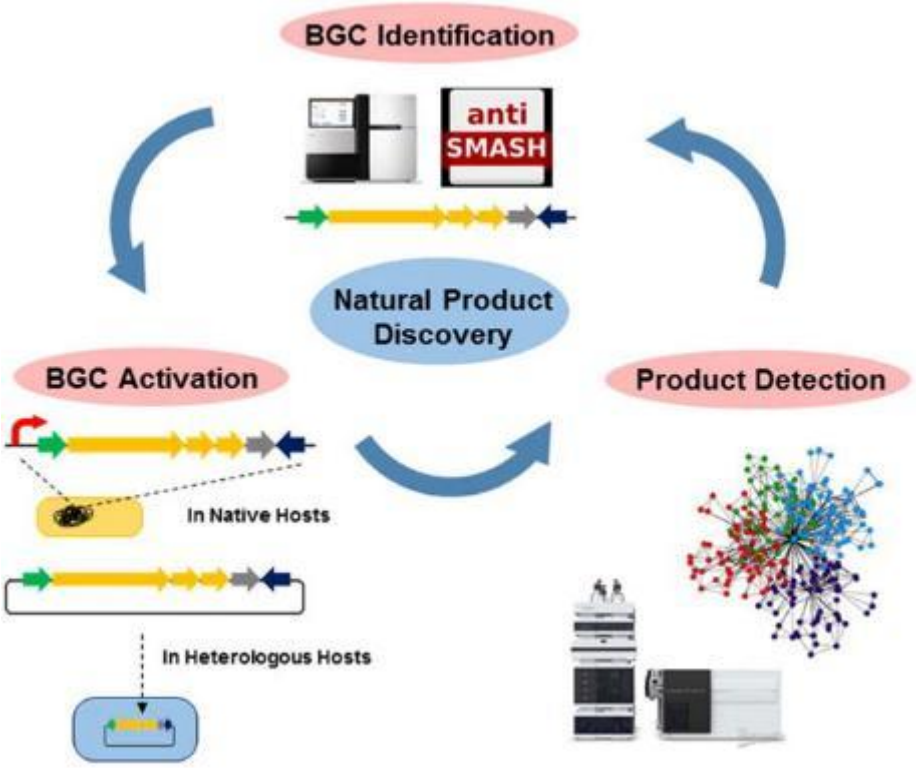


Figure 6: Overview of NP discovery. Identification of silent BGC with bioinformatic tools, activation by different strategies, product characterisation (Ren, Wang & Zhao, 2017)

2.6. Activation of silent BGCs

After the discovery of a silent BGC by means of bioinformatics, the next step is its activation to unravel the identity of the natural product specified by it. Several strategies exist for this purpose (Sekurova et al., 2019). In *Streptomyces* the production of secondary metabolites is controlled by both pleiotropic and pathway specific regulatory genes. This suggests that the production of a secondary metabolite under natural conditions requires specific external and/or internal signals. Those signals, for example environmental factors, are mostly unknown (Bibb & Hesketh, 2009). A change in growth conditions such as different fermentation media, change in pH or different cultivation temperatures that mimic environmental signals, co-cultivation with other microbes or chemical elicitors can lead to the activation of a cryptic BGCs (Zhu et al., 2014).

It is often difficult to cultivate the organism in which a new silent BGC was detected, because the best conditions for its growth and production of secondary metabolites are unknown and to discover those conditions can be a tedious and time-consuming task. The strategy of heterologous expression relies on cloning and expressing the BGC of interest in a host organism which is well studied and easier to handle (Liu et al. 2021). *Escherichia coli* and *Streptomyces* are suitable hosts for heterologous expression, but both bring their own disadvantages. *Escherichia coli* lacks specific precursors and sigma factors for the recognition of promoters, additionally there can be problems with protein folding and toxicity. *Streptomyces* spp. produce their own secondary metabolites and for this reason take away resources needed to synthesize the product of the introduced BGC (Sekurova et al., 2019). Activation of a silent BGC can be achieved by placing a specific positive regulatory gene under the control of a strong constitutive promoter which leads to its heightened expression. This process of promoter replacement is called refactoring (Myronovskyi & Luzhetskyy, 2016). Examples of such promoters are *ermEp** obtained from *Saccharopolyspora erythraea* (Bibb, Janssen & Ward, 1985) and *SF14P* obtained from phage I19 isolated from *Streptomyces ghanaensis* (Labes, Bibb & Wohlleben, 1997). By the overexpression of transcriptional activators or the deletion of negative regulatory genes activation of cryptic BGC can be achieved (Lu et al., 2017).

Another approach is ribosome or RNA polymerase engineering. It is based on the fact that specific mutations in ribosomes or RNA polymerase can lead to an elevated secondary metabolism and production of the compounds hidden in silent BGCs. The approach is rather

straight forward. The targeted organism is exposed to antibiotics and surviving colonies which have spontaneously developed resistances are isolated. Many antibiotics target the ribosome, and some RNA polymerase and therefore a resistance can indicate a ribosomal or RNA polymerase mutation (Zhu, Duan & Huang, 2019).

After a BGC is activated and a potentially new metabolite is produced, it must be isolated, and its structure elucidated. This process, however, relies heavily on a sufficient yield of the new metabolite as well as on high-resolution analytics. The production of many other compounds can obscure the new metabolite, especially if only low levels are produced (Ren, Wang & Zhao, 2017). A biosensor that can indicate whether a BGC was activated or not can help to reduce the analytical workload (Sun et al., 2017).

2.7. Biosensors

According to IUPAC (International Union of Pure and Applied Chemistry) a biosensor is defined as following: “A device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals” (IUPAC, 2025). It generally consists of two components, a bioreceptor like enzymes, RNA aptamers, DNA or antibodies, which interacts with the analyte, a transducer that transforms the biological signal into a physicochemical one (Aliet al., 2020). Often a third component, a signal processing unit which amplifies and presents the signal, typically in digital form, is added (Katey et al., 2023).

Biosensors play an important role in many different fields such as drug discovery, biomedical diagnosis, environmental monitoring, food control etc. (Bhalla et al., 2016) Simonian et al. (2001) presented a biosensor designed to detect pesticide residues, such as organophosphates, in water based on the inhibition of the enzyme acetylcholinesterase that leads to a pH change which is registered by a pH meter. Wang et al. (2023) introduced a biosensor able to detect arginine kinase, an allergen found in shellfish, through the binding to an aptamer and inducing a fluorescence resonance energy transfer (FRET) between the fluorescein labelled aptamer and a polydopamine particle. Pregnancy tests are an example of widely used and commonly known biosensors as they rely on the detection of human chorionic gonadotropin (hCG), a hormone produced with the onset of pregnancy. Antibodies labelled with a colloidal metal such as gold are transported by the urine applied to the test. They bind to hCG and are then captured by different antibodies in the testing zone. The accumulation of the metal particles then leads to a visual signal (Ehrenkranz, 2002). Other uses for biosensors

in biomedical diagnosis are the glucose monitoring in diabetes patients (Zhang et al., 2020), detection of basal cell cancer (Khani & Hayati, 2022), detection of human papillomavirus-16 (Pareek et al., 2023) and many more (Hemdan et al., 2024).

2.7.1. Biosensors in drug discovery

Biosensors in drug discovery can be used to signal whether a BGC is active or not and in the follow up to detect if the activation of a cryptic BGC was achieved. Widely used biosensors concepts are FRET-sensors, riboswitches and transcription factor (TF) based biosensors. They differ in their functional principle and application. TF based biosensors are suited best to indicate the activation of a silent BGC and production of a potentially new metabolite (Zhang, Jensen & Keasling, 2015). TFs are regulatory proteins that bind to DNA and can act as repressors or activators for transcription. The activity of TFs can be affected by an array of different signals such as interaction with small molecules, change in pH, change in temperature etc. (Mahr & Frunzke, 2015). By placing a reporter gene under the control of the TF, its activity can be made visible (Fernandez-Lopez et al., 2015). One of these TFs is the repressor TetR. Resistance to tetracycline in gram negative bacteria comes about through the efflux pump TetA, whose gene is repressed by TetR which is released from its operator when tetracycline binds, in the follow up TetA is expressed and transports tetracycline out of the cell (Berens & Hillen, 2003). Within a multitude of BGCs from many different *Streptomyces* genes encoding TetR-like repressors have been reported (Pei, Lei & Zhang, 2024), leading to the suggestion that they could play a role in sensing cluster-specific metabolites. It has been shown that some of these TetR-like repressors are able to bind the end products of BGC-specific pathways. When a reporter gene is placed under the control of the promoter/operator region controlled by a TetR-like repressor, a detectable signal, such as the production of a pigment, can be obtained when the metabolite encoded in the BGC binds to the TetR-like repressor and thereby releases it from its DNA binding site further indicating the production of said metabolite. Figure 7 shows this process: A BGC is activated, which leads to the production of a secondary metabolite it specifies. This metabolite binds TetR which then is released from its DNA-binding site and stops repressing the reporter gene. The reporter gene is expressed and generates a detectable signal (Sun et al., 2017). Examples for such reporter genes are *xylE* (Zukowski et al., 1983)*, bpsA* (Müller et al., 2012)*, gusA* (Platteeuw, Simons & de Vos, 1994) etc. The *bpsA* gene is responsible for the production of the blue pigment indigoidine (Müller et al., 2012).

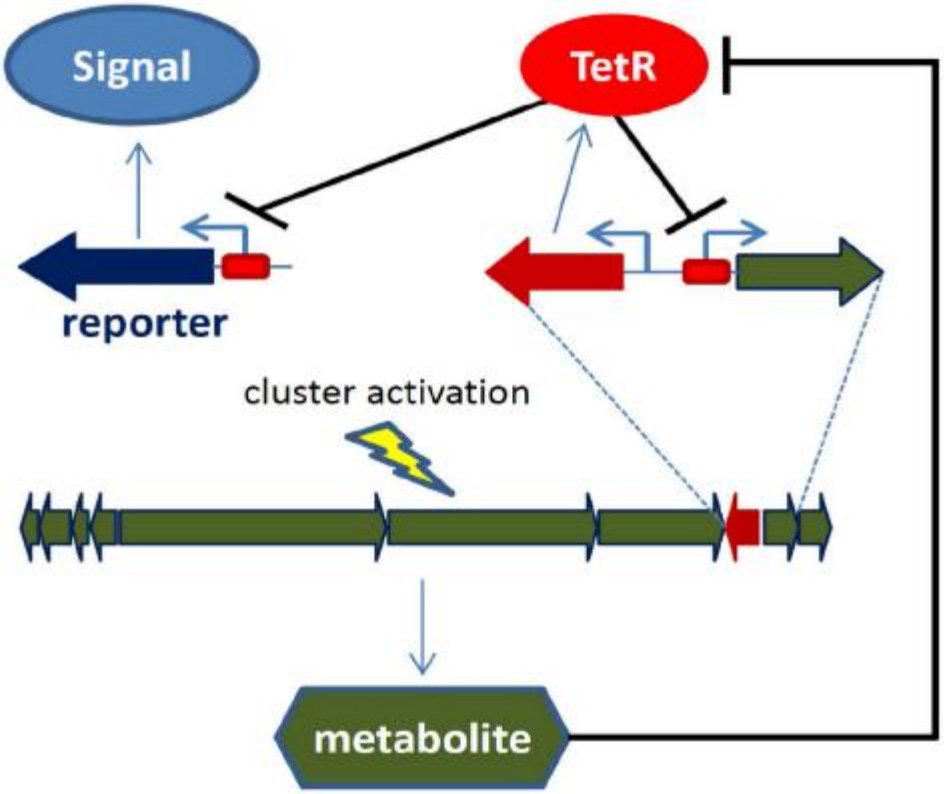


Figure 7: After activation of the silent BGC the product binds TetR. TetR stops repressing the reporter gene leading to a detectable signal (Sun et al., 2017)

2.8. Aim of this project

Streptomyces sp. RLA012 isolated from the rhizosphere of *Leontopodium nivale* subsp. *alpinum* (Oberhofer et al, 2019) was the subject in this project, in particular its BGC 2.34, which might specify biosynthesis of a novel natural product. The aim of this project was to use 5 TetR-like repressors from BGC 2.34 to construct biosensor systems based on the reporter gene *bpsA* to investigate the activity of this BGC. Further, overexpression vectors will be constructed with the aim to activate this BGC and induce production of its cognate metabolite, which in the end could lead to the discovery of a novel compound with potential to be developed into a new drug.

3. Results

3.1. antiSMASH data

antiSMASH analysis was performed by the lab and provided for this work. *Streptomyces* sp. RLA012 contains 45 distinct BGC. For this project region 2.34 was chosen. The region potentially contains 3 sets of genes encoding scaffold-building proteins: CC50 (T1PKS-NRPS-like-NRPS), CC51 (T1PKS-NRPS-like), CC52 (NRPS). It is not certain whether this region contains 1 big cluster or 2 separate clusters (Figure 8). The region shows 28% homology to the rimosamide BGC from *Streptomyces rimosus* subsp. *rimosus* ATCC 10970. Figure 9 shows the promoter/operator regions of 5 putative TetR like repressors, selected to create the biosensor system (Results 3.4.). Figure 10 shows the regulatory sequences selected to create overexpression vectors (Results 3.6.) and gene selected to create the knockout vector (Results 3.7.).

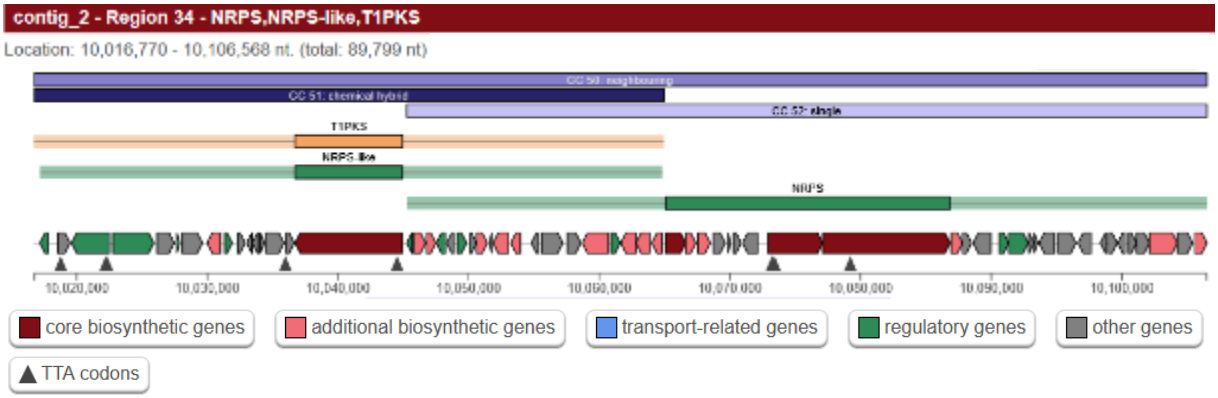


Figure 8: Region 2.34 of Streptomyces RLA012.

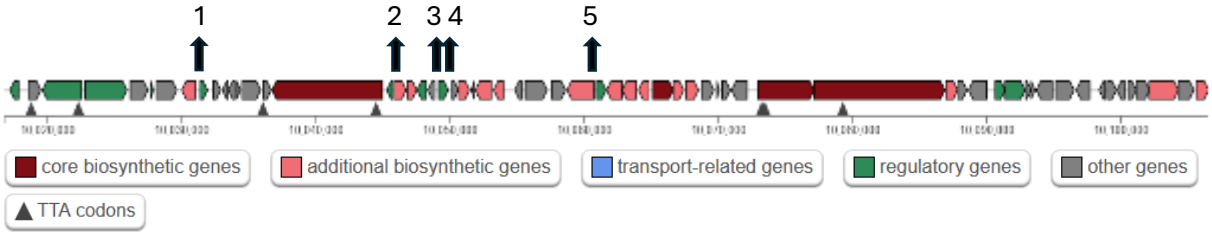


Figure 9: promoter/operator regions of 5 putative TetR like repressors, selected to create the biosensor system. Annotated in antiSMASH as: 1(8970)= TetR family transcriptional regulator, 2(8980)= transcriptional regulator, 3(8983)= response regulator, 4(8985)= response regulator, 5(8993)= TetR family transcriptional regulator

Figure 10: 1, 2, 3, 5 and 6 are regulatory genes selected to create overexpression vectors. Annotated in antiSMASH as: 1(8963)= autoinducer-binding transcriptional regulator, 2(8965)= LuxR family DNA-binding response regulator, 3(8966)= LuxR family transcriptional regulator, 5(8979)=transcriptional regulator, 6(8984)= response regulator. 5(8978)= T1PKS/NRPS-like shows the gene for which an intergenic region was amplified to create a knockout vector.

3.2. Growth experiments with *Streptomyces* sp. RLA012

1. Solid media

Growth experiments to gain spore suspensions were conducted on different media (ISP4, SFM, ISP5, 5326, 5081, 5402, 5336, ISP2, CP6, LA, Bennet´s; Materials 5.1.1.) according to Methods 6.3.1. It was established that ISP4 medium was best suited for growth.

Tabel 1: Results of growth experiments

Medium	Sporulation	Time to sporulation (d)
ISP2	+	9+
ISP4	+++	7-8
ISP5	-	
5081	-	
5326	++	8+
5336	+	11+
5402	-	
LA	-	
Bennet´s	-	
CP6	+	10
SFM	-	

2. Liquid media

Growth experiments in liquid culture were conducted in TSB, ISP4, LB, 2XYT, MYM, YEME (Materials 5.1.2) according to Methods 6.3.1. LB was established to be best suited.

Table 2: Results of growth experiments in liquid culture

Medium	Growth 2d/7d		Aggregates	Pigment 2d/7d	
TSB	-	++	small, round	-	++ redbrown
ISP4	-	+++	big, round	-	+ brown
LB	+	+++	small, round	-	+ brown
2XYT	-	+	very small	-	++ light brown
MYM	-	++	small, oblong	-	++brown
YEME	-	-	-	-	-

3.3. Biosensor systems

5 putative genes for TetR like repressors in the region of interest, BGC 2.34, were selected to place the reporter gene *bpsA* (indigoidine synthase) under the control of their promoter/operator regions (Results 3.1.). Some TetR like repressors are known to bind the end products and/or intermediates of BGC-specific biosynthetic pathways. After constructing biosensor vectors containing *bpsA* and the PCR-amplified promoter/operator regions and transforming *E. coli* cells with them, they were introduced to *Streptomyces* sp. RLA012 via conjugation, to test the activity of the BGC 2.34. With the introduction of overexpression vectors to recombinant strains it was attempted to activate the silent BGC.

3.4. Construction of biosensor vectors

3.4.1. Isolation of genomic DNA

To PCR-amplify the putative operator/promoter regions of the TetR like repressors, first isolation of the genomic DNA of *Streptomyces* sp. RLA012 was done as described in Methods 6.5.1. After isolation the DNA was checked on an agarose gel to test its integrity (Methods 6.6.1.).

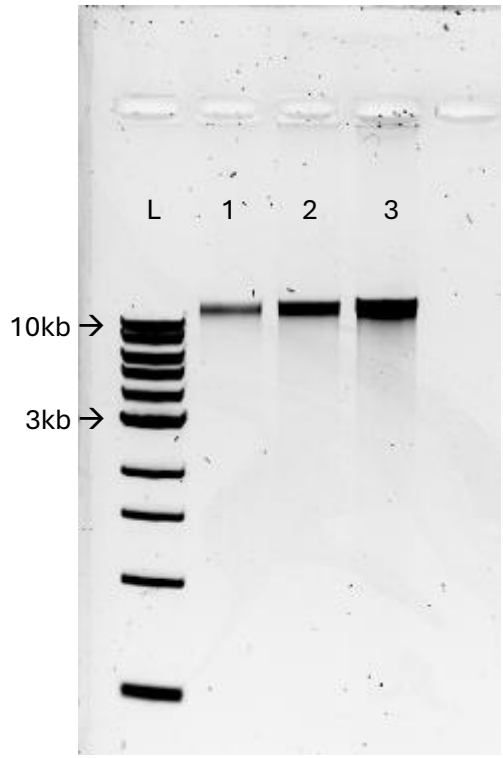


Figure 11: Lanes 1, 2 and 3 are 1µl, 2µl and 5µl of gDNA of Streptomyces sp. *RLA012, L is the ladder: Quick-Load® 1 kb DNA Ladder (Methods 6.6.1.)*

3.4.2. PCR for biosensor vectors

Primers needed to amplify promoter/operator regions of the 5 TetR-like repressor encoding genes were designed by Sergey Zotchev and synthesized at Eurofins. PCR to amplify the putative promoter/operator regions was performed as described in Methods 6.5.3. After PCR, gel electrophoresis was performed (Methods 6.6.1) to verify if correct products were generated. Then the correct bands were cut out and extracted from the gel as described in Methods 6.6.2.

Product 8993 could not be obtained in the first run, so annealing temperature was lowered to 65°C and the reaction repeated, then also that product could be amplified.

Table 3: 5 different promoter/operator regions were amplified and used to construct biosensor vectors

PCR product	Size (bps)	Product confirmed
8970	240	+
8980	158	+
8983	310	+
8985	276	+
8993	230	+

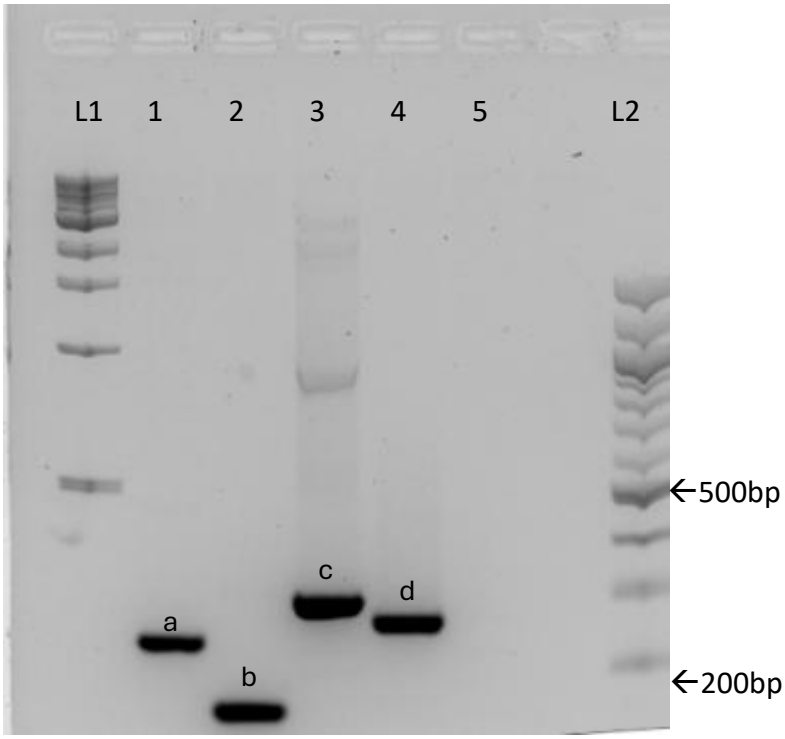


Figure 12: PCR reactions for biosensor vectors. 1a=PCR8970 (240 bps), 2b=PCR8980 (158 bps), 3c=PCR8985 (310 bps), 4d=PCR8993 (276 bps), L1= Quick-Load® 1 kb DNA Ladder, L2= Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.)

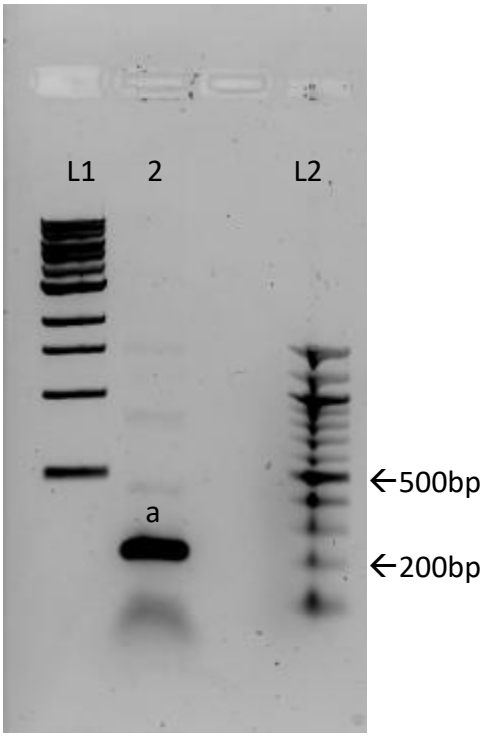


Figure 13: 1a=PCR8993 (240 bps), L1= Quick-Load® 1 kb DNA Ladder, L2= Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.)

3.4.3. Construction of biosensor vectors

The amplified promoter/operator regions were inserted to the plasmid pBGC1.9 provided by Olga Sekurova, by digesting the PCR products and the vector with *Bam*HI and *Spe*I restriction enzymes, followed by purification of digestion products and ligation. After ligating the vector fragment and the PCR products, the ligation mixtures were introduced into Escherichia coli, plasmids isolated from obtained clones, and thier restriction analyses were performed (Methods 6.5.4.) and checked by gel electrophoresis (Methods 6.6.1). Maps of all constructs can be found in Materials 5.3.

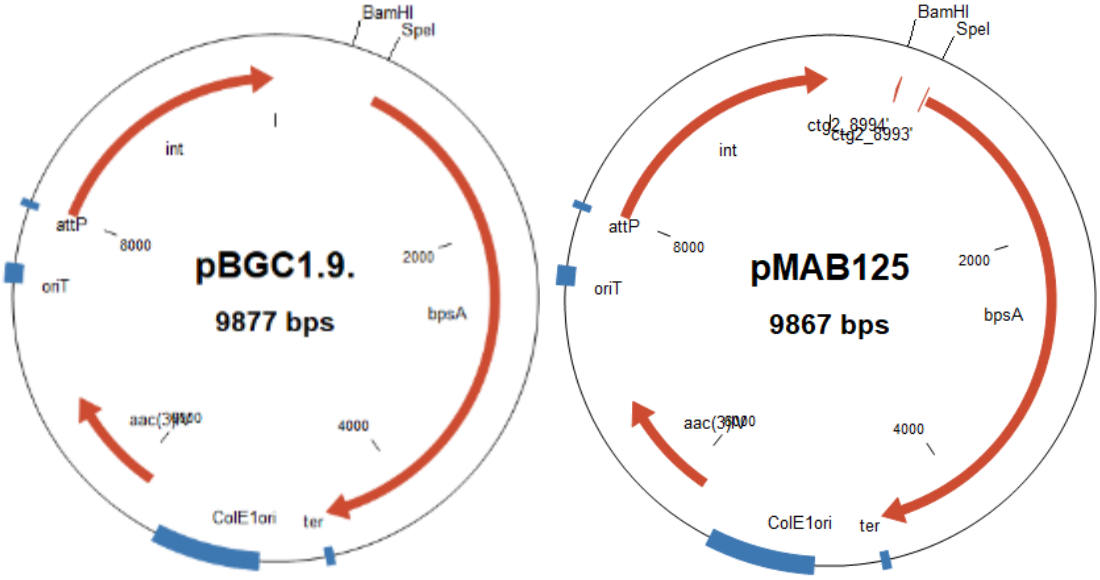


Figure 14: Vector maps (created using clone manager Methods 6.1.2.) of vector pBGC1.9. and pMAB125 containing inserted PCR fragment 8970 (ctg2_8994' - ctg2_8993') as an example for created biosensor vectors.

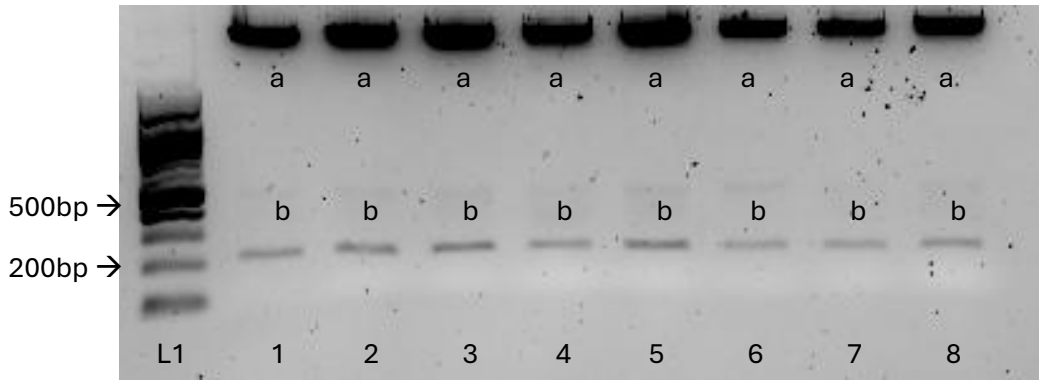


*Figure 15: Restriction analysis of constructed vector pMAB121. 1a-8a are pBGC1.9. cut with Bam*HI*/Spe*I *(9651 bps) and 1b-8b are the inserted PCR product 8970 (226 bps), L1=Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.).*

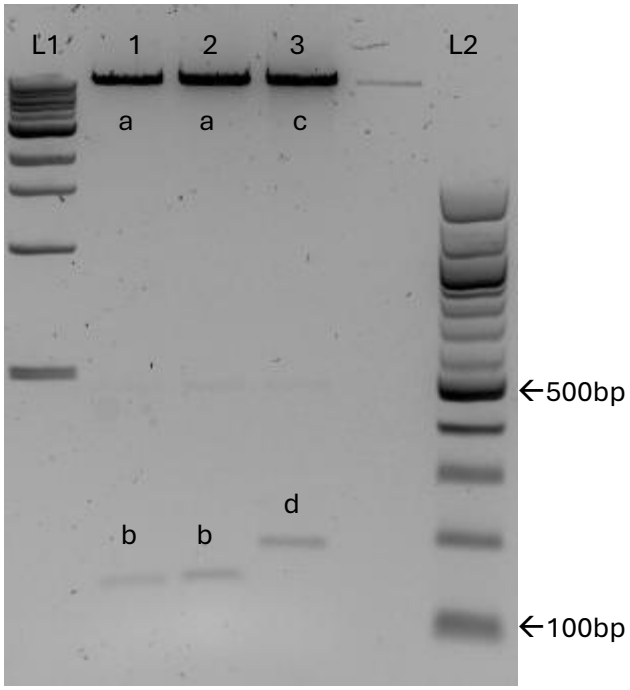


*Figure 16: Restriction analysis of constructed vector pMAB122. 1-2 are pMAB122 cut with Bam*HI*/Spe*I *and 3 is pBGC1.9. cut with BamHI/speI as control. 1a-2a are pBGC1.9. cut with Bam*HI*/Spe*I *1b-2b are inserted PCR product 8980 (144 bps). 3c is pBGC1.9. cut with Bam*HI*/Spe*I *and 3d is the resulting fragment (226 bps). L1= Quick-Load® 1 kb DNA Ladder, L2= Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.)*

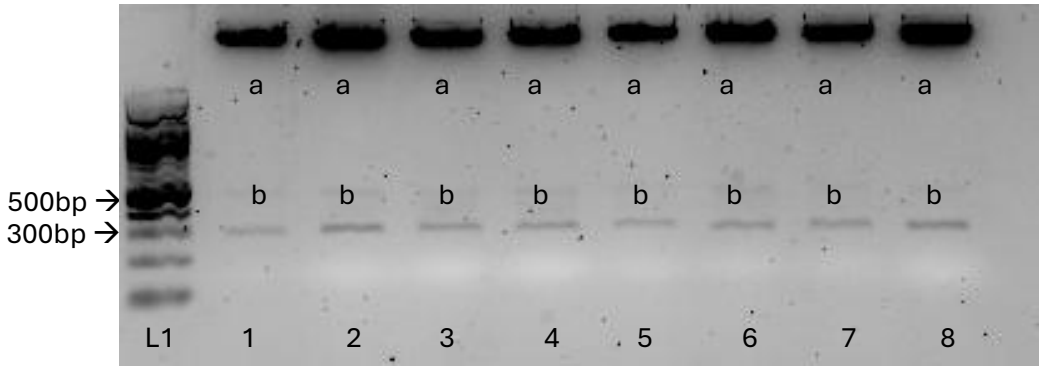


*Figure 17: Restriction analysis of constructed vector pMAB123. 1a-8a are pBGC1.9. cut with Bam*HI*/Spe*I *(9651 bps) and 1b-8b are the inserted PCR product 8983 (296bps), L1=Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.).*

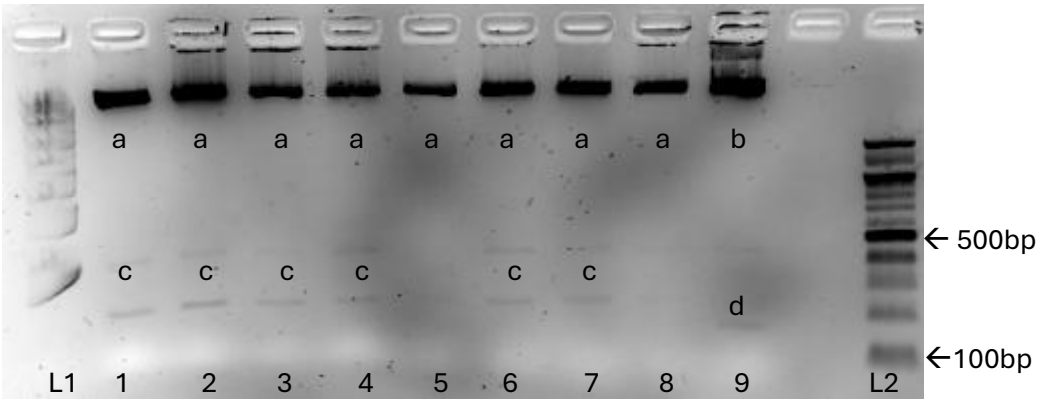


*Figure 18: Restriction analysis of constructed vector pMAB124. 1-8 are pMAB124 cut with Bam*HI*/Spe*I *and 9 is pBGC1.9. cut with Bam*HI*/Spe*I *as control. 1a-8a are pBGC1.9. cut with Bam*HI*/Spe*I *1c-7c are inserted PCR product 8985 (262 bps). 9b is pBGC1.9. cut with Bam*HI*/Spe*I *and 9d is the resulting fragment (226 bps). In lane 5 and 8 correct clones couldn´t be confirmed. L1= Quick-Load® 1 kb DNA Ladder, L2= Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.)*

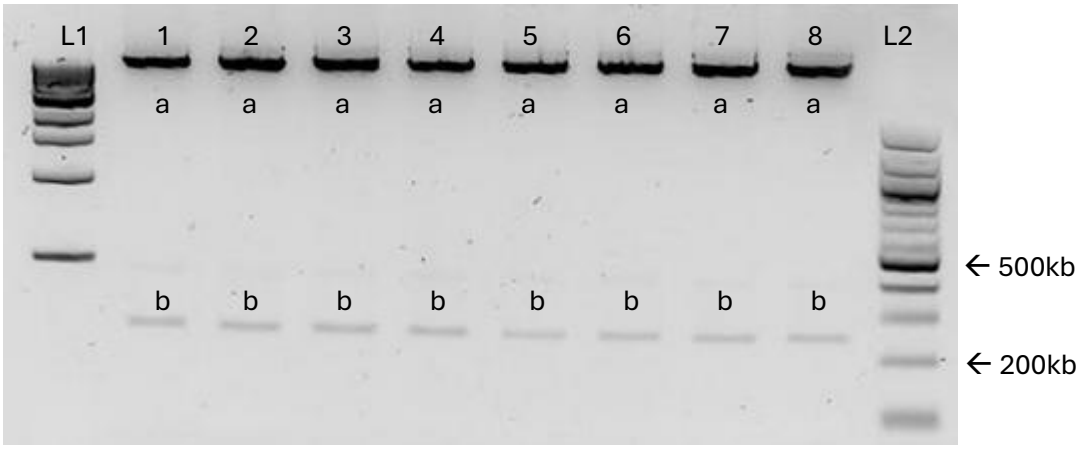


*Figure 19: Restriction analysis of constructed vector pMAB125. 1a-8a are pBGC1.9. cut with Bam*HI*/Spe*I *(9651 bps) and 1b-8b are the inserted PCR product 8993 (216 bps). L1= Quick-Load® 1 kb DNA Ladder, L2= Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.)*

3.5. Conjugation with biosensor vectors

All 5 successfully constructed biosensor vectors, positive control vector and negative control vector (see Materials 5.3. for a list of vectors and their properties) were introduced to *Streptomyces* sp. RLA012 via conjugation (Methods 6.5.7.). This resulted in the recombinant biosensor strains RB1, RB2, RB3, RB4, RB5, R240 (positive control), R152 (negative control). Properties of the strains can be found at Materials 5.2.1.

Single colonies of RB1, RB2, RB3, RB5 growing after the conjugation process had a brown colour like those of the negative control R152. Single colonies of RB4 showed a blue colour like those of RB240. This indicates the expression of the gene controlled by 8985 (Figure 9).

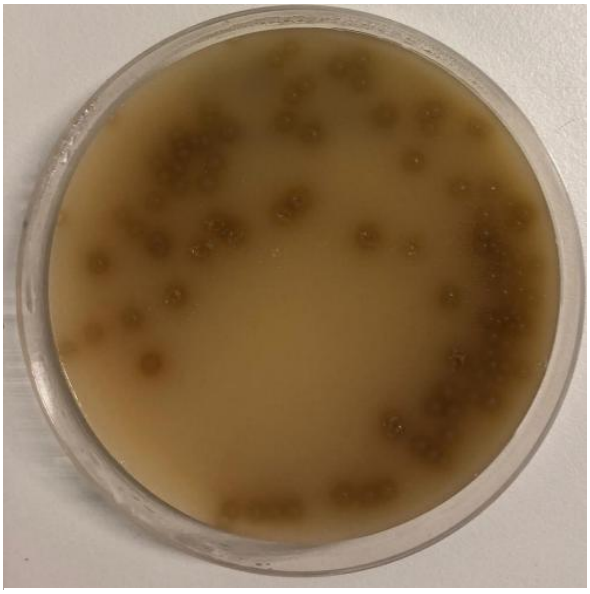


Figure 20: Single colonies of RB2 showing a brown colour

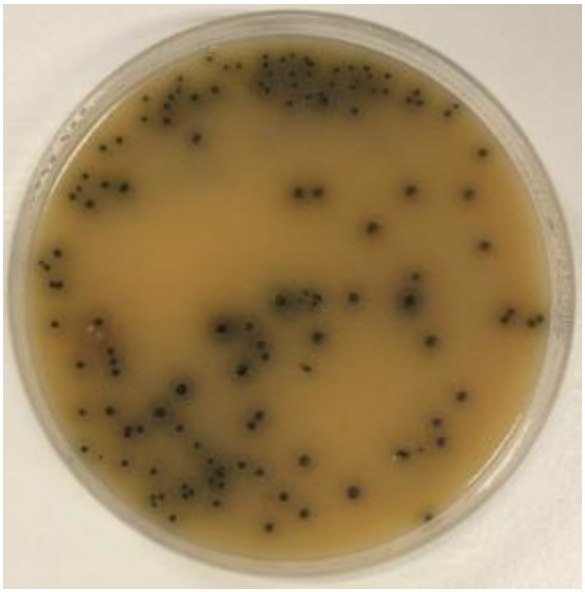


Figure 21: Single colonies of R240 showing dark brown/blue pigmentation.

3.6. Construction of overexpression vectors

To construct overexpression vectors, 5 putative regulatory genes identified using antiSMASH (Results 1.1.), were placed under control of the strong constitutive promoter PermE* to achieve overexpression of said regulatory sequences when the vectors are introduced into *Streptomyces* sp. RLA012.

3.6.1. PCR for overexpression vectors and knock out vector

Primers for amplification of regulatory genes and knockout DNA fragment were designed by Sergey Zotchev and synthesized at Eurofins. PCR to amplify the regulatory regions and the intragenic region of gene 8978 for the knockout vector (Results 3.7.) was performed as described in Methods 6.5.3. After PCR, gel electrophoresis was performed (Methods 6.6.1.) to verify if correct products were amplified. Then the correct bands were cut out and extracted from the gel as described in Methods 6.6.2. For gDNA the same samples can be seen in Results 3.4.1. were used.

Table 4: 5 different regulatory regions were amplified and used to construct overexpression vectors

PCR product	Size (bps)	Product confirmed
8963	767	+
8965	3102	+
8966	3318	+
8979	589	+
8984	839	+

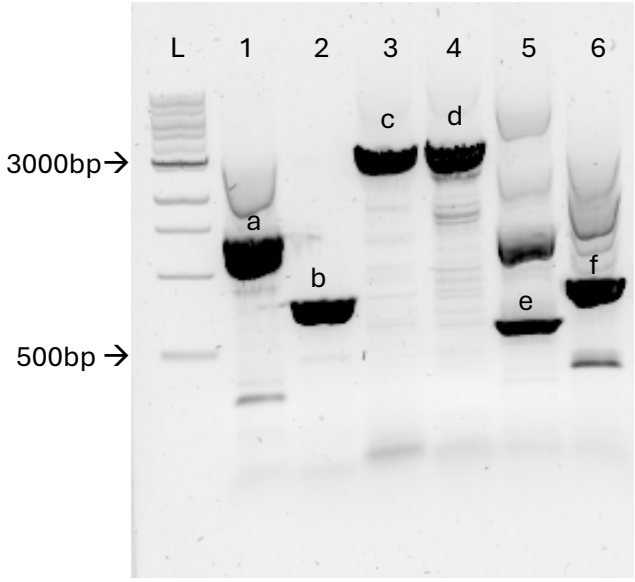


Figure 22: PCR reactions for overexpression vectors and knockout vector (Results 3.7.), 1a=PCR8978 (1336 bps) (Results 3.7.), 2b=PCR8963 (753 bps), 3c=PCR8965 (3088 bps), 4d=PCR8966 (3304 bps), 5e=PCR8979 (575 bps), 6f=PCR8984 (825 bps) L= Quick-Load® 1 kb DNA Ladder (Methods 6.6.1.)

3.6.2. Construction of overexpression vectors

To construct overexpression vectors plasmids pSOK911 and pSOK806 provided by Olga Sekurova were digested with *Eco*RI/*Sfi*I. Cutting pSOK806 resulted in 2 fragments (5383 bps, 371 bps) of which the smaller one (371 bps) contained the PermE* promoter. Cutting pSOK911 also resulted in 2 fragments (6728 bps, 110 bps). The 371bps fragment of pSOK806 and the 6728bps fragment of pSOK911 were then ligated (Methods 6.5.4.) to create plasmid pSOK912. The amplified regulatory genes were inserted to the plasmid pSOK912, by cutting the PCR products (Results 3.6.1.) and the plasmid with the restriction enzymes *Eco*RI/*Spe*I, followed by purification and ligation. After transforming the ligation mixture to E. coli and isolation of plasmids from obtained clones, their restriction analyses were performed (Methods 6.5.4.) and checked by gel electrophoresis (Methods 6.6.1). Maps of all constructs can be found in Materials 5.3.

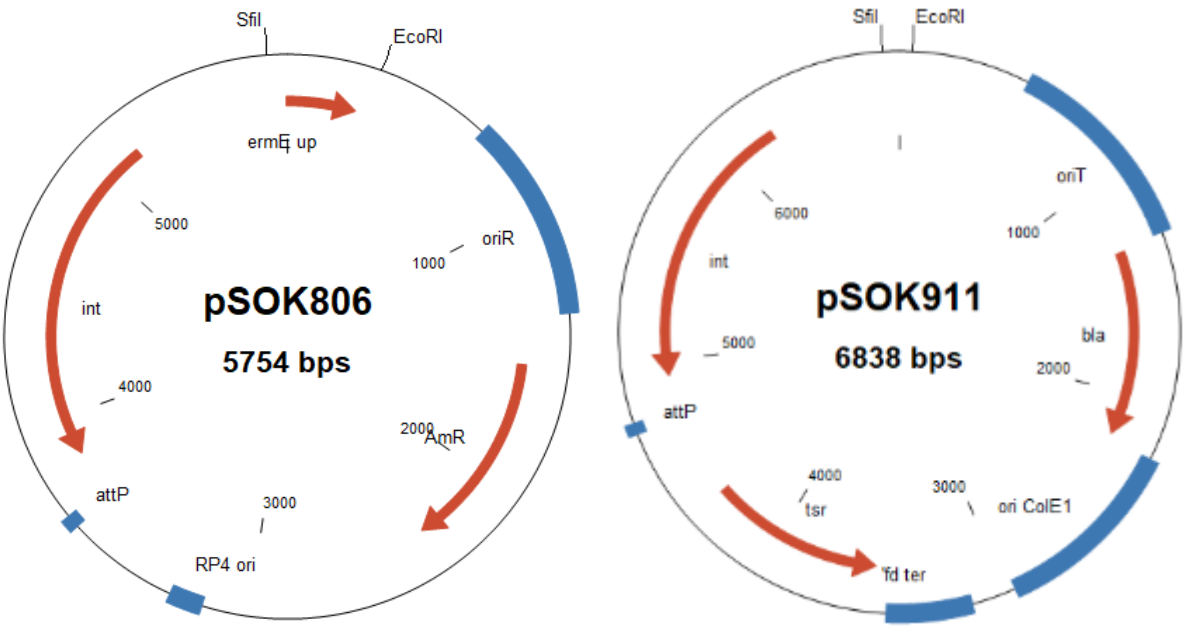


Figure 23: Vector maps of vectors pSOK806 and pSOK911

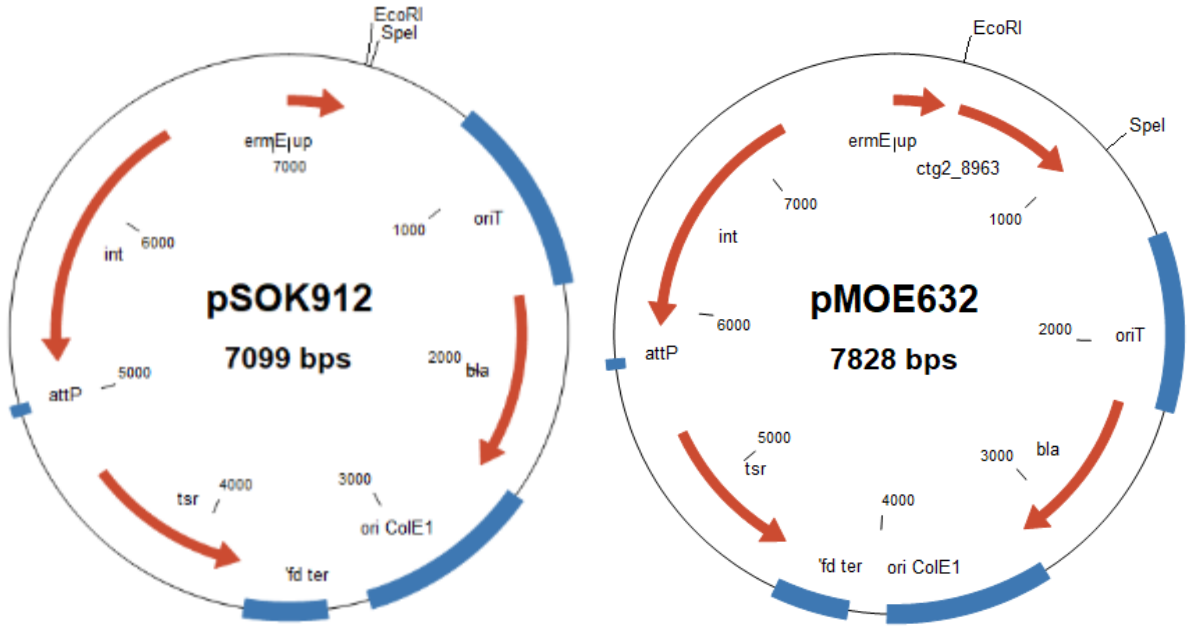


Figure 24: Vector maps (created using Clone Manager, Methods 6.1.2.) of vector pSOK912 and pMOE632 containing inserted PCR fragment 8963 (ctg2_8963) as an example for created overexpression vectors.

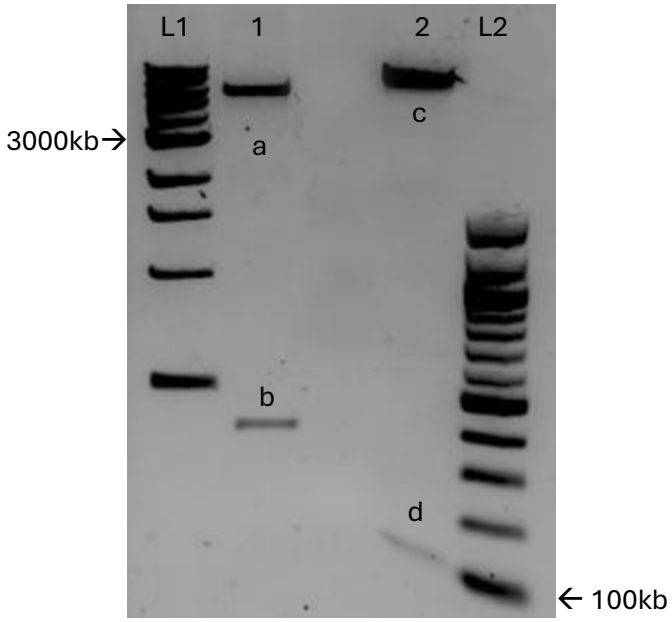


Figure 25: pSOK806 cut with EcoRI/SfiI in 1 and pSOK911 cut with EcoRI/SfiI in 2. 1a=5383 bps, 1b=371 bps, 2c=6728 bps, 2d=110 bps. Fragments 1b and 2c were then used for ligation to create pSOK912. L1= Quick-Load® 1 kb DNA Ladder, L2= Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.)

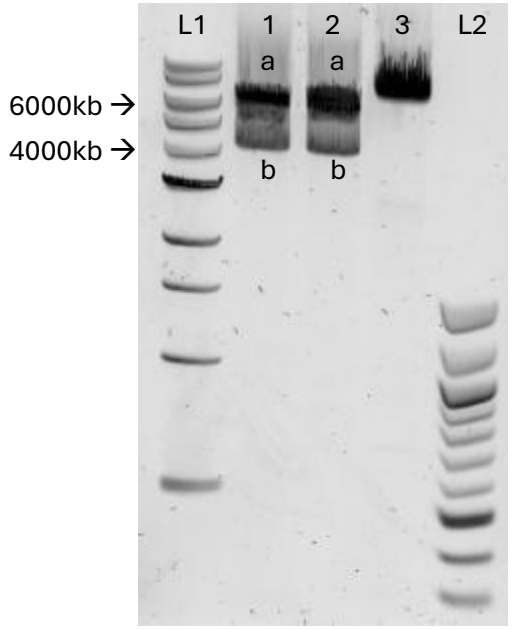


Figure 26: Restriction analysis of pMOE653 with MluI. Because EcoRI/SpeI showed star activity and correct clones couldn't be confirmed, MluI was chosen. This results in 2 fragments a1-a2=6046 bps and b1-b2=4117 bps. In lane 3, pSOK912 served as control. It has only 1 restriction site for MluI so it results in the linear plasmid, 3c=7099 bps. L1= Quick-Load® 1 kb DNA Ladder, L2= Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.)

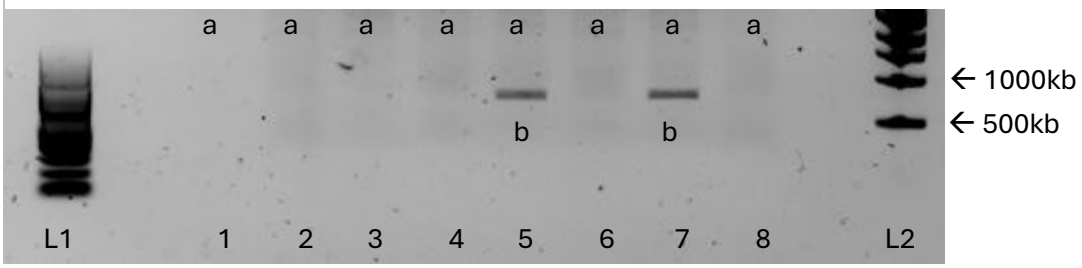


Figure 27: Restriction analysis of constructed vector pMOE632. 1a-8a are pSOK912 cut with EcoRI/SpeI (7075 bps), 5b and 7b are the inserted PCR product 8963 (753 bps). The correct clone couldn't be confirmed in lanes 1,2,3,4,6,8. L1= Quick-Load® 100 bp DNA Ladder, L2= Quick-Load® 1 kb DNA Ladder (Methods 6.6.1.)

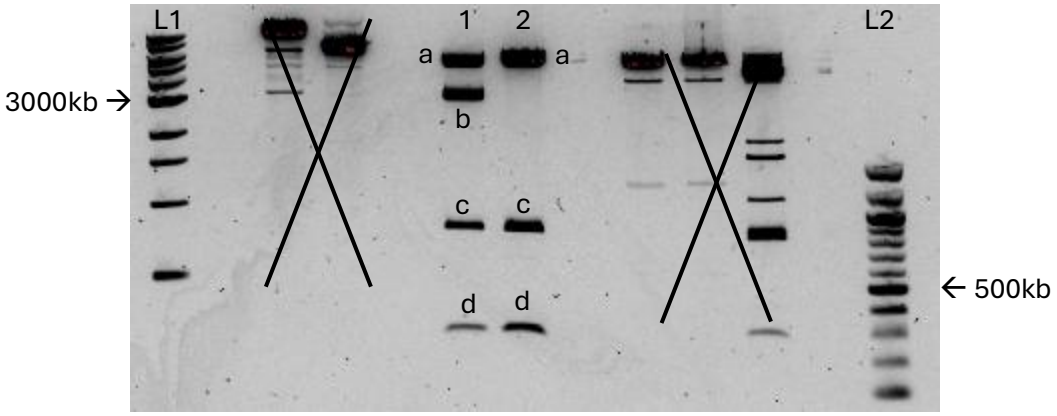


Figure 28: Restriction analysis of pMOE664 with NcoI. Because EcoRI/SpeI showed star activity and correct clones couldn't be confirmed, NcoI was chosen. This results in 4 fragments, 1a=6003 bps, 1b=3022 bps, 1c= 870 bps, 1d=284 bps. In lane 2 pSOK912 served as control, 2a=6003 bps, 2c=870 bps, 2d=284 bps. L1= Quick-Load® 1 kb DNA Ladder, L2= Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.)

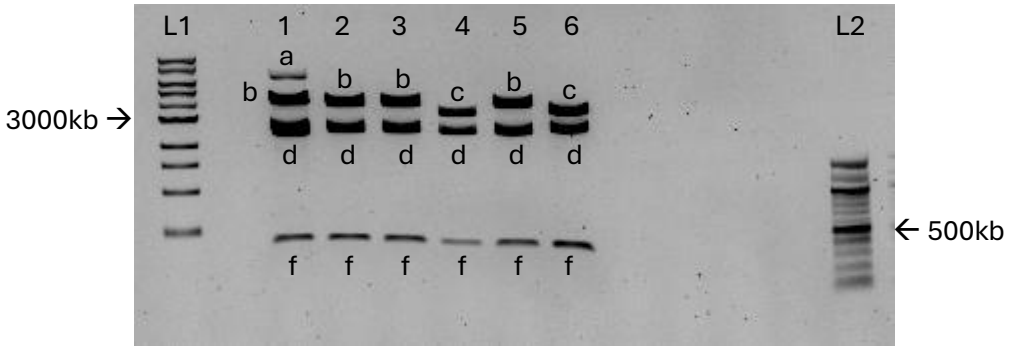


Figure 29: Restriction analysis of pMOE795 with EcoRV/SfiI. Because EcoRI/SpeI showed star activity and correct clones couldn't be confirmed, EcoRV/SfiI was chosen. This results in 3 fragments 2b,3b,5b (4510 bps), 2d,3d,5d (2773 bps) and 2f, 3f, 5f (367 bps). In lane 6 pSOK912 served as control, 6c=3959 bps, 6d=2773 bps, 6f=367 bps. In lane 5 correct clone couldn't be confirmed is it shows the same bands as the control. In lane 1 an additional band can be observed which also leads to its exclusion. L1= Quick-Load® 1 kb DNA Ladder, L2= Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.)

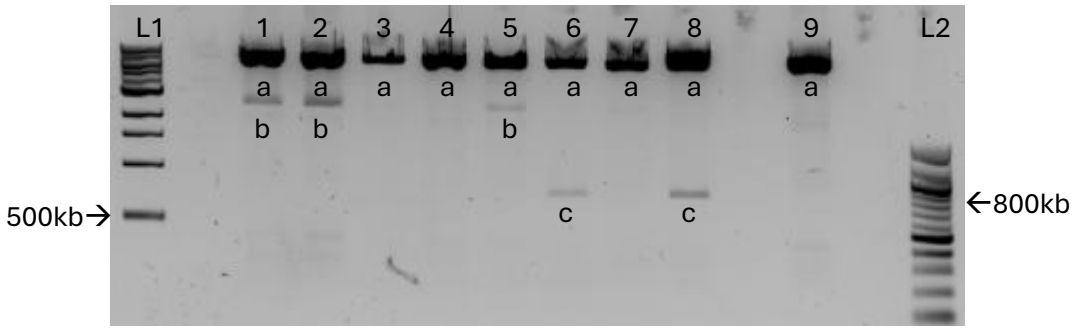


Figure 30: Restriction analysis of constructed vector pMOE846. 1a-8a are pSOK912 cut with EcoRI/SpeI (7075 bps), 6c and 8c are the inserted PCR product 8984 (825 bps). The correct clone couldn't be confirmed in lanes 1,2,3,4,5,7. 9 is pSOK912 as control. 1b,2b,5b are an unexpected band. L1= Quick-Load® 1 kb DNA Ladder, L2= Quick-Load® 100 bp DNA Ladder (Methods 6.6.1.)

3.7. Construction of a knockout vector

To verify if gene 8978 (Figure 10) is responsible for the production of a secondary metabolite that triggers the biosensor, resulting in indigoidine production by the recombinant strain RB4, an internal part of this gene was PCR amplified (Methods 6.5.3.), digested with *EcoR*I/*Nde*I and ligated with the pSOK911 fragment lacking integrase gene *int* to create a knockout vector. This vector could be used to knock out the gene 8978 via homologous recombination. If the indigoidine production would be abolished after knockout (Methods 6.5.7.) in strain RB4, this would confirm that gene 8978 is responsible for the production of a BGC 2.34-specific secondary metabolite or its precursor. For agarose gel of PCR reaction see Figure 22.

Table 5: intragenic region was amplified to create knockout vector

PCR product	Size (bps)	Product confirmed
8978	1336	+

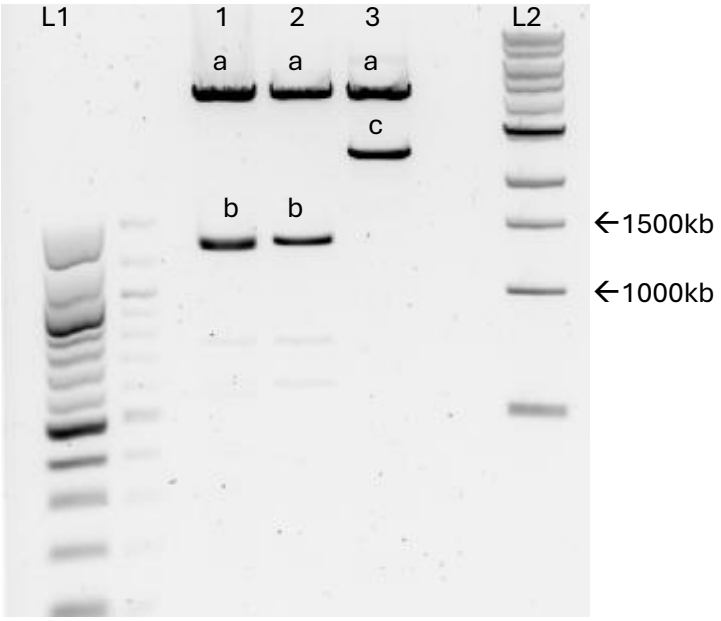


Figure 32: Restriction analysis of constructed vector pMKO781. 1a-3a are pSOK911 cut with EcoRI/NdeI (4478 bps), 1b and 2b are the inserted PCR product 8978 (1323 bps). 3 is pSOK911 cut with EcoRI/NdeI resulting in 2 fragments, 3a=4478 bps and 3c= 2380bps. L1=Quick-Load® 100 bp DNA Ladder, L2= Quick-Load® 1 kb DNA Ladder (Methods 6.6.1.).

3.8. Conjugation of overexpression vectors and knockout vector to biosensor strains

After the successful construction of the knockout and overexpression vectors attempts were made to introduce them to the *Streptomyces* biosensor strains via conjugation. To create control strains without overexpression vectors, the plasmid pSOK911 was used. Conjugation was performed under different conditions, but results could only be achieved using mycelium grown in liquid culture (Methods 6.5.7.). Still only 5 conjugations were successful. Created strains can be found at Materials 5.2.1. R43, R46, R4911 still showed indigoidine production when grown, R23 and R2911 did not.

Table 6: conjugation attempts of overexpression vectors and knockout vector to biosensor strains. +=successful, -=unsuccessful, /=not performed

	RB1	*RB2*	*RB3*	*RB4*	*RB5*
pMOE632	-	-	-	-	-
pMOE653	-	+ (*R23*)	-	+ (*R43*)	-
pMOE664	-	-	-	-	-
pMOE795	-	-	-	-	-
pMOE846	-	-	-	+ (*R46*)	-
pMKO781	/	/	/	-	/
pSOK911	-	+ (*R2911*)	-	+ (*R4911*)	-

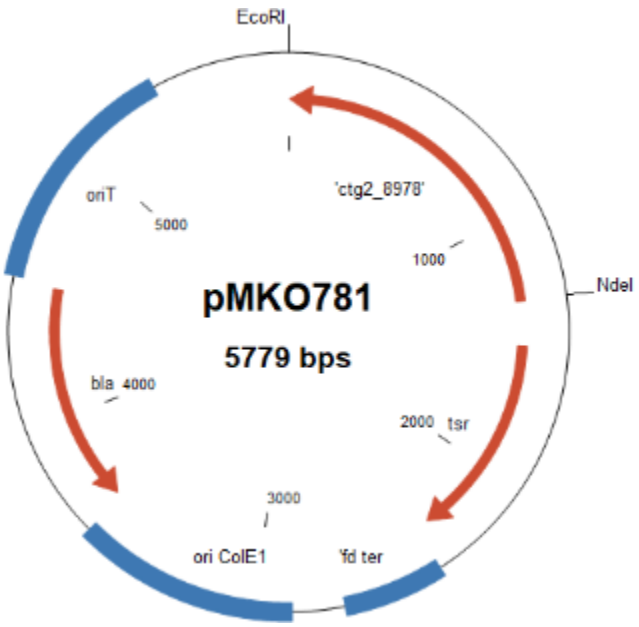


Figure 31: Vector maps (created using clone manager Methods 6.1.2.) of vector pSOK911 and knockout vector pMKO781 containing inserted PCR fragment 8978 ('ctg2_8978').

3.9. Fermentation

The strains resulting from the successful conjugations of biosensor strains with overexpression vectors were fermented in 3 different media (Materials 5.1.3.) for 3 and 7 days (Methods 6.3.6.). During this time, they were monitored to see if fermentation would lead to a colour change indicating indigoidine production. In Sm-17M after 3 and 7 days R23, R2911 and R43 showed a light brown colour and, R46 and R4911 were dark brown/purple (Figure 33 and 34), in PM4 after 3 and 7 days R23, R2911 and R43 showed a light brown colour, R46 and R4911 were dark brown (Figure 35 and 36), in 5228 after 3 days R23, R2911 and R43 showed a light brown colour and R46 and R4911 were dark brown/purple after 7 days R23 and R2911 were brown R43 was a darker brown and had a dark brown/purple colour (Figure 37 and 38). The strains R43, R46 and R4911 were expected to produce indigoidine as they produced it when grown on ISP4 plates. However, R43 seems to not have produced indigoidine in any of the 3 media. Due to the production of brown to dark brown metabolites like melanin it was hard to discern whether ingoidine was produced or not. The seeding cultures all had a light brown colour.

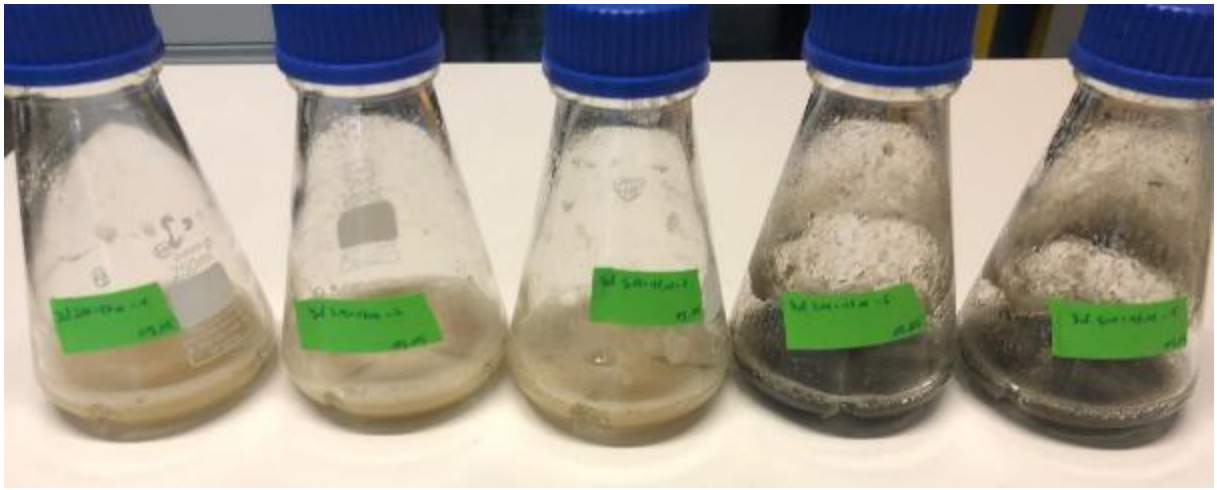


Figure 33: 3 days fermentation cultures in SM-17M, left to right: R23, R2911, R43, R46, R4911. Possible indigoidine production in R4911 and R46.

Figure 34: 7 days fermentation cultures in SM-17M, left to right: R4911, R46, R43, R2911, R23. Possible indigoidine production in R4911 and R46.

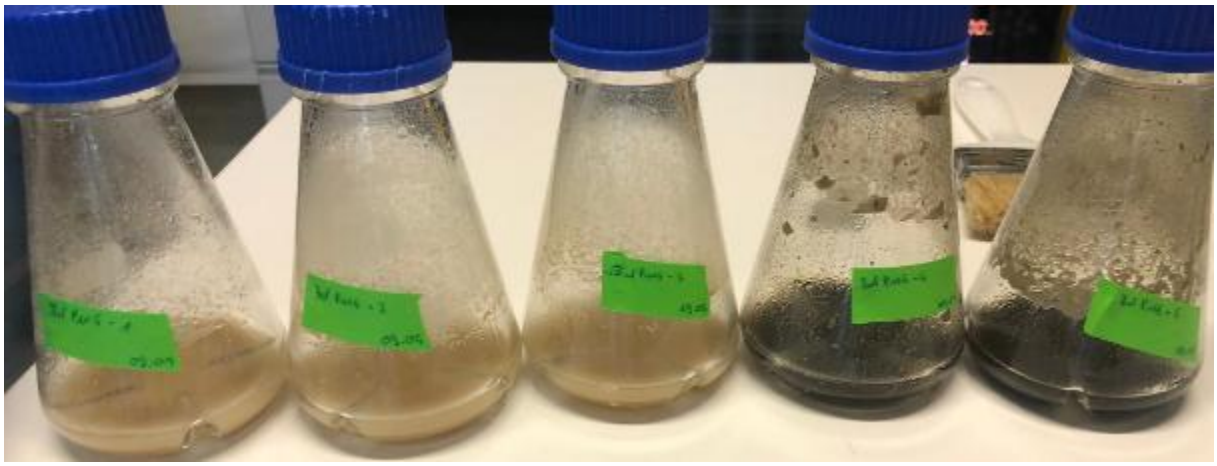


Figure 35: 7 days fermentation cultures in PM4, left to right: R23, R2911, R43, R46, R4911. Possible indigoidine production in R4911 and R46.

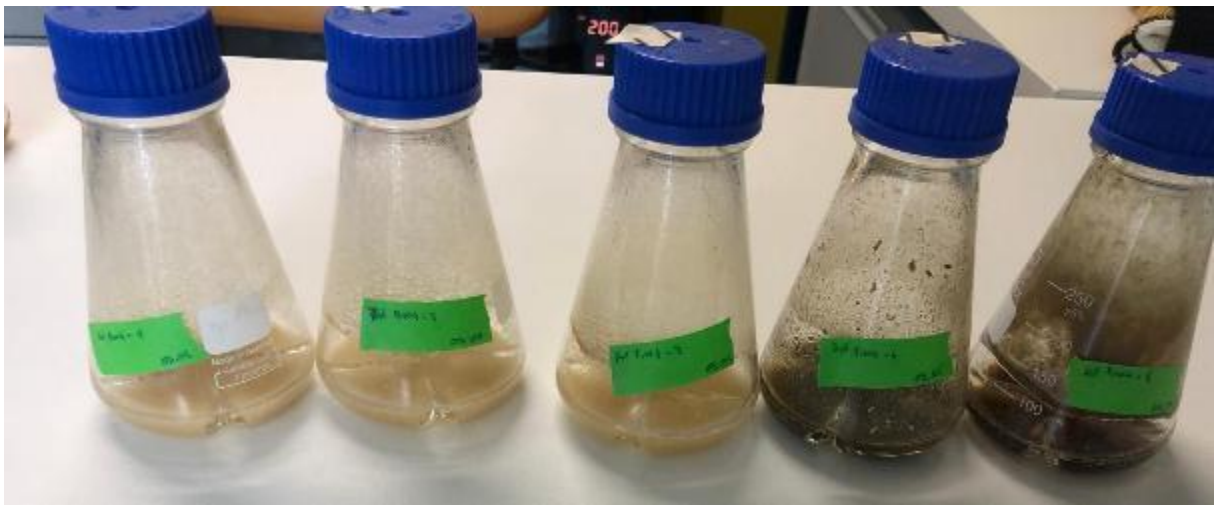


Figure 36: 7 days fermentation cultures in PM4, left to right: R23, R2911, R43, R46, R4911. Possible indigoidine production in R4911 and R46.

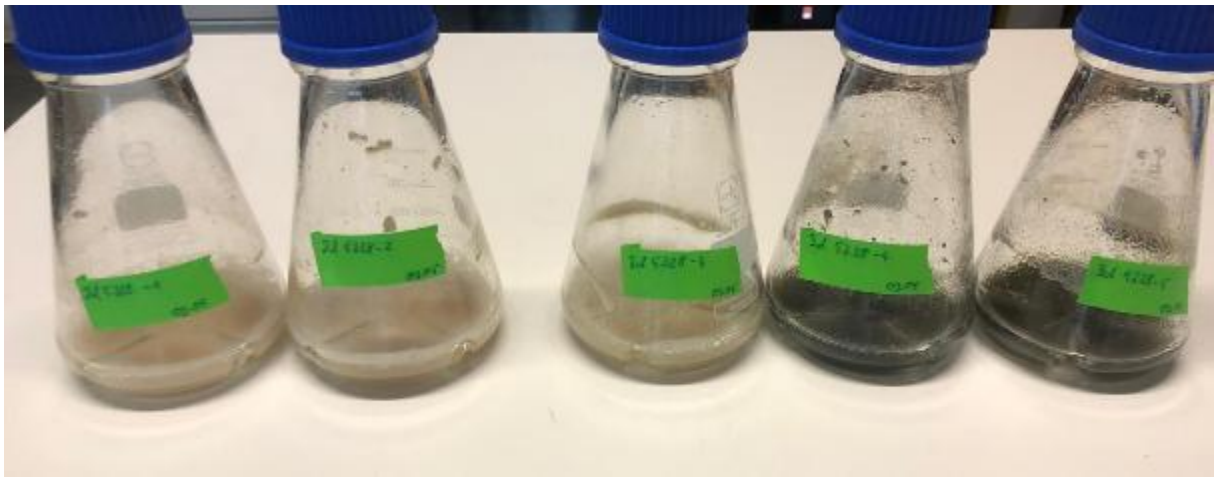


Figure 37: 3 days fermentation cultures in 5228, left to right: R23, R2911, R43, R46, R4911. Possible indigoidine production in R4911 and R46.

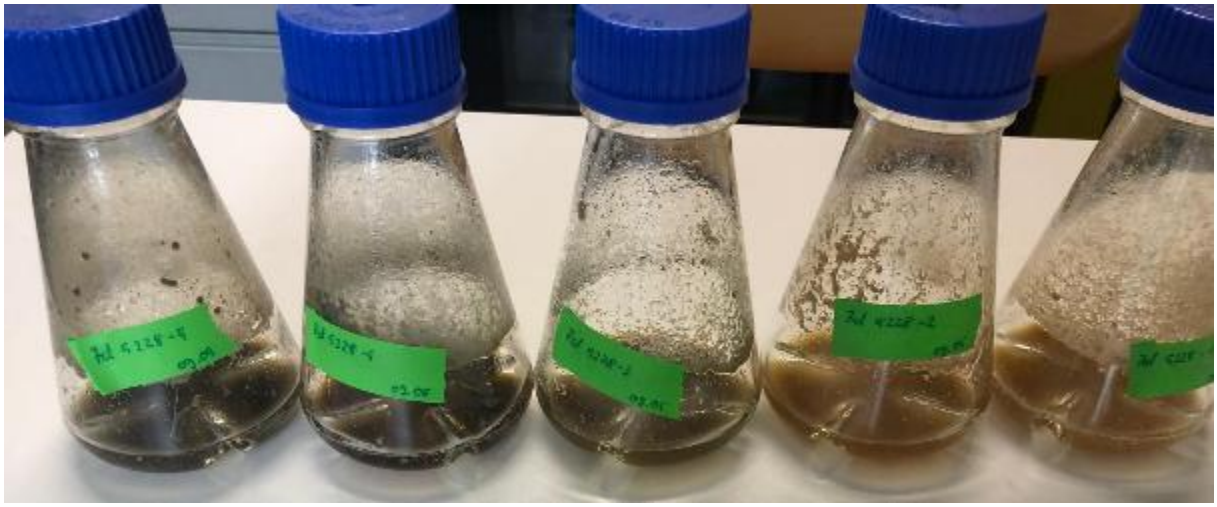


Figure 38: 7 days fermentation cultures in 5228, left to right: R4911, R46, R43, R2911, R23. Possible indigoidine production in R4911, R46 and R43

3.10. Extracts

Methanolic extracts of all fermentation cultures were created as described in Methods 6.7.1. and tested for antimicrobial properties via disc diffusion assay (Methods 6.7.2).

Table 7: Methanolic extracts that were created from fermentation cultures.

Extract name	Days of fermentation	Fermentation medium
3dPM4R23 (3d11)	3	PM4
3dPM4R2911 (3d12)	3	PM4
3dPM4R43 (3d13)	3	PM4
3dPM4R46 (3d14)	3	PM4
3dPM4R4911 (3d15)	3	PM4
3d5228R23 (3d21)	3	5228
3d5228R2911 (3d22)	3	5228
3d5228R43 (3d23)	3	5228
3d5228R46 (3d24)	3	5228
3d5228R4911 (3d25)	3	5228
3dSM17MR23 (3d31)	3	SM-17M
3dSM17MR2911 (3d32)	3	SM-17M
3dSM17MR43 (3d33)	3	SM-17M
3dSM17MR46 (3d34)	3	SM-17M
3dSM17MR4911 (3d35)	3	SM-17M
7dPM4R23 (7d11)	7	PM4
7dPM4R2911 (7d12)	7	PM4
7dPM4R43 (7d13)	7	PM4
7dPM4R46 (7d14)	7	PM4
7dPM4R4911 (7d15)	7	PM4
7d5228R23 (7d21)	7	5228
7d5228R2911 (7d22)	7	5228
7d5228R43 (7d23)	7	5228
7d5228R46 (7d24)	7	5228
7d5228R4911 (7d25)	7	5228
7dSM17MR23 (7d31)	7	SM-17M
7dSM17MR2911 (7d32)	7	SM-17M
7dSM17MR43 (7d33)	7	SM-17M
7dSM17MR46 (7d34)	7	SM-17M
7dSM17MR4911 (7d35)	7	SM-17M

3.11. Disc diffusion assays

Disc diffusion assays (Methods 6.7.2.) were performed for all created methanolic extracts to check growth inhibition of different test organisms (Materials 5.2.3.). 1 extract (3dPM4R43) showed some growth inhibition of *Micrococcus luteus* and *Bacillus subtilis*. (Figures 39 and 43).

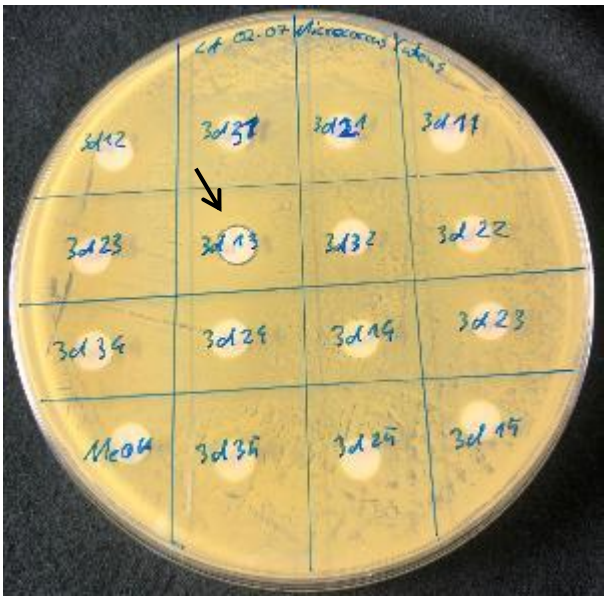


Figure 39: Disc diffusion assay of extracts from 3 days fermentation cultures against Micrococcus luteus. 1 extract (3dPM4R43) shows a small growth inhibition.

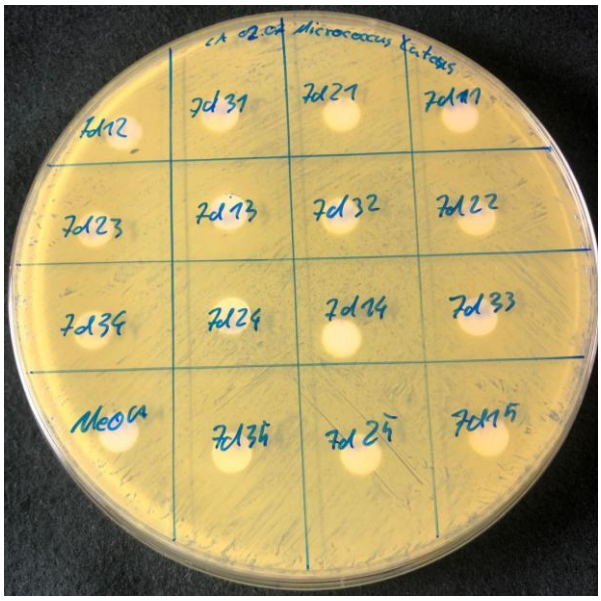


Figure 40: Disc diffusion assay of extracts from 7 days fermentation cultures against Micrococcus luteus. None of the extracts show signs of growth inhibition.

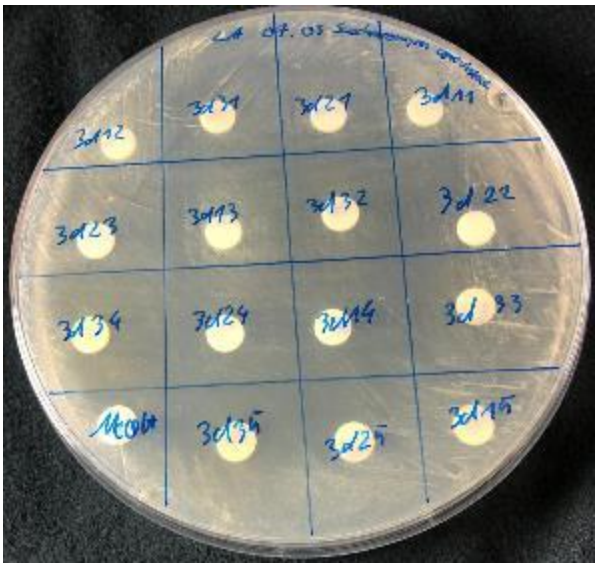


Figure 41: Disc diffusion assay of extracts from 3 days fermentation cultures against Saccharomyces cerevisiae. None of the extracts show signs of growth inhibition.

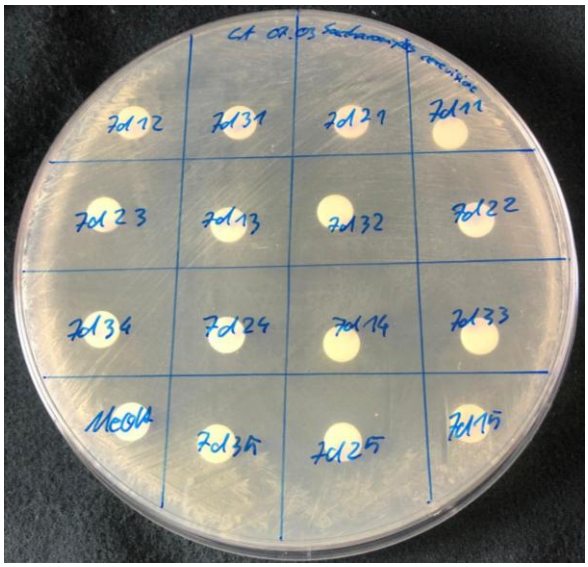


Figure 42: Disc diffusion assay of extracts from 3 days fermentation cultures against Saccharomyces cerevisiae. None of the extracts show signs of growth inhibition.

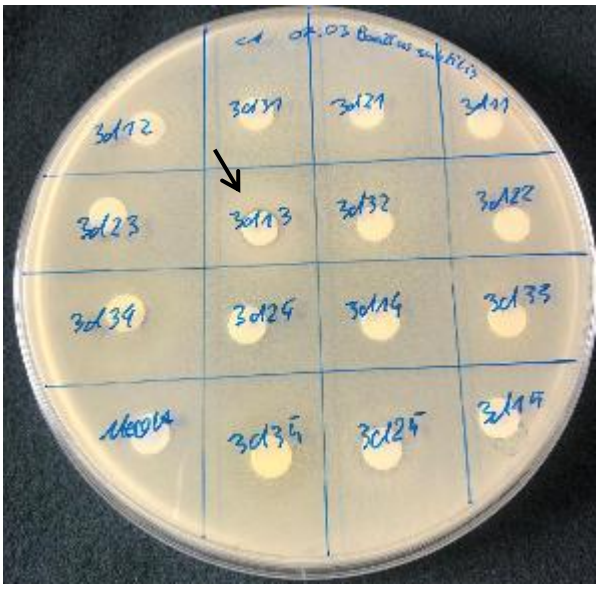


Figure 43: Disc diffusion assay of extracts from 3 days fermentation cultures against Bacillus subtilis. 1 extract (3dPM4R43) shows a light growth inhibition.

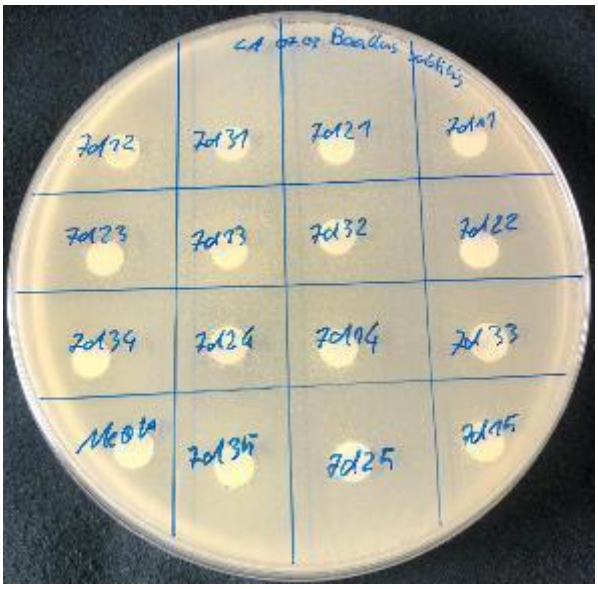


Figure 44: Disc diffusion assay of extracts from 3 days fermentation cultures against Bacillus subtilis. None of the extracts show signs of growth inhibition.

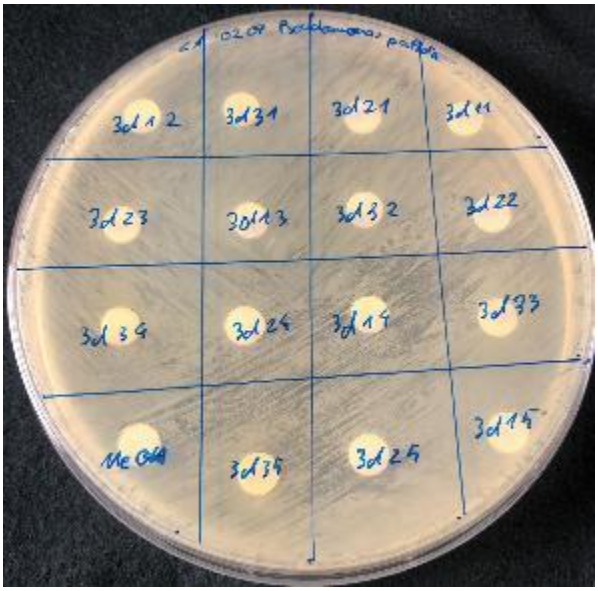


Figure 45: Disc diffusion assay of extracts from 3 days fermentation cultures against Pseudomonas putida. None of the extracts show signs of growth inhibition.

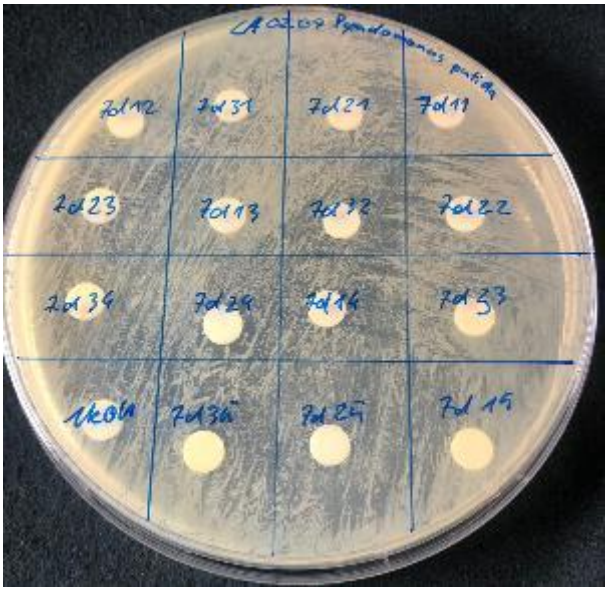


Figure 46: Disc diffusion assay of extracts from 3 days fermentation cultures against Pseudomonas putida. None of the extracts show signs of growth inhibition.

3.12. HPLC analysis of methanolic extracts

It was decided to perform HPLC analysis for the methanolic extracts gained from the 3 days fermentation cultures as none of the extracts from the 7 days fermentation cultures showed bioactivity in the disc diffusion assays (Results 3.11.). HPLC analysis was performed according to Methods 6.7.3. The chromatograms of the extracts from the strains carrying an overexpression vector were compared to the control strains carrying pSOK911. R43 showed a very different pattern in all 3 media when compared to R4911 (Figure 50). It is possible that this is due to the production of indigoidine which can change the metabolic pattern however it is more likely that the differences arise from contamination with another species of *Streptomyces*. For this reason, it was compared to R2911. In Figure 54 the peaks 1 and 2, only visible in the chromatogram of 3dPM4R43, may correlate to the bioactivity of the extract (Results 3.11). Figures 47-54 show the different comparative chromatograms.

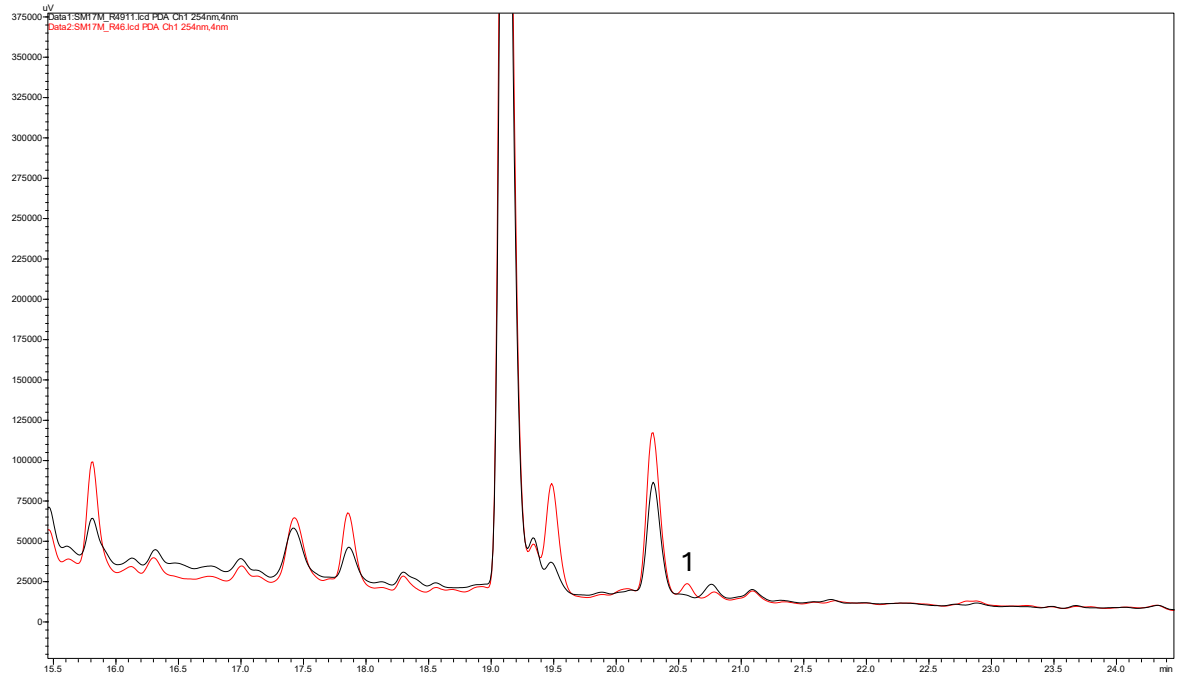


Figure 47: Comparison of 3dSM17R4911=control (black) and 3dSM17R46 (red), PDA 254 nm. Some peaks are bigger in the chromatogram of 3dSM17R46 and peak 1 at 20.6 min can only be seen in the chromatogram of 3dSM17R46.

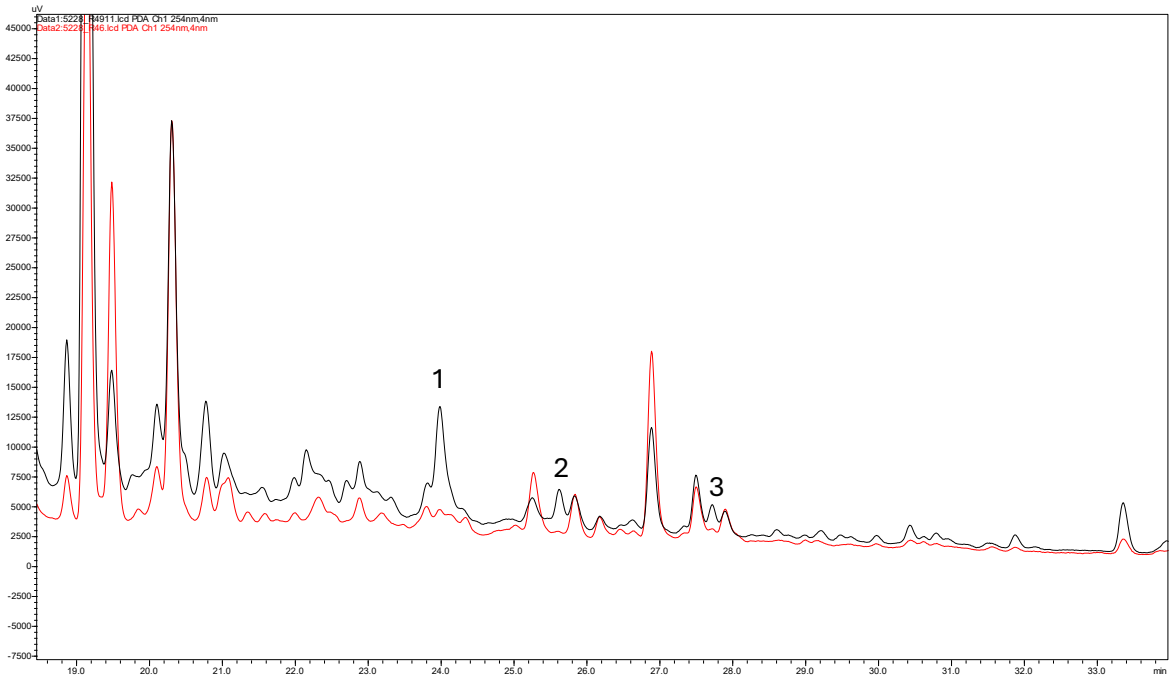


Figure 48: Comparison of 3d5882R4911=control (black) and 3d5882R46 (red), PDA (photodiode array) 254 nm. Some peaks are either bigger or smaller in the chromatogram of 3d5882R911. Peaks 1,2 and 3 at 24 min 25.6 min and 27.7 min can only be seen in the chromatogram of 3d5882R4911.

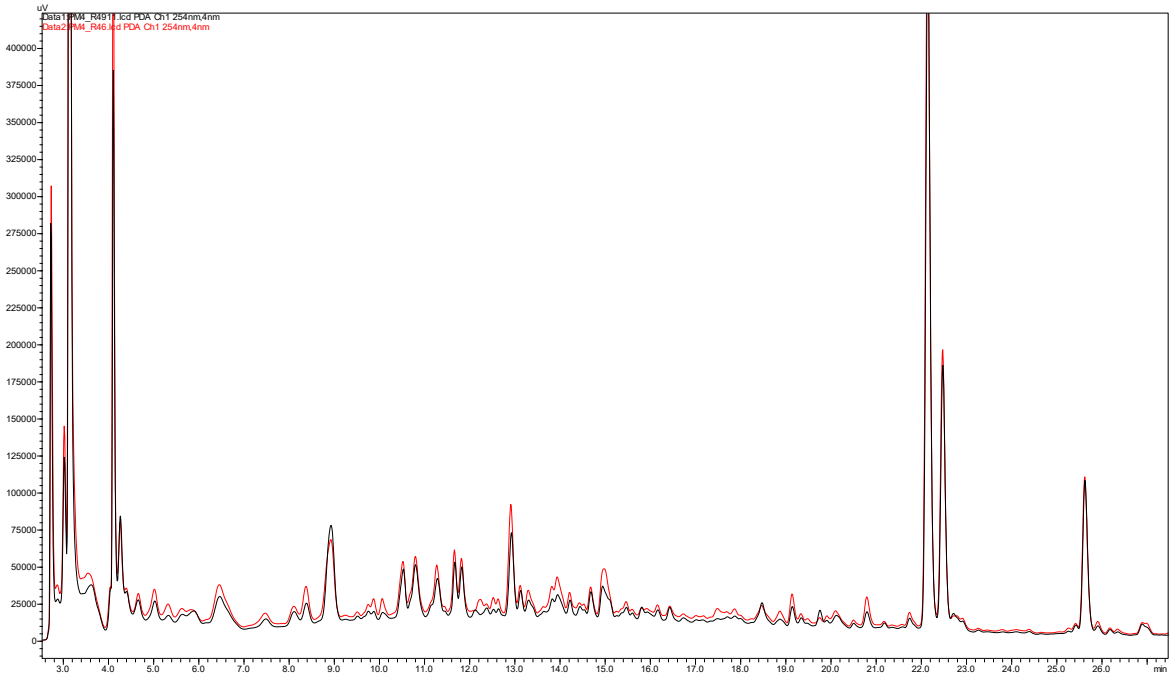


Figure 49: Comparison of 3dPM4R4911=control (black) and 3dPM4R46 (red), PDA 254 nm. The 2 chromatograms show no relevant differences.

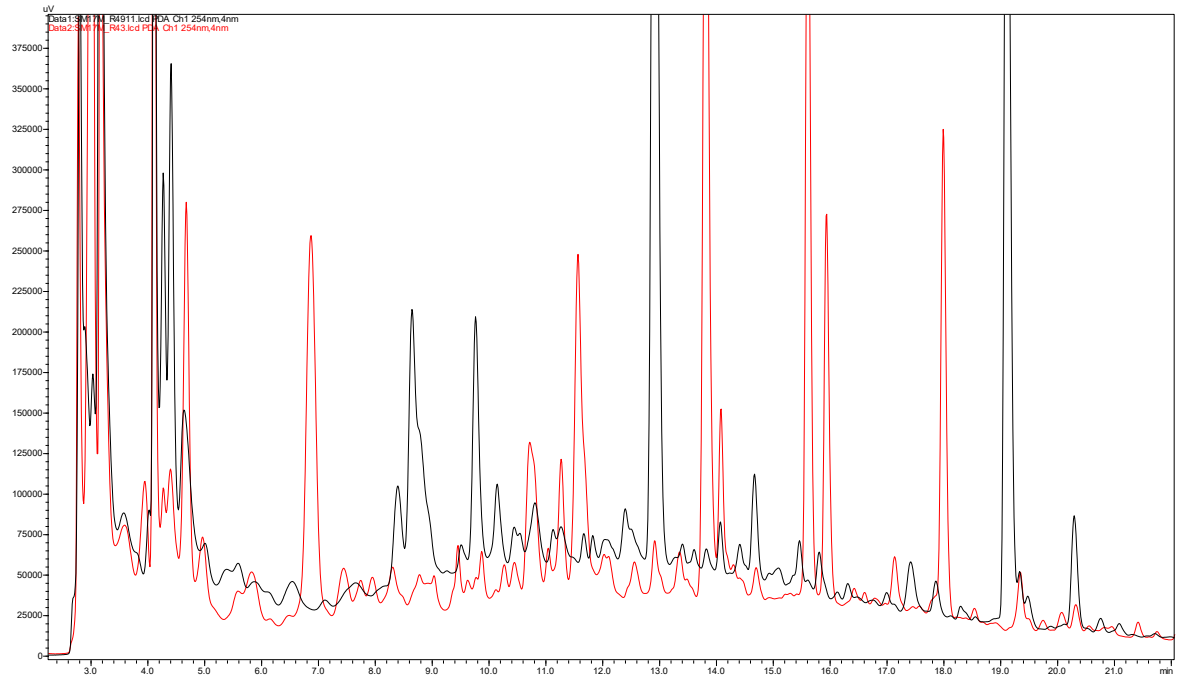


Figure 50: Comparison of 3dSM17R4911=control (black) and 3dSM17R43 (red), PDA 254nm. Both chromatograms show a very different pattern of peaks. This could be due to the production of ingoidine in the fermentation culture of 3dSM17R4911 and no production of indigoidine in the fermentation culture of 3dSM17R43 (Figure 33) as the production of ingoidine can change the metabolic pattern of strains. Another reason for this could be contamination with other Streptomyces. The same could be observed when comparing the chromatograms of 3dPM4R4911 to 3dPM4R43 and 3d5228R4911 to 3d5228R43 (not shown).

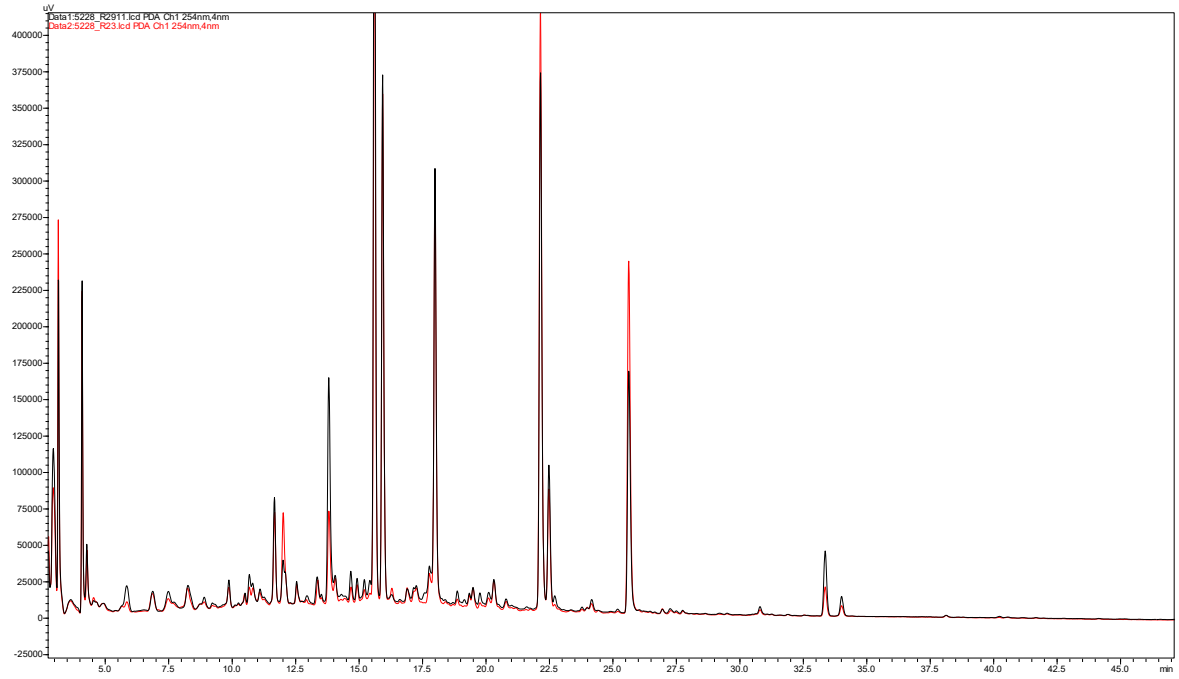


Figure 51: Comparison of 3d5228R2911=control (black) to 3d5228R23 (red), PDA 254 nm. The 2 chromatograms show no relevant differences. The same is true for when comparing the chromatogram of 3dPM4R2911 to 3dPM4R23 and 3dSM17R2911 to 3dSM17R23 (not shown).

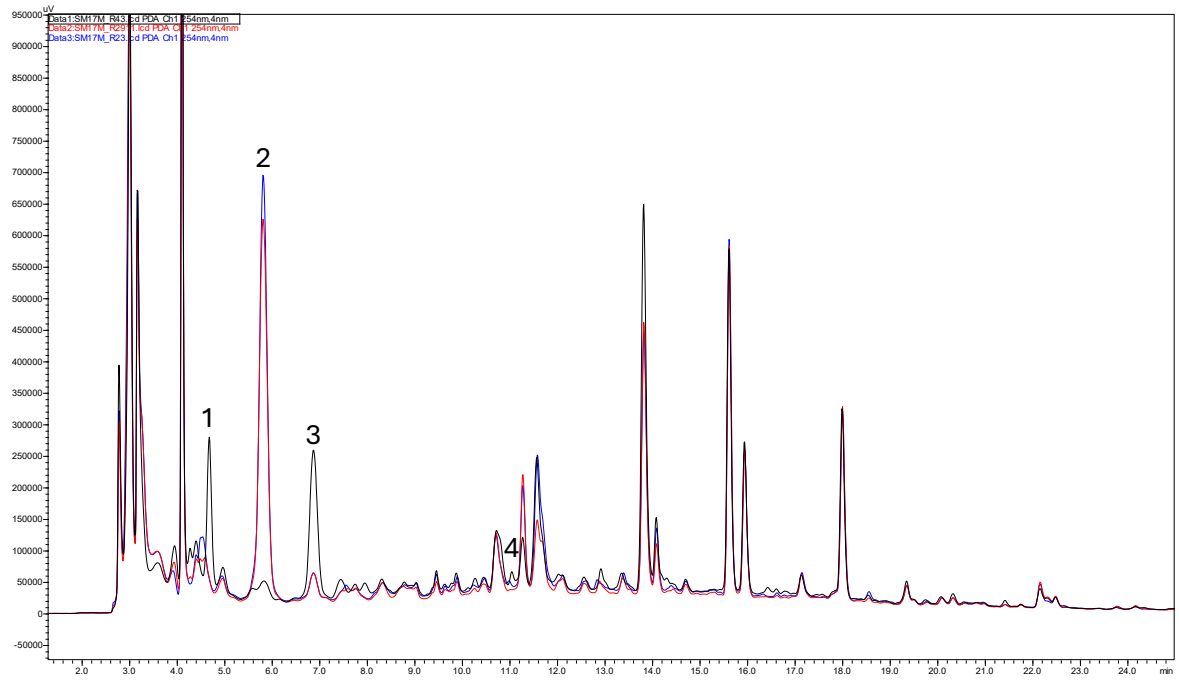


Figure 52: Comparison of 3dSM17R43 (black) to 3dSM17R2911= control (red) and 3dSM17R23 (blue), PDA 254 nm. In the chromatogram of 3dSM17R43 peaks 1, 3 and 4 at 4.6 min, 6.9 min and 11 min can be seen and peak 2 at 5.8min disappears.

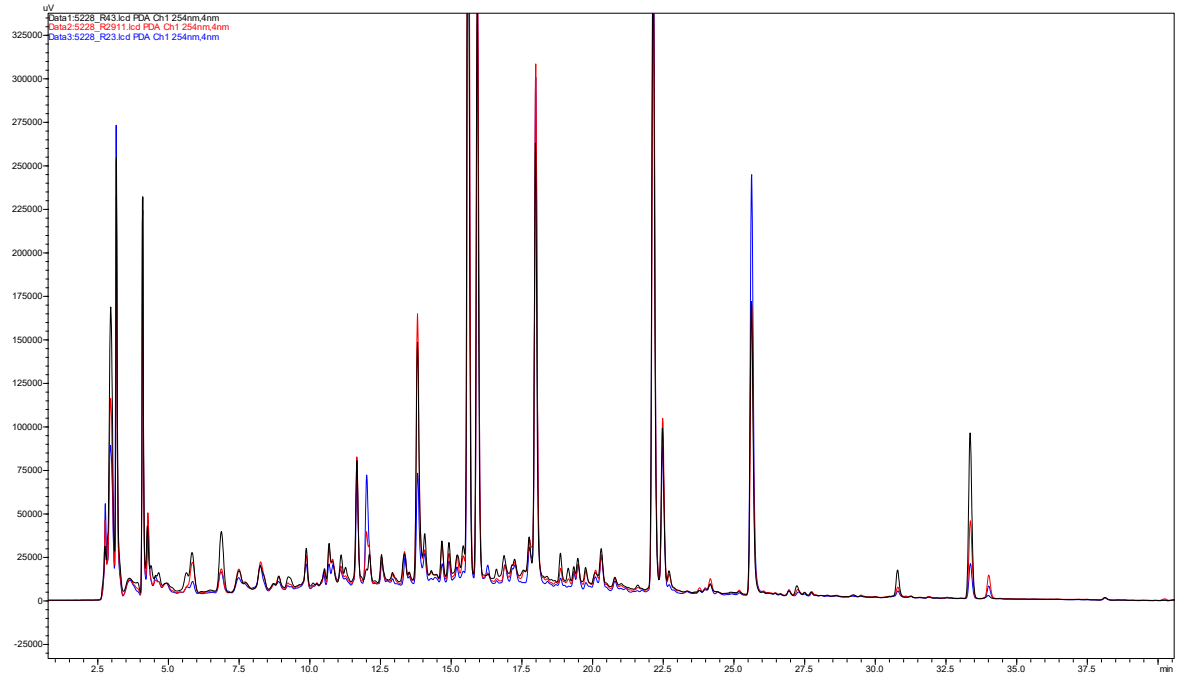


Figure 53: Comparison of 3d5228R43 (black) to 3d5228R2911= control (red) and 3d5228R23 (blue), PDA 254 nm. The 2 chromatograms show no relevant differences.

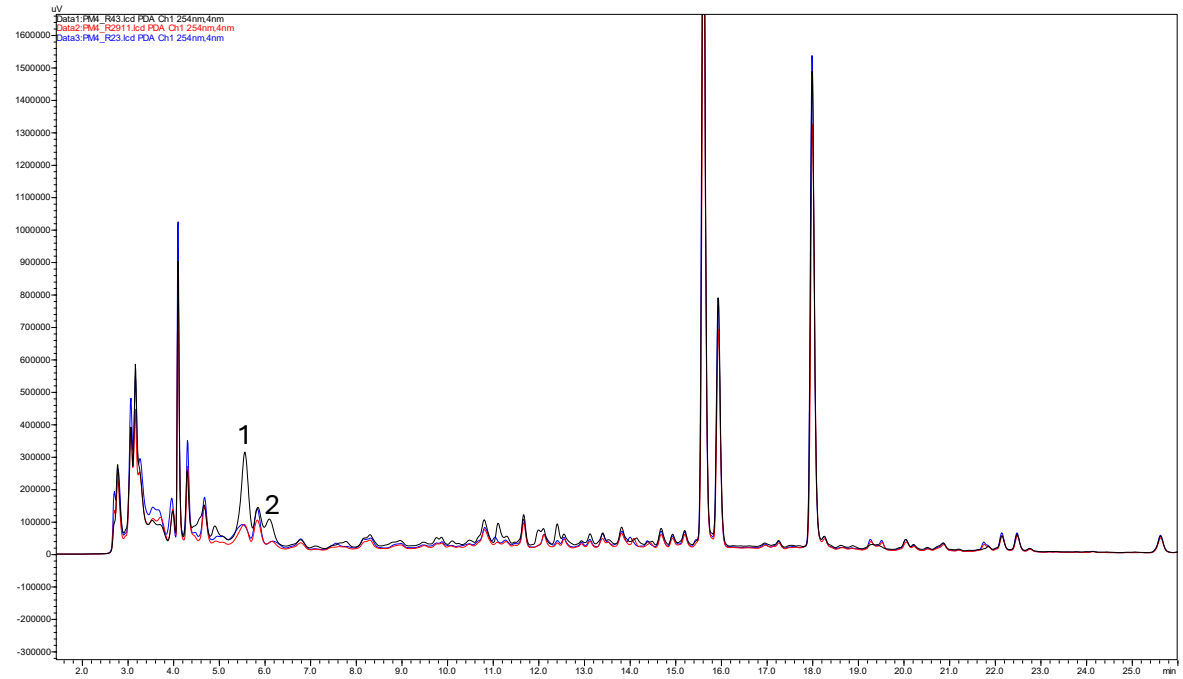


Figure 54: Comparison of 3dPM4R43 (black) to 3dPM4R2911= control (red) and 3dPM4R23 (blue), PDA 254 nm. In the chromatogram of 3dPM4R43 peak 1 at 5.6 min is bigger compared to the other chromatograms and peak 2 at 6.1 min only appears in the chromatogram of 3dPM4R43.

3. Discussion

The strain *Streptomyces* sp. RLA012 was isolated from the rhizosphere of the medicinal plant *Leontopodium nivale* subsp. *alpinum* (Oberhofer et al, 2019). In this project, BGC 2.34 identified using antiSMASH (Medema et al., 2011) (Results 3.1.) was chosen to create a biosensor system by placing the reporter gene *bpsA*, which is responsible for the production of the blue pigment indigoidine, under the control of promoter/operator regions of TetR like repressors associated with the BGC of interest. Using this approach 5 recombinant strains, as well as a negative and a positive control strain, were created of which 1 (RB4) showed indigoidine production (Results 3.5.).

To prove that indigoidine production is the result of the activity of the BGC of interest a knockout vector was constructed containing an intragenic region of gene 8978 (Figure 10 a res). It was expected that conjugants of RB4 with this knockout vector would cease to produce indigoidine. However, no conjugants could be obtained from the conjugation attempts and therefor this assumption could not be confirmed. To achieve BGC activation, 5 overexpression vectors, containing regulatory regions found in the BGC under the control of the strong promoter PermE*, were constructed (Results 3.6.). The goal was to transfer each overexpression vector and the empty control vector to each recombinant biosensor strain. Those attempts proved mostly unsuccessful and of the 30 attempted conjugations only 5 were successful, resulting in the strains R43, R46, R4911, R23, R2911 (Results 3.8.). Different conditions (temperature of heat shock, heat shock vs. no heat shock, mycelium vs. spores) for conjugation were tested, and best results were obtained when using the protocol found at Methods 6.5.7. Optimization of conjugation conditions could lead to successful conjugation and higher efficiency. The concentration of MgCl2, temperature of heat shock, the ratio of donor to recipient cells and the overlaying time of antibiotics are parameters that can influence the results of conjugation (Zhang et al., 2019). Zhang shows that for the strain of *Streptomyces* he was working with, optimal overlaying time of antibiotics when using spores as recipients was nearly double the time than when using mycelium and that a too long overlaying time can lead to false positives. This matches with observations that were made in this work as false positives were a recurring problem. When working with mycelium as recipient the same overlaying time of antibiotics was used as when working with spores. A change of this parameter and experiments with other parameters might lead to successful conjugations. When working with pSET152 based plasmids in creating the biosensor strains better results

were achieved than when working with pSOK911 based plasmids to create the overexpression vectors and knockout vector. Using a different vector to create overexpression vectors and knockout vector could lead to easier conjugation.

The long time it took for *Streptomyces* sp. RLA012 to sporulate made experiments time extensive and when faced with contaminations a lot of time was lost to get rid of them and to repeat the experiments. This suggests that the growing conditions were still not ideal and improving them could help to reduce this problem. For this reason, more media and different growing temperatures should be tested.

During this project, contaminations in cultures of *Streptomyces* sp. RLA012 and the created recombinant strains occurred frequently. Contaminations can stem from a variety of sources such as the air, human skin and breath, not sufficiently sterilised materials, media, containers or devices. The origin of contaminations could not always be discerned. Even after cleaning spore suspensions according to Methods 6.3.4. and the creation of new spore suspensions, contamination in liquid cultures, derived from the cleaned spore suspensions, could be observed. The reason for this could be that contaminants were not growing on the solid media or masked by *Streptomyces* sp. RLA012. This often led to delays and difficulties with conducting experiments. This proves that it is absolutely crucial to follow aseptic working protocols and working in sterile environments, to gain usable and reproducible results.

The recombinant strains resulting from transferring the overexpression and control vectors were fermented in 3 different media. In fermentation cultures strain R43 stopped producing indigoidine. R43 showed a very different chromatographic pattern than R46 and R4911 and was more comparable to R23 and R2911. This could be due to 2 reasons. Either indigoidine production changed the second metabolism or due to contamination with other strains of *Streptomyces*. It is more likely and more in agreement with the rest of the project that the reason for the different patterns is contamination. To prove this the fermentation, extraction and HPLC should be repeated with new cultures of the recombinant strains. It would be even better to repeat the conjugations with new cultures of the biosensor strains to exclude the possibility of a hidden contamination.

Due to the production of dark metabolites, it was difficult to discern whether indigoidine was produced or not, as the brown colour masked the blue colour (Results 3.9.). A possibility to evade this problem could be the deletion of the BGC responsible to produce melanin in *Streptomyces* sp. RLA012.

Dsic diffusion assays were performed against 4 different test organisms for all the extracts created out of the fermentation cultures. Out of them only 1 extract showed bioactivity against 2 test organisms. This bioactivity however was rather weak and resulted in just a little growth inhibition (Results 3.11.). The bioactivity of extract 3dPM4R43 can be linked to peaks 1 and 2 in Figure 54. To identify which compound is responsible for the bioactivity, to know if it can be linked to region 2.34 or to any other previously silent BGC in the genome of *Streptomyces* sp. RLA012 and if it were a previously unknown compound would need further investigations. The possibility of the substance responsible for the bioactivity stemming from the contamination with another strain of *Streptomyces* calls for the repeat of fermentation, extraction and HPLC.

Due to the difficulties with conjugations a different approach, other than overexpressing regulatory genes, could lead to different results when trying to activate this silent BGC. Different approaches could be heterologous expression of BGC 2.34 in a different *Streptomyces* host that is easier to handle than *Streptomyces* sp. RLA012 (Liu et al. 2021), ribosome engineering (Zhu, Duan & Huang, 2019) or to work with chemical elicitors or co-cultivation (Abdelmohsen et al., 2015).

With the aid of biosensors, the analytical workload after experiments trying to activate silent BGCs can be reduced, in this project however they could not unfold their full potential as most of the conjugation attempts with a knockout vector and overexpression vectors failed not giving the biosensor system a possibility to shine.

4. Materials

5.1 Media, solutions and buffers

5.1.1. Solid media for *Streptomyces*

- SFM
  - Soy flour 20g
  - Mannitol 20g
  - Agar 20g
  - H_2O to 1000 ml

- ISP4
  - Soluble starch 10g
  - Dipotassium phosphate 1g
  - Magnesium sulfate 1g
  - Sodium chloride 1g
  - Ammonium sulfate 2g
  - Calcium carbonate 2g
  - Iron(II) sulfate heptahydrate 1g
  - Manganese(II) chloride tetrahydrate 1g
  - Zinc sulfate 1g
  - Agar 20g
  - dH_2O to 1000ml

- ISP5 (5323)
  - L-Asparagine 1g
  - Glycerol 10g
  - Dipotassium phosphate 1g
  - Salt solution 1ml
    - Iron(II) sulfate heptahydrate 1g
    - Manganese(II)chloride tetrahydrate 1g
    - Zinc sulfate heptahydrate 1g
    - dH_2O to 100ml
  - Agar 20g
  - dH_2O to 1000ml

- 5326
  - Yeast extract 4g
  - Malt extract 10g
  - Glucose 4g
  - Agar 15g
  - dH_2O to 1000ml

- Czapek Dox (5081)
  - Sucrose 30g
  - Sodium nitrate 3g
  - Dipotassium phosphate 1g
  - Magnesium sulfate heptahydrate 0,5g
  - Iron(II) sulfate heptahydrate 0,01g
  - Potassium chloride 0,5g
  - Agar 20g
  - dH_2O to 1000ml

- PYS Agar (5402)
  - Peptone 15g
  - Yeast extract 5g
  - Sodium chloride 5g
  - Agar 15g
  - dH_2O to 1000ml

- 5336
  - Soluble starch 10g
  - Caseine 1g
  - Dipotassium phosphate 0,5g
  - Magnesium sulfate heptahydrate 5g
  - Agar 20g
  - dH_2O to 1000ml

- ISP2
  - Yeast extract 4g
  - Malt extract 10g
  - Dextrose 4g
  - Agar 20g
  - dH_2O to 1000ml

- Bennett`s
  - Yeast extract 1g
  - Beef extract 1g
  - N-Z Amine type A 2g
  - Glucose 10g
  - Agar 15g
  - dH_2O to 1000ml

- CP6
  - Corn steep liquor 10g
  - Soluble starch 10g
  - Calcium carbonate 3g
  - Sodium chloride 3g
  - Ammonium sulfate 3g
  - Agar 20g
  - H_2O to 1000 ml

- LA
  - Tryptone 10g
  - Yeast extract 10g
  - Sodium chloride 5g
  - Agar 10g
  - dH_2O to 1000 ml

5.1.2. Liquid media for *Streptomyces*

- YEME
  - Yeast extract 3g
  - Malt extract 3g
  - Peptone 5g
  - Glucose 10g
  - Sucrose 340g
  - dH_2O to 1000 ml
  - after autoclaving 2.5M magnesium chloride hexahydrate 2ml

- TSB
  - Tryptic soy broth 30g
  - dH_2O to 1000ml

- ISP4
  - Soluble starch 10g
  - Dipotassium phosphate 1g
  - Magnesium sulfate 1g
  - Sodium chloride 1g
  - Ammonium sulfate 2g
  - Calcium carbonate 2g
  - Iron(II) sulfate heptahydrate 1g
  - Manganese(II) chloride tetrahydrate 1g
  - Zinc sulfate 1g
  - dH_2O to 1000ml

- LB
  - Tryptone 10g
  - Yeast extract 5g
  - Natrum chloride 10g
  - dH_2O to 1000ml

- MYM
  - Maltose 4g
  - Yeast extract 4g
  - Malt extract 10g
  - dH_2O to 1000ml

- 2XYT
  - Tryptone 16g
  - Yeast extract 10g
  - Sodium chloride 5g
  - dH_2O to 1000ml

5.1.3. Fermentation media

- SM-17M
  - Glucose 2g
  - Glycerol 20g
  - Soluble starch 2g
  - Soy flour 5g
  - Peptone 5g
  - Yeast extract 5g
  - Sodium chloride 5g
  - Calcium carbonate 2g
  - dH_2O to 1000ml

- PM4
  - Glucose 15g
  - Soy flour 15g
  - Corn steep liquor 5g
  - Calcium carbonate 2g
  - dH_2O to 1000ml

- 5288
  - Glycerol 15g
  - Soy flour 10g
  - Sodium chloride 5g
  - Calcium carbonate 1g
  - Cobalt(II) chloride hexahydrate 0.001g
  - dH_2O to 1000ml

5.1.4. Media for *E. coli* and test organisms

- LA
  - Tryptone 10g
  - Yeast extract 10g
  - Sodium chloride 5g
  - Agar 10g
  - dH_2O to 1000ml

- LB
  - Tryptone 10g
  - Yeast extract 5g
  - Natrum chloride 10g
  - dH_2O to 1000ml

- TSB
  - Tryptic soy broth 30g
  - dH_2O to 1000ml

- YPD
  - Yeast extract 10g
  - Peptone 20g
  - Glucose 20g
  - dH_2O to 1000ml

5.1.5. Solutions and buffers

- 1M $MgCl_2$ stock solution (conjugation)
  - Magnesium chloride 95.2g
  - dH_2O to 1000ml

- 20% Glycerol stock solution (preparation of cell suspensions)
  - Glycerol 200ml
  - dH_2O to 1000ml

- 0.1M NaOH stock solution
  - Sodium hydroxide 4g
  - dH_2O to 1000ml

- 10x TBE buffer (gel electrophoresis)
  - Tris base 108g
  - Boric acid 55g
  - 0.5M EDTA (pH 8) 51ml
  - dH_2O to 1000ml

- 1x TBE buffer (gel electrophoresis)
  - 10x TBE buffer 100ml
  - dH_2O to 1000ml

- 0.8-1.6% Agarose gel (gel elctrophoresis)
  - Agarose 8-16g
  - 10,000 GelRed® (Biotium) 50µl
  - 1x TBE buffer 1000ml

- 50mM $CaCl_2$ stock solution (competent cells)
  - Calcium chloride 7.34g
  - dH_2O to 1000ml

- 10% Gylcerol 50mM $CaCl_2$ stock solution (competent cells)
  - Calcium chloride 7.34g
  - Glycerol 100ml
  - dH_2O to 1000ml

- Antibiotic stock solutions

Table 8: Antibic stock solutions

Antibiotic	Concentration stock (mg/ml)	Concentration medium (µg/ml)	solvent
Apramycin (Am)	100	50-100	dH_2O
Chloramphenicol (Cml)	30	30	96% ethanol
Kanamycin (Kann)	30	30	dH_2O
Nalidixic acid (Nal)	30	30	0.1M sodium hydroxide
Thiostrepton (Thio)	30	15-30	DMSO
Ampicillin (Amp)	100	100	dH_2O

5.2. Organisms and Vectors

5.2.1. *Streptomyces*

Table 9: Streptomyces strains and mutants used and created during this project

Strain	Properties	Source
RLA012	WT	provided by the lab from the rhizosphere of *Leontopodium nivale* subsp. *Alpinum* (Oberhofer et al, 2019)
RB1	RLA012 harboring pMAB121	this project
RB2	RLA012 harboring pMAB122	this project
RB3	RLA012 harboring pMAB123	this project
RB4	RLA012 harboring pMAB124	this project
RB5	RLA012 harboring pMAB125	this project
R152	RLA012 harboring pSET152	this project
R240	RLA012 harboring pBCB240	this project
R23	RB2 harboring pMOE653	this project
R43	RB4 harboring pMOE653	this project
R46	RB4 harboring pMOE846	this project
R2911	RB2 harboring pSOK911	this project
R4911	RB4 harboring pSOK911	this project

5.2.2. Escherichia coli

Two strains of *E. coli* have been used in this project:

- Cloning: *E. coli XL-1blue* genotype: *recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17* (r_k^-, m_k^+), *supE44*, *relA1*, *lac* $^-$, λ^- [F', *proAB*. *lacI* ^{q} *ZΔM15*, Tn*10*(tet_R)] (Bullock et. Al, 1987)
- Conjugation: *E. coli ET12567 (pUZ8002)* genotype: *F–, dam–, dcm–, hsdR– hsdM–, recF143, galK2, galT22, ara14, leuB6, thi–1, lac–, supE44, rpsL20 (*Str_R*), [pUZ8002, *Kan_R*, *Cml_R*)]* (MacNeil et al., 1992)

Table 10: E. coli strains and the vector they carry used and created during this project

Strain	Vector	Source
XL-1blue	pPBGC1.9	provided by the lab
XL-1blue	pMAB121	this project
XL-1blue	pMAB122	this project
XL-1blue	pMAB123	this project
XL-1blue	pMAB124	this project
XL-1blue	pMAB125	this project
ET12567	pMAB121	this project
ET12567	pMAB122	this project
ET12567	pMAB123	this project
ET12567	pMAB124	this project
ET12567	pMAB125	this project
ET12567	pBCB240	provided by the lab
ET12567	pSET152	provided by the lab
XL-1blue	pSOK911	provided by the lab
ET12567	pSOK911	This project
ET12567	pSOK806	provided by the lab
XL-1blue	pSOK912	this project
ET12567	pSOK912	this project
XL-1blue	pMOE632	this project
XL-1blue	pMOE653	this project
XL-1blue	pMOE664	this project
XL-1blue	pMOE795	this project
XL-1blue	pMOE846	this project
XL-1blue	pMKO781	this project
ET12567	pMOE632	this project
ET12567	pMOE653	this project
ET12567	pMOE664	this project
ET12567	pMOE795	this project
ET12567	pMOE846	this project
ET12567	pMKO781	this project
ET12567	pOTRE012	Provided by the lab

5.2.3. Bioassay test organisms

Table 11: Microorganisms used for bioactivity testing

Organism	Antibiotic target	Source
Bacillus subitilis	Gram positive bacteria	provided by the lab
Micrococcus luteus	Gram positive bacteria	provided by the lab
Pseudomonas putida	Gram negative bacteria	provided by the lab
Saccharomyces cerevisiae	Fungi	provided by the lab

5.3. Vectors

Table 12: Vectors used and created during this project

Vector (plasmid)	Genotype	Function	Length (bps)	Source
pPBGC1.9	bpsA, ter, ColE1ori, aac(3)IV, oriT, attP, int	Cloning of Biosensor vectors	9877	provided by the lab
pMAB121	ctg1_2664" - ctg1_2663', bpsA, ter, ColE1ori, aac(3)IV, oriT, attP, int	Biosensor vector	9807	This project
pMAB122	ctg2_8979' - ctg2 8980, bpsA, ter, ColE1ori, aac(3)IV, oriT, attP, int	Biosensor vector	9795	This project
pMAB123	ctg2_8984' - ctg2_8983', bpsA, ter, ColE1ori, aac(3)IV, oriT, attP, int	Biosensor vector	9947	This project
pMAB124	'ctg2_8984, bpsA, ter, ColE1ori, aac(3)IV, oriT, attP, int	Biosensor vector	9913	This project
pMAB125	ctg2_8994' - ctg2_8993', bpsA, ter, ColE1ori, aac(3)IV, oriT, attP, int	Biosensor vector	9867	This project
pSET152	ColE1ori, aac(3)IV, oriT, attP, int	Negative control vector	5715	This project
pBCB240	ColE1ori, aac(3)IV, oriT, attP, int, ter bpsA	Positive control vector	9905	This project
pSOK911	oriT, bla, ori ColE1, 'fd ter, tsr, attP, int	Cloning/ negative control vector	6838	provided by the lab
pSOK806	oriR, AmR, RP4 ori, attP, int, PermE*	Used to gain PermE*	5754	provided by the lab
pSOK912	oriT, bla, ori ColE1, 'fd ter, tsr, attP, int, PermE*	Vector for overexpression	7099	This project
pMOE632	oriT, bla, ori ColE1, 'fd ter, tsr, attP, int, PermE*, ctg2_8963	Overexpression vector	7828	This project

pMOE653	oriT, bla, ori ColE1, 'fd ter, tsr, attP, int, PermE* ctg2_8965	Overexpression vector	10163	This project
pMOE664	oriT, bla, ori ColE1, 'fd ter, tsr, attP, int, PermE* 'ctg2_8966'	Overexpression vector	10379	This project
pMOE795	oriT, bla, ori ColE1, 'fd ter, tsr, attP, int, PermE* ctg2_8979	Overexpression vector	7650	This project
pMOE846	oriT, bla, ori ColE1, 'fd ter, tsr, attP, int, PermE* ctg2_8984	Overexpression vector	7900	This project
pMKO781	oriT, bla, ori ColE1, 'fd ter, tsr, 'ctg2_8978'	Knock-out vector	5779	This project
pOTRE012	'otrR', 'dnaE1', ColE1ori, aac(3)IV, oriT, attP, int	Mutator	10210	provided by the lab

5.4. Primers

5.4.1. Primers for Biosensor vectors

Restriction sites are underlined (BamHI=GGATCC, SpeI=ACTAGT)

- BGC2.34_BS8970

BGC2.34_BS8970 forward primer

5'-GATCGGATCCATCCAGTCTAGGAGCAGTGGC-3' (31)

BGC2.34_BS8970 reverse primer

5'- GATCACTAGTGGCTCGGCCGAGTTCACG-3' (28)

→ PCR product: 240 bps (226 bps cut)

- BGC2.34_BS8980

BGC2.34_BS8980 forward primer

5'-GATCGGATCCGTGGTGACGAGGTCACCTG-3' (29)

BGC2.34_BS8980 reverse primer

5'-GATCACTAGTGCACGGAGATCTCCAGGGAGG-3' (31)

→ PCR product: 158 bps (144 bps cut)

- BGC2.34_BS8983

BGC2.34_BS8983 forward primer

5'-GACTACTAGTCAACTGCGTAACGGCGCG-3' (28)

BGC2.34_BS8983 reverse primer

5'-GATCGGATCCGCATACATGACAAGACCATTCGC-3' (33)

→ PCR product: 310 bps (296 bps cut)

- BGC2.34_BS8985

BGC2.34_BS8985 forward primer

5'-GATCGGATCCCAGTCGTTGACCCGTTTCC-3' (29)

BGC2.34_BS8985 reverse primer

5'-GACTACTAGTGTCCGTCATCATGTTCACTCCTCG-3' (34)

→ PCR product: 276 bps (262 bps cut)

- BGC2.34_BS8993

BGC2.34_BS8993 forward primer

5'-GATCGGATCCTGGTCGTGGCATTGATTCACTCC-3' (33)

BGC2.34_BS8993 reverse primer

5'-GATCACTAGTATGCGCGTCCTCGCAATG-3' (28)

→ PCR product: 230 bps (216 bps cut)

5.4.2. Primers for overexpression vectors

Restriction sites are underlined (EcoRI=GAATTC, SpeI=ACTAGT)

- BGC2.34_OE8963

BGC2.34_OE8963 forward primer

5'-GATCGAATTCGAGACGACCTCCGTCGTGAGTTG-3' (33)

BGC2.34_OE8963 reverse primer

5'-GATCACTAGTAAACCGGGACAGTTGCCCAG-3' (30)

→ PCR product: 767 bps (753 bps cut)

- BGC2.34_OE8965

BGC2.34_OE8965 forward primer

5'-GATCGAATTCGTGCCACACCGTGAACACC-3' (29)

BGC2.34_OE8965 reverse primer

5'-GATCACTAGTCCGTCGATCATCGAGAGGTGGC-3' (32)

→ PCR product: 3102 bps (3088 bps cut)

- BGC2.34_OE8966

BGC2.34_OE8966 forward primer

5'-GATCGAATTCGAACAATGGCTGCTCCGTCCG-3' (31)

BGC2.34_OE8966 reverse primer

5'-GATCACTAGTGACGGTCACTGTACTGCGTCC-3' (31)

→ PCR product: 3318 bps (3304 bps cut)

- BGC2.34_OE8979

BGC2.34_OE8979 forward primer

5'-GATCGAATTCAAGACCGCACTTCCGCGAC-3' (29)

BGC2.34_OE8979 reverse primer

5'-GATCACTAGTGATGCAGGTGCAAGGCCTGC-3' (30)

→ PCR product: 589 bps (575 bps cut)

- BGC2.34_OE8984

BGC2.34_OE8984 forward primer

5'GATCGAATTCTGGTCGCGTACGGGACCTG--3' (29)

BGC2.34_OE8984 reverse primer

5'-GATCACTAGTGGAAACGGGTCAACGACTGGG-3' (31)

→ PCR product: 839 bps (825 bps cut)

5.4.3. Primers for knock-out vector

Restriction sites are underlined (EcoRI=GAATTC, ndeI=CATATG)

- BGC2.34_OE8978

BGC2.34_OE8978 forward primer

5'-GATCGAATTCGAAGTCCGTACGGTTCAGCAG-3' (31)

BGC2.34_OE8978 reverse primer

5'-GATCCATATGACACCGCGTGTTCGTCCTC-3' (29)

→ PCR product: 1336 bps (1323 bps cut)

5.5. Kits

- Monarch® Spin gDNA Extraction Kit (New England Biolabs)

  → genomic DNA extraction
- Wizard® Plus SV Minipreps DNA Purification System (Promega)

  → plasmid DNA isolation
- Monarch® DNA Gel Extraction Kit (New England Biolabs)

  → DNA recovery from gel
- Q5® High-Fidelity 2X Master Mix (New England Biolabs)

  → PCR

5. Methods

6.1. In silico methods

6.1.1. Genome analysis performed with antiSMASH

The genomic sequence of *Streptomyces sp. RLA012* was uploaded to antiSMASH (Medema et al., 2011) and analysed by this software. It is a tool able to predict BGCs in the genome of bacteria. The output consists of the predicted cluster type e.g. NRPS, PKS, the position in the genome, associated genes as well as their enzyme class, function and domains, a putative product class and homology to any known BGC.

AntiSMASH is an open-source software and freely available. The online version can be accessed at https://antismash.secondarymetabolites.org.

6.1.2. Software

- Clone Manager (Sci Ed software): Clone manager is a software for the analyzation and visualization of DNA sequences. In this project it was used for cloning simulations and design of vector maps.
- Image Lab Software (Bio-Rad): Image Lab is a software used for the analyzation and documentation of gels and blots. It was used to check and take pictures of gels after gel electrophoresis.
- Lab Solutions (Shimazdu): Lab Solutions is a software for the control, data collection and analyzation for HPLC systems and other analytical instruments. It was used to gain and analyse HPLC chromatograms.

6.2. Preparation of media, solutions and buffers

6.2.1. Preparation of media

Ingredients where weighed, dissolved and/or mixed in beakers and put into autoclavable bottles. All media were autoclaved for 20min at 121°C and stored at room temperature. Media containing agar was melted in a microwave oven before usage. If antibiotics were needed, they were added right before usage. Prepared plates were stored at +4°C. Media recipes can be found at Materials 5.1.

6.2.2. Preparation of solutions and buffers

For solutions except 10x TBE buffer and 0.8-1.6% Agarose gel ingredients were weighed, dissolved and/or mixed in beakers, put into autoclavable bottles, autoclaved for 20min at 121°C and stored at room temperature. Ingredients for 10x TBE buffer were weighed, dissolved and stored at room temperature. Ingredients for 0.8-1.6% Agarose gel except 10,000 Gel Red were mixed and dissolved in a microwave, 10,000 Gel Red was added afterwards. It was stored at 60°C to stay liquid until used. Recipes can be found at Materials 5.1.5.

6.2.3. Preparation of antibiotic stock solutions

Antibiotics were weighed and dissolved in their corresponding solvent. Then solutions were sterile filtered and stored as aliquots at -20°C. Concentrations and solvents can be found at Materials 5.1.5.

6.3. Cultivation and storage of *Streptomyces*

6.3.1. Growth experiments

For growth experiments 100-200µl of spore suspension of *Streptomyces sp. RLA012* was spread on 10cm or 6cm plates containing 25-30ml or 15-20ml of different media, incubated at 28°C and checked each day for spore development.

For growth experiments in liquid media 100µl of spore suspension of *Streptomyces* were added to 10ml to different media in a sterile 100ml flask, incubated at 200rpm and 28°C (Incubator: Infors HT Multitron Pro, Infors HT) and checked daily.

6.3.2. Cultivation

For the growth of *Streptomyces sp. RLA012* 100-200µl of spore suspension was spread on 10cm or 6cm plates containing 25-30ml or 15-20ml ISP4 and incubated at 28°C. After 9-12 days spores were collected and spore suspensions prepared. For the growth of recombinant strains carrying a resistance marker the corresponding antibiotic was added to the medium. See Materials 5.1.5 for concentrations of antibiotics in the media.

6.3.3. Preparation of spore suspensions

Using a 10ml pipette 4ml of 20% glycerol stock solution for 10cm plates and 2ml for 6cm plates were pipetted onto a plate with sporulated *Streptomyces* RLA012. The tip of the pipette was then used to gently scratch the plate to remove the spores from mycelium and suspend them in the glycerol solution. The suspension was then filtered through a sterile syringe containing cotton wool. Aliquots of the spore suspension were stored in cryo tubes at -80°C.

6.3.4. Cleaning of spore suspensions

As spore suspensions frequently showed signs of contamination with either *E. coli* or other unknown microorganisms it was needed to clean them. This was done by transferring 100µl of spore suspension to an Eppendorf® tube containing 900µl of dH_2O (dilution -1), then transferring 100µl of dilution -1 to an Eppendorf® tube containing 900µl of dH_2O (dilution -2) and repeating this step until dilution -5. 100µl of dilutions -4 and -5 were then spread on ISP4 plates with or without added antibiotics. Single sporulated colonies were then either transferred to fresh plates and cultivated at 28°C to gain new spore suspensions.

6.3.5. Cultivation of *Streptomyces sp. RLA012* for DNA isolation and conjugation using mycelium

100µl of spore suspension were added to 10ml LB in a sterile 100ml flask and incubated at 200rpm and 28°C. After 3 days 1ml of the culture was used for DNA isolation.

6.3.6. Fermentation

100µl of spore suspension of recombinant strains were added to 15ml TSB in a sterile 250ml flask and incubated at 200rpm and 28°C for 2 days to start the seeding cultures. 30ml of fermentation medium in a sterile 250ml flask was inoculated with 3ml of the seeding culture and fermented for 3 or 7 days at 200rpm and 28°C (Incubator: Infors HT Multitron Pro, Infors HT).

6.4. Cultivation and storage of *E. coli*

6.4.1. Cultivation

To cultivate *E. coli* 100µl of spore suspension were spread on 10cm plates containing 25-30ml of LA and incubated at 37°C for 24h. If necessary, antibiotics were added to the medium.

6.4.2. Cultivation for plasmid DNA isolation

After incubating a plate with *E.Coli* for 24h at 37°C a single colony was picked with a sterile toothpick and put in a sterile culture tube containing 2ml of LB and appropriate antibiotics and incubated at 37°C and 200rpm for 24h.

6.4.3. Preparation of cell suspensions

After incubating a plate with *E.coli* for 24h at 37°C a single colony was picked with a sterile toothpick and put in a sterile culture tube containing 2ml of LB and appropriate antibiotics and incubated at 37°C and 200rpm for 24h. The culture was centrifuged at 13,400rpm (Centrifuge: MiniSpin®, Eppendorf) to gain a cell pellet. After removing the supernatant, the pellet was resuspended in 1,5ml 20% glycerol stock solution and stored in at -80°C in Cryo tubes.

Table 33: Strains of E. coli and corresponding antibiotics

Strain	Volume of antibiotc stock solution per 1ml of medium
E. coli XL-1blue WT (wild type)	No antibiotics
E. coli ET12567 WT	1µl Kan, 1µl Cml
E. coli XL-1blue (biosensor vectors)	1µl Am
E. coli ET12567 (biosensor vectors)	1µl Am, 1µl Kan, 1µl Cml
E. coli XL-1blue (overexpression and knockout vectors)	1µl Amp
E. coli ET12567 (overexpression and knockout vectors)	1µl Amp, 1µl Kan, 1µl Cml

6.4.4. Cultivation and storage of bioassay test organisms

Liquid cultures of test organisms were grown by inoculating 2ml of their corresponding medium with 100µl of cell suspensions and cultivated for overnight at 200rpm and their needed temperature. The cultures were either used to perform a disc diffusion assay or to create stocks. To create stocks the cultures were centrifuged at 13,400rpm, after removal of the supernatant, resuspended in 1,5ml 20% glycerol stock solution and stored as aliquots in Cryo tubes at -80°C.

Table 14: Media and growing temperature for test organisms

Organism	Medium	Temperature (°C)
Bacillus subitilis	LA	37
Micrococcus luteus	TSB	30
Pseudomonas putida	LA	37
Saccharomyces cerevisiae	YPD	28

6.5. Molecular biology methods

6.5.1. Genomic DNA isolation of *Streptomyces*

Genomic DNA of *Streptomyces sp. RLA012* was gained by using the Monarch® Spin gDNA Extraction Kit (New England Biolabs). 1ml of a culture grown as described at 1.3.4. was used.

1) The culture was centrifuged, the supernatant removed and resuspended in 100 µl cold PBS by pipetting up and down.
2) 1 µl proteinase K and 3 µl RNase A were added and mixed by vortexing (Vortex: MIX ARGOlab Vortex Mixer, ARGOlab) briefly.
3) 100 µl Cell lysis buffer were added and vortexed immediately.
4) The mixture was incubated at 56°C (Heating block: AccuBlock™ Digital Dry Baths, Labnet) and vortexed every 30s.
5) 400 µl gDNA binding buffer were added to the sample and mixed thoroughly by pulse-vortexing for 5-10 seconds.
6) The mix was transferred to a gDNA purification column pre-inserted into a collection tube, without touching the upper column area.
7) The mix was centrifuged for 3 minutes at 1,000rpm to bind the gDNA and then for 1 minute at 13,400rpm to clear the membrane. The flowthrough and collection tube were discarded.
8) The column was transferred to a new collection tube and 500 µl gDNA wash buffer were added. After closing the cap, the mixture was inverted a few times and immediately centrifuged for 1min at 13,400rpm. Afterwards the flowthrough was discarded.
9) After reinserting the column into the collection tube 500µl gDNA wash buffer were added and the mixture was centrifuged for 1min at 13,400rpm. Afterwards flowthrough and the collection tube were discarded.
10) The gDNA purification column was placed in a DNase-free 1.5 ml microfuge tube, 35-100 µl preheated (60°C) gDNA Elution Buffer were added and incubated at room temperature for 1min.
11) As last step it was centrifuged at 13,400rpm for 1min to elute the gDNA and the column discarded. The gDNA was stored at-20°C.

6.5.2. Plasmid DNA isolation from *E. coli*

To gain isolated plasmid DNA Wizard® Plus SV Minipreps DNA Purification System (Promega) was used. A culture grown as seen at 1.4.2. was used.

1) 2ml of culture were centrifuged for 3 min. at 13 000 rpm.
2) The pellet was resuspended with 250µl of Cell Resuspension Solution.
3) 250µl of cell lysis Solution were added and inverted 4 times.
4) 10µl of alkaline protease solution were added and inverted 4 times. The mixture was incubated at room temperature for 5min.
5) 350µl of neutralization solution were added and inverted 4 times.
6) The mixture was centrifuged at 13,400rpm for 10min.
7) The spin column was inserted into collection tube.
8) The cleared lysate was decanted into the spin column and centrifuged at 13,400rpm for 1min. The flowthrough was discarded.
9) 750µl of wash solution were added and centrifuged at 13,400rpm for 1min. The flowthrough was discarded.
10) 250µl of wash solution were added and centrifuged at 13,400rpm for 1min. The flowthrough was discarded.
11) It was centrifuged at 13,400rpm for 2min.
12) The spin column was transferred to a sterile 1.5ml microcentrifuge tube.
13) 100µl of nuclease-free water were added to the Spin Column and centrifuge at 13,400rpm for 1 min at room temperature.
14) The column was discarded and the DNA stored at -20°C.

6.5.3. PCR

PCR-reactions were performed to multiply specific parts of the genome prior identified with antiSMASH to create PCR products to insert them into plasmids creating different vectors. Q5® High-Fidelity 2X Master Mix (New England Biolabs) was used to perform PCR reactions. They were carried out on a Mastercycler® nexus X2 (Eppendorf). Between the addition of the individual components to the reaction mix, an Eppendorf® PCR (Eppendorf) cooler was used keep the reaction mix cold to prevent unspecific binding leading to wrong products. PCR products were stored at -20°C. Primer sequences, restriction sites, etc. (see below)

Table 15: Setup of PCR reactions

Component	Volume (µl)
DNA	1
Forward primer	0.5
Reverse primer	0.5
Q5® High-Fidelity 2X Master Mix	12.5
dH_2O	To 25

Table 16: PCR program used for the creation of biosensor vectors

Stage	Temperature (°C)	Time (s)
Initial denaturation	98	60
Denaturation	98	30
Annealing	68	60
Elongation	72	60
Final elongation	72	240
Hold	4	∞

Table 17: PCR program used for the creation of overexpression and knock out vectors

Stage	Temperature (°C)	Time (s)
Initial denaturation	98	60
Denaturation	98	30
Annealing	66	60
Elongation	72	180
Final elongation	72	240
Hold	4	∞

6.5.4. Restriction digestion and ligation

As a first step to cloning restriction digestions with restriction enzymes (pure or diluted 1:10), that cut DNA at a specific site was performed. This was done with plasmid DNA and DNA fragments gained from PCR to make the DNA ends compatible for ligation. Restriction digestion was also performed to find the correct clones after ligation. Plasmid DNA and PCR products were incubated with restriction enzymes in a water bath or in the PCR device at 37°C for 1-3h. After restriction the enzymes were either deactivated at an enzyme specific temperature or the reaction was used instantly for gel electrophoresis. The compatible ends of vector and insert were joined together by T4-DNA ligase. Concentrations of vector and insert DNA were examined by gel electrophoresis and used in a ratio of 1:4-10. Reactions were carried out overnight over an ice bath.

Table 18: Restriction digestion

Substance	Vol. for plasmid DNA (µl)	Vol. for PCR products (µl)
DNA	2-7	5
Enzyme 1	0,5 (2 diluted)	0,5 (2 diluted)
Enzyme 2	0,5 (2 diluted)	0,5 (2 diluted)
Cut Smart buffer (10x)	2	2
H_2O	To 20	To 20

Table19: Ligation reaction

Substance	Volume (µl)
Plasmid DNA	1
PCR product	4-10
T4-DNA ligase	1
T4-DNA ligase buffer	2
H_2O	To 20

6.5.5. Preparation of competent cells

Competent *E. coli* cells were created to introduce constructed plasmids by transformation. *E. coli XL-1bue* was used for cloning and *E. coli ET12567* for conjugation. They were created via the calcium chloride methode.

1) *E. coli ET12567 (pUZ8002)* cells were plated on an LA plate containing 30µg/ml Kan and 30µg/ml Cml and incubated overnight at 37°C.
2) A single colony was picked up with a sterile toothpick and transferred to a sterile containing 2ml LB, 30µg/ml Kan and 30µg/ml Cml and incubated overnight at 37°C and 200rpm.
3) 0.5ml of the overnight culture were added to 50ml of LB, 30µg/ml Kan and 30µg/ml Cml in a sterile 200ml flask and incubated at 37°C and 200rpm.
4) The OD_{600} was measured regularly by UV/Vis spectrophotometry (LB as blank) and when it reached 0.4-0.5 the culture was placed in an ice bath for 20min.
5) The culture was placed in a 50ml falcon tube and centrifuged at 6000rpm for 8min at 4°C. After the supernatant was removed.
6) The cell pellet was resuspended in 20ml of ice cold 50mM $CaCl_2$ solution and incubated on ice for 20min
7) The mixture was centrifuged at 6000rpm for 8min at 4°C. After the supernatant was removed.
8) The cells were resuspended in 2.5ml of ice cold 10% gylcerol 50mM $CaCl_2$ solution.

9) Eppendorf® Tubes were frozen in liquid nitrogen.
10) The resuspended cell mixture was put into the frozen Eppendorf® Tubes as aliquots of 100µl and stored at -80°C immediately. For recipes of media and solutions see Materials 5.1.

6.5.6. Transformation of *E. coli*

1) 100µl of competent cells in Eppendorf® Tubes were slowly melted on ice. 1µl of plasmid DNA or 10µl of ligation mixture were added and stirred very gently with the tip of the pipette.
2) The mixture was incubated on ice for 15-30min.
3) Heat shock was performed on a heating block at 42°C for 1min.
4) The tubes were instantly put back on ice for 5min.
5) 600µl of LB medium were added and then incubated at 37°C and 200rpm for 40min.
6) The cells were grown on plates according to Methods 6.4.1.

6.5.7. Conjugation to *Streptomyces*

To perform conjugation and introduce vectors to *Streptomyces*, *E. coli ET12567* was used. This strain carries the helper plasmid pUZ8002 which has a function for intergeneric transfer, it allows the introduction of any plasmid carrying RK2 *oriT*. Conjugation was performed in 2 different ways, to introduce biosensor vectors a method using spore suspensions (Methods 6.3.3.) of *Streptomyces* was used and to introduce overexpression and knock- out vector a method using mycelium grown in liquid culture (Methods 6.3.2.) was used.

- Using spore suspensions

1) *E. coli ET12567* cells carrying the desired plasmid were cultivated according to Methods 6.4.1.
2) Approximately 2cm^2 of a plate carrying were scratched of with a loop and transferred to Eppendorf® Tube containing 500µl of 2XYT. By pipetting up and down with the pipette the cells were evenly dispersed in the medium.
3) 50-90µl of *Streptomyces* spore suspension were added to 350µl of 2XYT, vortexed and heat shocked at 50°C for 7min.
4) The mixture was left to cool until reaching room temperature and then 100µl of the *E. coli* suspension were added.

5) Then the mixture was centrifuged at 13,400rpm for 1min and 250µl of the supernatant removed.
6) After resuspending the pellet in the remaining medium the suspension was spread on dry room temperature 10cm^2 SFM plates supplemented with 2.5ml 1M $MgCl_2$ stock solution.
7) Plates were incubated at 28°C for at least 24h or until first signs of growth could be seen.
8) Afterwards antibiotic selection was performed: 1ml of dH_2O was supplemented with 0.5µl per ml of SFM of Am stock solution to select transconjugants and 1µl per ml of SFM of Nal stock solution against *E. coli*. First Nal was added then Am, if it came to precipitation 0.1M NaOH stock solution was added in steps of 15µl and vortexed until the solution appeared clear.
9) 1ml of this solution was pipetted onto the plates, carefully spread to leave no dry sections and then left to dry completely (at least 30min).
10) Plates were then incubated at 28°C and after 6-8 days single transconjugants appeared. They were then transferred to ISP4 plates containing 0.5µl of Am stock solution per ml of ISP4 and 1µl of Nal stock solution per ml of ISP4 using a sterile toothpick.
11) Plates were again incubated at 28°C and after 9-12 days sporulation occurred.
12) Media with spores was cut out of the plates and transferred to 2mL Soft tissue homogenizing CK14 tubes (Bertin Technologies) containing 500µl 2XYT and homogenized (Precellys 24, Bertin technologies)).
13) The homogenized mixture was then spread on ISP4 plates containing 0.5µl of Am stock solution per ml of ISP4.
14) After incubating these plates at 28°C for 9-12 days spores could be collected.

- Using mycelium

1) *E. coli ET12567* cells carrying the desired plasmid were cultivated according to Methods 6.4.1.
2) Approximately 2cm^2 of a plate carrying were scratched of with a loop and transferred to Eppendorf® Tube containing 500µl of 2XYT. By piping up and down with the pipette the cells were evenly dispersed in the medium.
3) A liquid culture grown as can be seen at Methods 6.3.5. was transferred to a sterile 50ml tube and centrifuged at 4000rpm (Centrifuge 5810 R, Eppendorf) for 5min.
4) The supernatant was removed, 5ml of dH_2O added, briefly vortexed and again centrifuged a 4000rpm for 5min. Then again, the supernatant was removed.

5) Step 4 was repeated and 5ml 2XYT added. The suspension was then put into sterile Eppendorf® tubes as aliquots of 250µl.
6) 100µl of the *E. Coli* suspension were added.
7) Then the mixture was centrifuged at 13,400rpm for 1min and 100µl of the supernatant removed.
8) After resuspending the pellet in the remaining medium the suspension was spread on dry room temperature 10cm^2 SFM plates supplemented with 1M $MgCl_2$.
9) Plates were incubated at 28°C for at least 24h or until first signs of growth could be seen.
10) Afterwards antibiotic selection was performed: 1ml of dH_2O was supplemented with 0.5µl per ml of SFM of Thio stock solution to select transconjugants and 1µl per ml of SFM of Nal stock solution against *E. coli*. First Nal was added then Thio, if it came to precipitation 0.1M NaOH stock solution was added in steps of 15µl and vortexed until the solution appeared clear.
11) 1ml of this solution was pipetted onto the plates, carefully spread to leave no dry sections and then left to dry completely (at least 30min).
12) Plates were then incubated at 28°C and after 6-8 days single transconjugants appeared. They were then transferred to ISP4 plates containing 0.5µl per ml of ISP4 of Thio stock solution and 1µl per ml of ISP4 of Nal stock solution using a sterile toothpick.
13) Plates were again incubated at 28°C and after 9-12 days sporulation occurred.
14) Media with spores was cut out of the plates and transferred to 2mL Soft tissue homogenizing CK14 tubes (Bertin technologies) containing 500µl 2XYT and homogenized (homogenizer: model?).
15) The homogenized mixture was then spread on ISP4 plates containing 0.5µl of Am stock solution per ml of ISP4.
16) After incubating these plates at 28°C for 9-12 days spores could be collected.

6.6. Gel electrophoresis

6.6.1. Gel electrophoresis

To carry out restriction analysis, check if PCR reactions led to desired products, verify the integrity of genomic DNA and to isolate PCR products and fragments of plasmids gel electrophoresis was performed. 0.8-1.6% Agarose gels (Materials 5.1.5) were cast and left to dry at room temperature. Higher concentration gels were used for smaller fragments. After

the removal of the comb the gel was ready to apply the samples. On the left and right reference ladders Quick-Load® 1 kb DNA Ladder and Quick-Load® 100 bp DNA Ladder (New England Biolabs) were applied. Samples were mixed with blue or purple DNA Gel Loading Dye (6X) (Thermo Scientific™). After loading all the samples, the gel was run in 1xTBE buffer (Materials 5.1.5) at 100-120V for 40-180min. The addition of Gel Red® (Biotium) allows the detection of DNA under UV light. To view the gels and take pictures the device ChemiDoc™ Touch Imaging System (Bio-Rad) was used. To view pictures and process them Lab Solutions (Shimazdu) was used.

Table 20: volumes of samples applied for gelelectrophoresis

Sample	Volume (total volume) analysis (µl)	Volume (total volume) preparation (µl)
Genomic DNA	1 (10)	/
Plasmid DNA	1-2 (10)	/
PCR products	10 (10)	10 (10)
Vector fragments	1-2 (10)	20 (20)

Figure 55: Quick-Load® 1 kb DNA

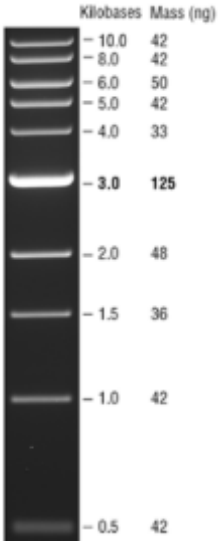
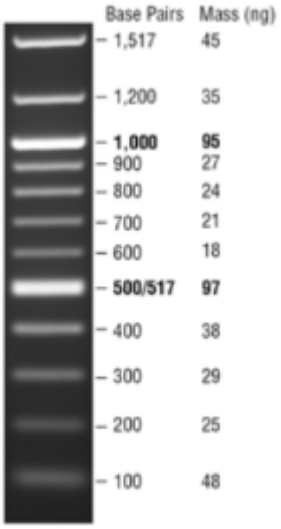


Figure 56: Quick-Load® 100 bp DNA



6.6.2. Purification from gel

To recover PCR products and vector fragments from a gel after gel electrophoresis Monarch® DNA Gel Extraction Kit (New England Biolabs) was used

1) A gel piece containing the desired DNA fragment was excised with a scalpel under the light of the ChemiDoc™, put into a 1,5ml Eppendorf® tube and weighed.
2) 4 volumes (e.g. 400µl for 100mg of gel) of gel dissolving buffer were added to the tube.

3) The sample was kept on a heating block at 50°C for 5-10min and periodically inverted to make sure gel slice dissolved completely.
4) The spin column was inserted into the collection tube and spun for 1min at 13,400rpm. The flow through was discarded.
5) The column was reinserted, 200µl of DNA wash buffer added and spun for 1min at 13,400rpm.
6) Step 5 was repeated.
7) The column was transferred to a new 1,5ml Eppendorf® tube.
8) 12µl of DNA elution buffer or nuclease free water were added to the centre of the matrix. After waiting for 1min the sample was centrifuged for 1min at 13,400rpm to elute the DNA.

6.7. Preparation of extracts and analysis

6.7.1. Creating extracts from fermentation cultures

1) Fermentation was performed as can be seen at Methods 6.3.6.
2) The whole culture was transferred to a 100ml round bottom flask and frozen on an ethanol bath.
3) The flasks were attached to the freeze dryer and frozen for 24h.
4) The material was dissolved in 30ml of methanol on a shaker for 1h.
5) The solution was transferred to a 50ml falcon tube and centrifuged at 4000rpm for 10min.
6) The supernatant was transferred to a 50ml round bottom flask and the methanol was evaporated using the rotary evaporator (Heidolph VV2011, heidolph).
7) The pellet was resuspended in 1.5 methanol, centrifuged at 13,400 for 1min ant stored at -20°C.

6.7.2 Disc diffusion assay

To test the bioactivity of the gained extracts, disc diffusion assays were performed against different test organisms (Materials 5.2.3.).

1) 15cm plates containing LA medium were prepared. The plate was divided into 16 squares (1 square for each extract and control, control: methanol).
2) 300µl of test organism cell suspension was spread onto the plates.
3) 30µl of extract were pipetted onto paper discs and then placed on their corresponding square with sterile tweezers.

4) Plates were then incubated at the test organism specific growing temperature and examined each day for the appearance of a growth inhibiting zone around the paper discs.

6.7.3. High performance liquid chromatography (HPLC)

To analyse the extracts HPLC was performed on a Shimazdu system with a photo diode array detector (190nm-800nm) and an evaporative light scattering detector. As stationary phase the following column was used: (Luna Omega C18, 4.6x150mm, 5µm, Phenomenex™) Column oven temperature was 25°C. A mixture of 2 solvents (Solvent A = 0.1% aqueous solution of formic acid; solvent B = acetonitrile) served as mobile phase. The mobile phase had a flow rate 0.5ml/min. A gradient elution was performed, increasing the amount of solvent B from 5% to 95% over 45min. After reaching the final concentration of solvent B the column was washed re-equilibrated with the mixture containing the starting concentration of 5% solvent B.

6. List of abbreviations

Am=Apramycin

Amp=Ampicillin

BGC=Biosynthetic gene cluster

Cml=Chloramphenicol

FRET=Fluorescence resonance energy transfer

hCG=Human chorionic gonadotropin

HPLC=High performance liquid chromatography

IUPAC =International Union of Pure and Applied Chemistry

Kan=Kanamycin

Nal=Nalidixic acid

NRP=Non ribosomal peptide

NRP=Non ribosomal peptide synthase

PDA=Photodiode array

PK=Polyketide

PKS= Polyketide synthase

TF=Transcription factor

Thio=Thiostrepton

WT=Wild type

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