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Table of content:

List of Abbreviations.....	3
1. Zusammenfassung	4
1. Abstract	6
2. Aim of the Work	7
3. Introduction	9
3.1. Secondary Metabolites	9
3.2. Antibiotics.....	9
3.2.1. The Need for new Antibiotics	11
3.3. Actinobacteria as a rich Source of Antibiotics.....	12
3.3.1. Marine Actinomycetes	13
3.3.2. Streptomyces spp.	14
3.4. Biosynthesis of Secondary Metabolites in Actinomycetes	16
3.5. Identification and Activation of Silent Gene Clusters	18
3.5.1. Identification of BGCs by Genome Mining	18
3.5.2. Methods for the Activation of Silent Gene Clusters	19
3.5.3. The useful Reporter Gene <i>bpsA</i>	20
3.6. This Project.....	22
4. Results	23
4.1. Genome Analysis by antiSMASH	23
4.2. Design of the Biosensor System.....	27
4.3. Selected BGCs and Intergenic Regions.....	28
4.4. Construction of Positive and Negative Control Vector.....	31
4.4.1. Positive Control Vector.....	32
4.4.2. Negative Control Vector.....	34
4.5. Primer design	36
4.6. PCR and Construction of Promoter Probe Vectors	36
4.6.1. Isolation of Genomic DNA	36
4.7. PCR and Construction of Knock-out Vectors.....	43
4.7.2. Construction of Knock-out Vectors.....	45
4.8. Conjugation	45
4.9. Fermentation Cultures	46
4.9.1. Ethanol-shock.....	49
4.10. Extraction of SMs from Fermentation Cultures.....	50
4.11. Growth Inhibition Assays	50
4.12. Comparative HPLC Analysis of Crude Extracts	52
4.13. HRMS Analysis	53

5. Discussion.....	57
6. Material.....	61
6.1. Organisms and Vectors	61
6.1.1. <i>Streptomyces</i>	61
6.1.2. <i>Escherichia coli</i>	61
6.1.3. Bioassay Test Organisms	62
6.1.4. Vectors.....	63
6.2. Media, Solutions and Buffers	64
6.2.1. Media used for <i>Streptomyces</i>	64
6.2.2. Media for <i>E. coli</i> and Bioassay Test Organisms	66
6.2.3. Stock Solutions and Buffers	66
6.3. Primers.....	67
6.4. Kits	68
7. Methods	69
7.1. In Silico Methods.....	69
7.1.1. Bioinformatic Genome Analysis by antiSMASH	69
7.1.2. Software used in this Project	69
7.2. Preparation of Media and Antibiotic Stock Solutions	69
7.2.1. Preparation of Media	69
7.2.2. Preparation of Antibiotic Stock Solutions	69
7.3. Cultivation and Storage of <i>Streptomyces</i> and <i>E. coli</i>	70
7.3.1. Cultivation and Storage of <i>Streptomyces</i>	70
7.3.2. Cultivation of <i>Streptomyces</i> Strains for total DNA Isolation	70
7.3.3. Fermentation Cultures of <i>Streptomyces</i> Strains	70
7.3.4. Cultivation and Storage of <i>E. coli</i>	71
7.3.5. Cultivation and Storage of Bioassay Test Organisms	71
7.4. Molecular Biological Methods	72
7.4.1. Genomic DNA Isolation from <i>Streptomyces</i> Strains	72
7.4.2. Plasmid DNA Isolation from <i>E. coli</i>	73
7.4.3. Primer Design	73
7.4.4. PCR	73
7.4.5. PCR-product Purification.....	74
7.4.6. Cloning	75
7.4.7. Preparation of Competent Cells.....	75
7.4.8. Transformation of <i>E. coli</i>	76
7.4.9. Conjugation to <i>Streptomyces</i>	76
7.5. Analytical and Preparative Gel Electrophoresis.....	77
7.6. Extraction and Analysis of Secondary Metabolites	78
7.6.1. Extraction of SMs from Fermentation Cultures	78

7.6.2. Disc Diffusion Assay.....	79
7.6.3. High Performance Liquid Chromatography (HPLC)	79
7.6.4. High Resolution Mass Spectrometry (HRMS).....	80
8. References	81
9. Acknowledgements	88
10. Appendix	89
10.1. Primers for Promoter Probe Vectors.....	89
10.2. Primers for Knock-out Vectors.....	92

List of Abbreviations

AM Apramycin
AMP Ampicillin
BGC Biosynthetic gene cluster
BLAST Basic Local Alignment Search Tool
CML Chloramphenicol
ELSD evaporate light scattering detector
HF High Fidelity
HPLC High-performance liquid chromatography
HPLC-MS High-performance liquid chromatography- mass spectrometry
KO Knock-out
KAN Kanamycin
NAL Nalidixic acid
NADPH Nicotinamide adenine dinucleotide phosphate
NMR Nuclear magnetic resonance spectroscopy
NRPS Non-ribosomal peptide synthase
OD Optical density
SM Secondary metabolite
PCR Polymerase chain reaction
PDA photo diode array
PKS Polyketide synthase
WT Wild type

1. Zusammenfassung

Pflanzen, Tiere und Mikroorganismen stellen eine reiche Quelle für Sekundärmetaboliten dar. Viele dieser natürlichen Produkte, darunter Antibiotika, spielen in der Medizin neben anderen Bereichen wie zum Beispiel in der Landwirtschaft eine wichtige Rolle. Seit Alexander Flemings Entdeckung des Penicillins 1928 und dem später folgenden, sogenannten goldenen Zeitalter der Antibiotika Forschung und der erfolgreichen Anwendung vieler der dabei gefundenen Wirkstoffe in der Therapie von infektiösen Erkrankungen, ist es leider auch schon zu Resistenzbildungen von Mikroorganismen gegen viele dieser Wirkstoffe gekommen.

Um die schweren Folgen einer post-antibiotischen Ära, in der vormals leicht zu behandelnde Infektionen durch z.B. kleine Verletzungen und Wunden wieder zum Tod führen können, da Therapiemöglichkeiten, aufgrund der mangelnden Zahl an Arzneistoffen, fehlen, zu verhindern, gilt es den Fokus der Forschung vermehrt auf das Ziel der Entdeckung neuer Antibiotika zu richten. Durch die hohe Wiederfindungsrate von bioaktiven Substanzen aus terrestrischen Quellen und der hohen Biodiversität im Meer, wurde die Erforschung mariner Lebensräume interessanter. Gleichzeitig stehen mehr, schnellere und günstigere Methoden zur Sequenzierung von Genomen zur Verfügung und die daraus resultierende stetig steigende Anzahl sequenzierter Genome, die Dank der wiederum verbesserten bioinformatischen Methoden präziser analysiert werden können, zeigen, dass ein enormes, meist verborgenes Potential, in Form von viel mehr biosynthetischen Genclustern in Chromosomen von Mikroorganismen, vorhanden ist, sie die darin kodierten Produkte aber unter normalen Kulturbedingungen wegen mangelnder umweltbedingter Reize nicht produzieren.

Um dieses Potential auszuschöpfen und Gencluster zu aktivieren, von denen vermutet wird, dass sie für die Bildung von unbekannten Komponenten verantwortlich sind, können verschiedene Methoden angewendet werden. In dieser Diplomarbeit wurde das Ziel der Entdeckung neuer natürlicher Substanzen, mit Hilfe rekombinanter DNA Technologie und eines damit konstruierten Reporter Systems, verfolgt. Es wurden drei *Streptomyces* Stämme verwendet, die aus drei verschiedenen Schwämmen des Trondheim Fjords in Norwegen isoliert wurden. Mit Hilfe der Software antiSMASH wurde das Genom dieser Aktinobakterien auf das Vorhandensein von biosynthetischen Genclustern untersucht und jene Cluster ausgewählt, die für die Bildung von eher unbekannten Sekundärmetaboliten verantwortlich sind. Um die Aktivität von vermutlichen Promotor Regionen in diesen Clustern zu ermitteln, wurden zuerst Primer entworfen und die entsprechenden Promotor Regionen unter Verwendung dieser Primer mittels PCR hergestellt. Nach dem Einbau dieser Promotoren in Plasmide, die ein ebenfalls eingebautes Reportergen namens *bpsA*, das für die Produktion von Indigoidin verantwortlich ist, enthielten, wurden diese Vektoren durch Konjugation von *E. coli* in die *Streptomyces* Stämme überführt und die Aktivität der Promotor Regionen aufgrund der Farbänderung des Fermentationsmediums durch Expression von *bpsA* beobachtet.

Die Verwendung von verschiedenen Nährmedien zur Fermentation und die Anwendung eines Ethanol- Schocks sollten die inaktiven Promotoren aktivieren und diese Aktivierung und die eventuell daraus folgende Bildung von Sekundärmetaboliten mittels Reporter nachgewiesen werden. Aus Kulturmedien mit einer Farbänderung wurden Extrakte hergestellt, die mittels HPLC und HPLC-MS analysiert und mit Hilfe von Wachstumshemmungstests auf Bioaktivität überprüft wurden.

1. Abstract

Plants, animals and microorganisms are a rich source of natural products, namely secondary metabolites. Some of these natural products can be used as drugs for the treatment of diseases, e.g. antibiotics for infections. The discovery of new anti-infectives is necessary due to the growing number of resistant pathogens. Despite a lack of medical treatments caused by a reduced number of available antibiotics, the list of newly discovered compounds that can be used for such purposes is short. One problem is frequent rediscovery of already known compounds. However, new approaches can be used to find unknown bioactive compounds using genomics, next to common screening methods. Bioinformatics-guided analysis of genome sequences of some bacteria revealed a vast potential for secondary metabolite production. In bacteria the genes for secondary metabolite biosynthesis are often physically clustered together. Under standard laboratory conditions most of these clusters remain silent, so it is necessary to activate them for the production of potentially new compounds.

Genetic manipulations by molecular biological methods and the use of different fermentation media can force the production of putative new substances. In this work, marine actinomycetes from the genus *Streptomyces*, which represent a rich source for bioactive secondary metabolites were used to discover new natural products. The draft genome sequences of three different strains isolated from three different sponges from Norwegian fjord were analyzed by a bioinformatics tool called antiSMASH. Different biosynthetic gene clusters, which have, according to the *in-silico* analysis, a higher chance to produce an unknown compound, were chosen for this project. Putative promoter regions of the genes, which are responsible for the building of scaffold structures of secondary metabolites, were amplified by PCR.

Those PCR products were introduced into plasmids upstream of the reporter gene *bpsA*, a NRPS encoding for indigoidine-synthase, responsible for production of a blue pigment indigoidine. After the introduction of those vectors in the *Streptomyces* strains by conjugation from *Escherichia coli*, fermentations in different media were performed. The observation of a color-change in the media used for fermentation, with and without ethanol shock, and compared to a control, indicated the activity of the promoter region and thus, the activation of the cluster. The latter may suggest the production of secondary metabolites, some of which are maybe previously undetected compounds. Fermentation cultures with a visible color difference, compared to a control, were extracted. The extracts were tested for bioactivity by growth inhibition assays. HPLC and HPLC-MS analysis resulted in known and putative new substances, which are interesting for further analyzation.

2. Aim of the Work

In this work, three *Streptomyces* strains (ADI 95-17, GBA 94-01 and PVA 91-01) isolated from samples of three different sponges (*Antho dichotoma*, *Geodia barretti* and *Phakellia ventilabrum*), which were collected at the Tautra ridge (Trondheim fjord, Norway) by members of the Norwegian University of Science and Technology, were chosen for a reporter-guided detection of the activation of silent gene clusters for the discovery of novel secondary metabolites. To first explore the genomic potential of these strains, bioinformatic analysis of genome sequences for biosynthetic gene clusters should be carried out.

Promoter regions of biosynthetic genes, which are responsible for the building of scaffold structures of secondary metabolites, are then chosen as targets. These promoter regions are synthesized by PCR using primers, which include restriction sites for endonucleases to be capable for a further insertion of these PCR-products into plasmids to create promoter probe vectors. These vectors include the reporter gene *bpsA*, which leads to indigoidine production when expressed, downstream of inserted promoter regions. Used plasmids are capable to integrate themselves into the genome of *Streptomyces* strains and should be transferred to them by conjugation from *Escherichia coli*. Introduction of these promoter probe vectors is performed to detect promoter activity by a color change in the fermentation media due to the production of indigoidine. Silent promoters, which show no color change of the media, are attempted to activate in different fermentation media and by ethanol-shock.

A resulting color change of the media due to promoter activation suggests the production of possibly unknown secondary metabolites. To proof if the gene expression and the production of secondary metabolites is the result of the awakening of a silent cluster by triggering promoter activity, knock-out vectors are constructed for the insertion into strains which show a color change of the media. After the preparation of extracts, comparative high-performance liquid chromatography (HPLC) can be applied to detect differences of secondary metabolite production in mutant and knock-out strains. Extracts should be tested for bioactivity by disc diffusion method and if promising, further analyzed by mass spectrometry.

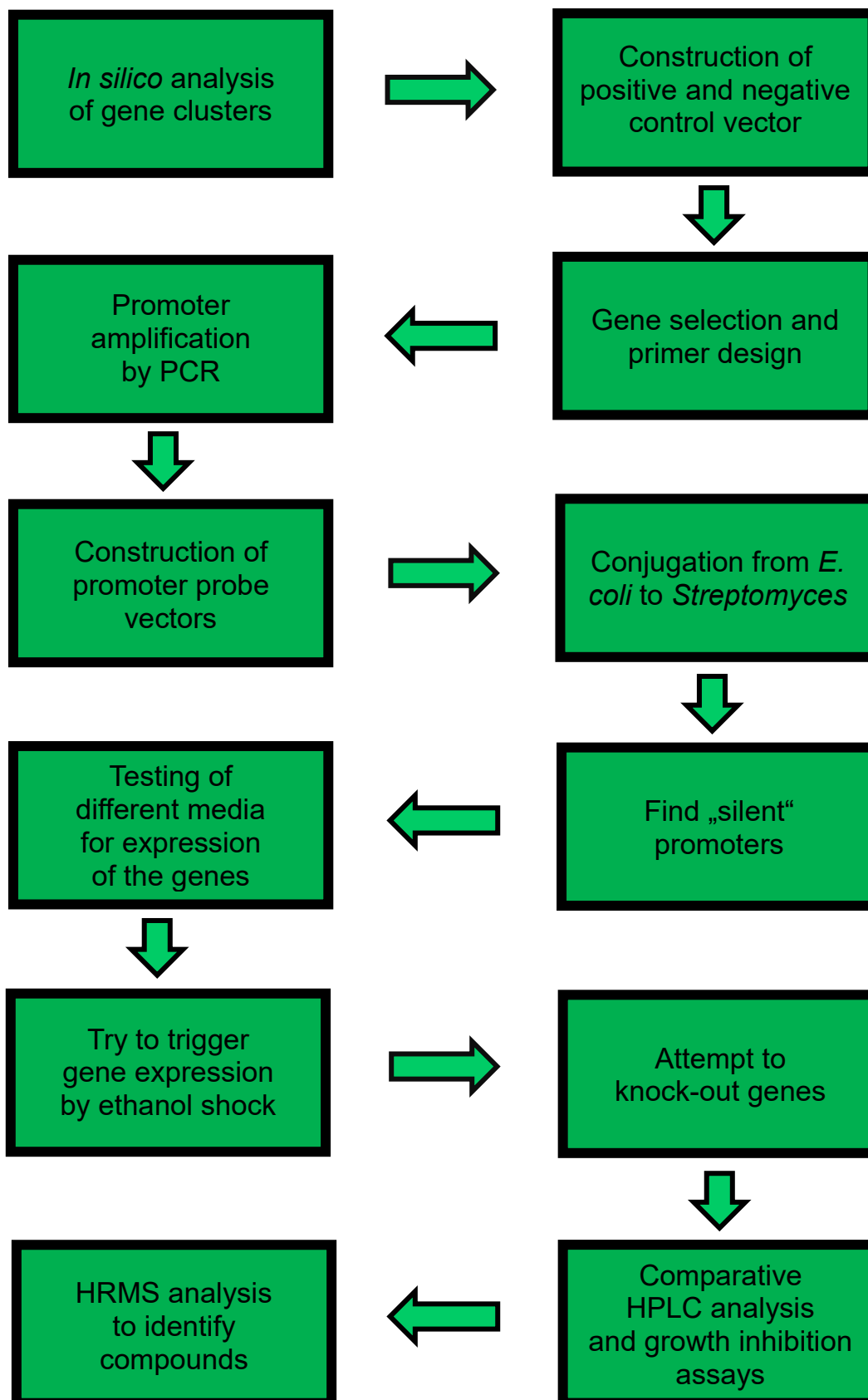


Figure 1: Overview of the workflow for this diploma project.

3. Introduction

3.1. Secondary Metabolites

Secondary metabolites are natural products produced by microorganisms, plants and animals which are not considered essential for organism growth and reproduction.

Nevertheless, they appear to play a significant role for those organism's adaption to the environment. Those substances have a low molecular weight and they have a plethora of different molecular structures and thus many different functions.¹ In nature, those substances are produced to promote survival functions and used as competitive weapons against other bacteria, fungi, plants, insects, and large animals, but also as metal transporting agents, as agents of symbiosis between microbes and plants, insects and higher animals, as sexual hormones and as differentiation effectors.² Various microbial communities exist as a multi-cellular network, and microbes have to evolve continuously upon encountering the constant environmental threat and the diverse selection pressure. When it comes to events such as a limitation of nutrients, microorganisms start to produce natural products to combat these environmental threats to ensure their survival.³ Secondary metabolites generated by microbes, which are of interest for medical purposes, include anti-infectives, comprising antibiotics against bacteria, antimycotics and antivirals, as well as antiparasitic agents, cytotoxic compounds, immunosuppressants and cholesterol lowering drugs.⁴

In agriculture, some microbial natural products are used as bioherbicides, plant growth regulators, biopesticides and bio insecticides, moreover they are also used as biofuels.^{5 6}

In the natural habitat secondary metabolites like antibiotics are produced at concentrations much lower than therapeutic doses, thus ways to boost the formation of these natural products are needed if they are to be produced at industrial scale.⁷

3.2. Antibiotics

Anti-infective treatments are utilized for thousands of years and are documented for ancient societies like in Egypt, Greece, the Roman Empire and China. For example, the antibiotic properties and the wound healing of honey were already known.⁸ Since the modern era of antibiotics started with the serendipitous discovery of the fungus derived β -lactam antibiotic penicillin by Sir Alexander Fleming in 1928.⁹ In 1945, Fleming, Florey and Chain got the Nobel Prize in physiology or medicine for the discovery of penicillin. Their work mainly contributed to the development of this natural product to a suitable treatment for infectious diseases.

Since antibiotics were prescribed to treat infections in the 1940s, millions of lives have been saved. In the United States for example, life expectancy increased from 47 years in 1900 to 74 years for males and to 80 years for females in the year 2000.¹⁰

Unfortunately, we are heading for a post-antibiotic era, because resistance occurs to nearly all antibiotics that have been developed and without intervention, minor infections and small injuries will once again be fatal.¹¹ Antibiotics target bacterial vital cellular functions, causing microbial cell death or the cessation of growth. These agents act by mechanisms (Figure 2) like the inhibition of cell wall synthesis (e.g. β -lactams and glycopeptides), protein biosynthesis, mainly via interaction with ribosomal subunits (e.g. aminoglycosides and macrolides), DNA replication (e.g. quinolones), RNA synthesis (e.g. rifampicin), interfering with other essential cellular functions or metabolic pathways (e.g. folic acid antagonists) and by disruption of the bacterial membrane structure (e.g. daptomycin).¹²

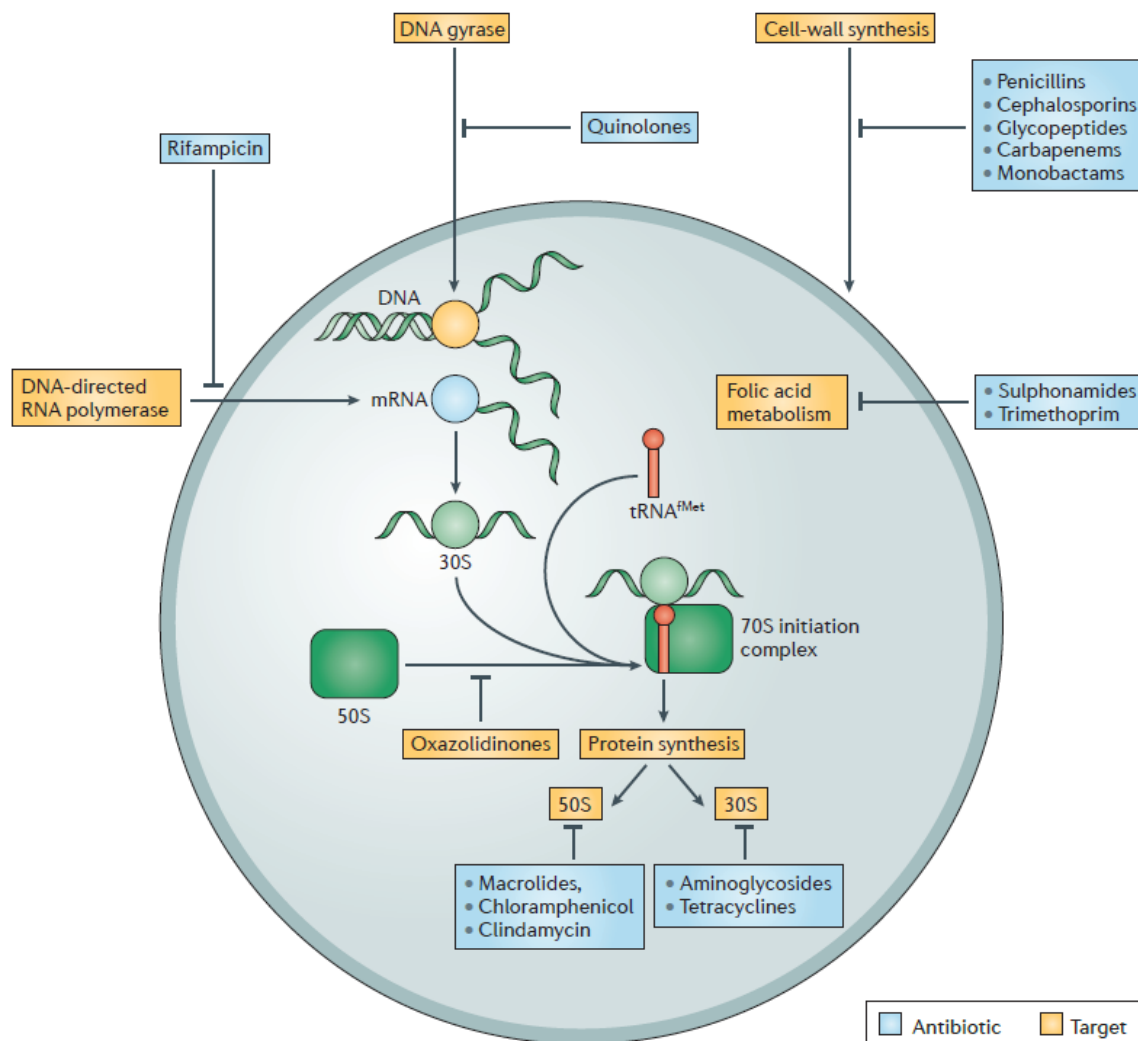


Figure 2: Targets of antibiotics. The main targets of antibiotics are cell wall synthesis, disruption of bacterial membrane, protein biosynthesis, RNA synthesis, DNA replication and metabolic enzymes.¹³

3.2.1. The Need for new Antibiotics

The emergence and rapid spread of antibiotic resistance among pathogens is a major threat to our health system. Multidrug resistant bacterial pathogens such as the so-called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) can cause untreatable infectious diseases.¹⁴ According to the review on antimicrobial resistance by Jim O'Neill¹⁵ the number of deaths caused by infectious diseases will increase from 700,000 to 10 million annually by 2050, thereby exceeding the number of deaths caused by cancer, which is actually the main cause of mortality.

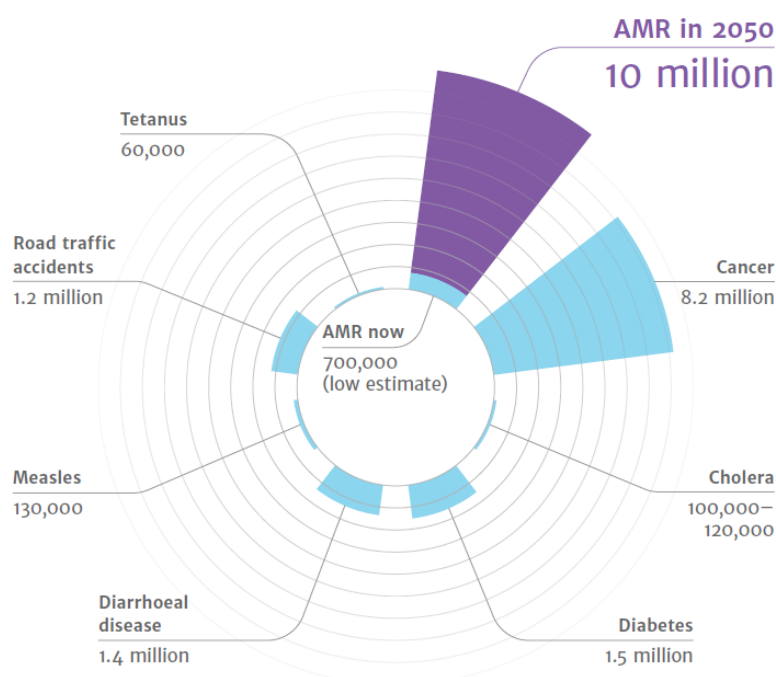


Figure 3: Worldwide deaths caused by antimicrobial resistance increase.¹⁵

Emergence and spread of antibiotic resistance, are caused by inconsiderate prescription of antibiotics by physicians, the intense use of antibiotics in animal husbandry and agriculture and the use of antimicrobial soaps and other products containing disinfectants.¹⁶ Microorganisms can acquire resistance (Figure 4) due to inactivation of antibiotics by enzymes, efflux mechanisms, which pump the antibiotic out of the cells, decreased uptake of the antibiotic, modification of the target to decrease binding efficiency of the antibiotic to the target, overproduction of the target and bypassing the essentiality of the target.¹⁷

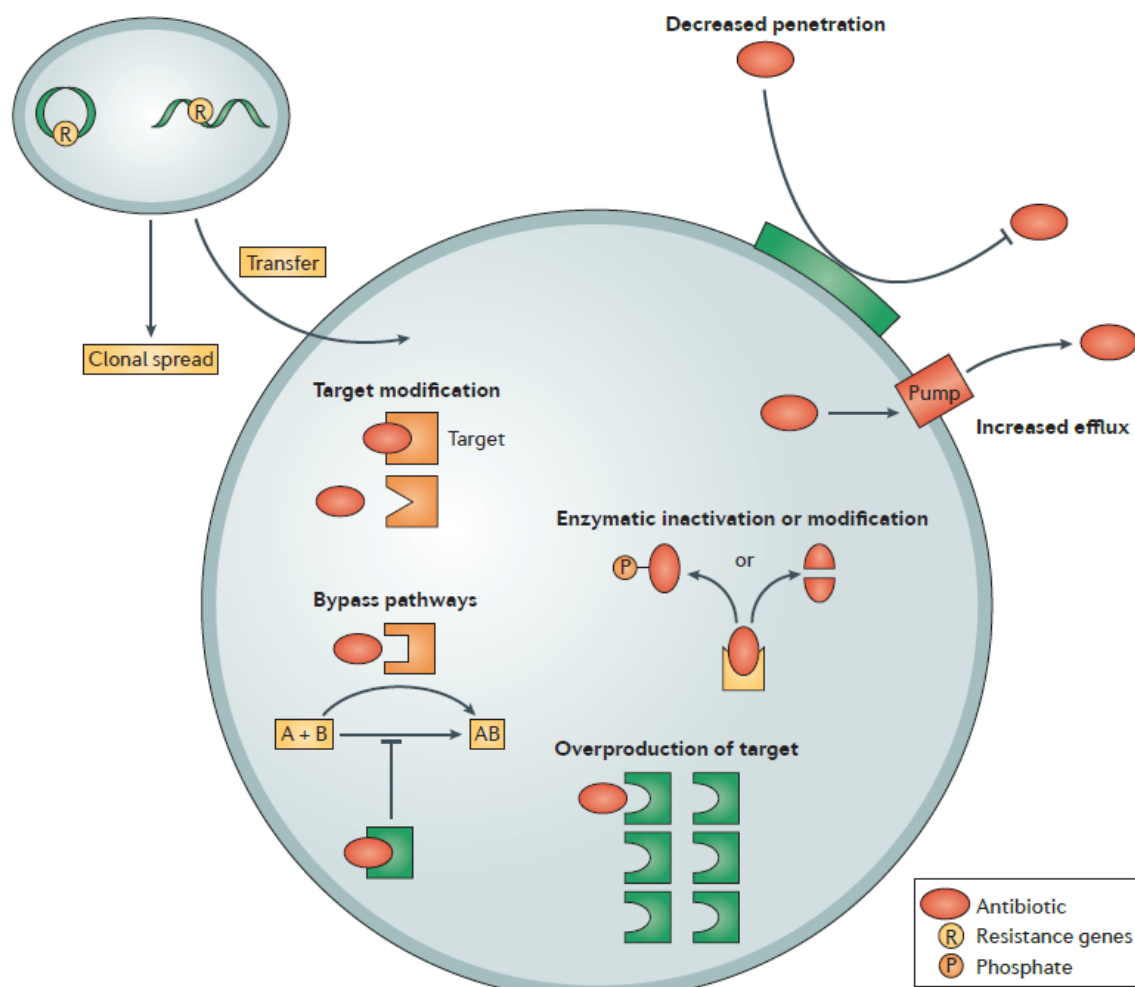


Figure 4: Mechanisms of how microorganisms can acquire antibiotic resistance.¹³

3.3. Actinobacteria as a rich Source of Antibiotics

Actinobacteria are Gram-positive, often filamentous bacteria with a high GC content in their DNA. They are widely distributed in both terrestrial and aquatic ecosystems, mainly in soil, decomposing biomaterials consisting of complex mixtures of polymers in dead plants, animals and fungal materials.¹⁸ Due to the diverse secondary metabolism these bacteria generate a large number of compounds, which are of major importance for biotechnology, agriculture and especially medicine, because they produce about two-thirds of all naturally derived antibiotics in current clinical use. The discovery of actinomycin from *Streptomyces antibioticus* in 1940, streptothricin from *Streptomyces lavendulae* in 1942, and especially streptomycin from *Streptomyces griseus* in 1943, present the first antibiotics isolated from Actinobacteria, all by the group of Selman Waksman. These microorganisms, particularly various *Streptomyces* species, have been a rich source of natural products and play an important role for the discovery of novel bioactive compounds.³

The major classes of antibiotics (Figure 5) produced by actinomycetes are aminoglycosides, angucyclines, ansamycines, anthracyclines, β -lactams, chloramphenicol, glycopeptides, lantibiotics, lipopeptides, macrolides, oxazolidinones, streptogramins and tetracyclines.³

New anti-infective and yet uncharacterized natural products¹⁹ can be found by using high-throughput fermentation, mining genomes for cryptic pathways, pleiotropic and pathway specific methods to activate secondary metabolite production etc.²⁰

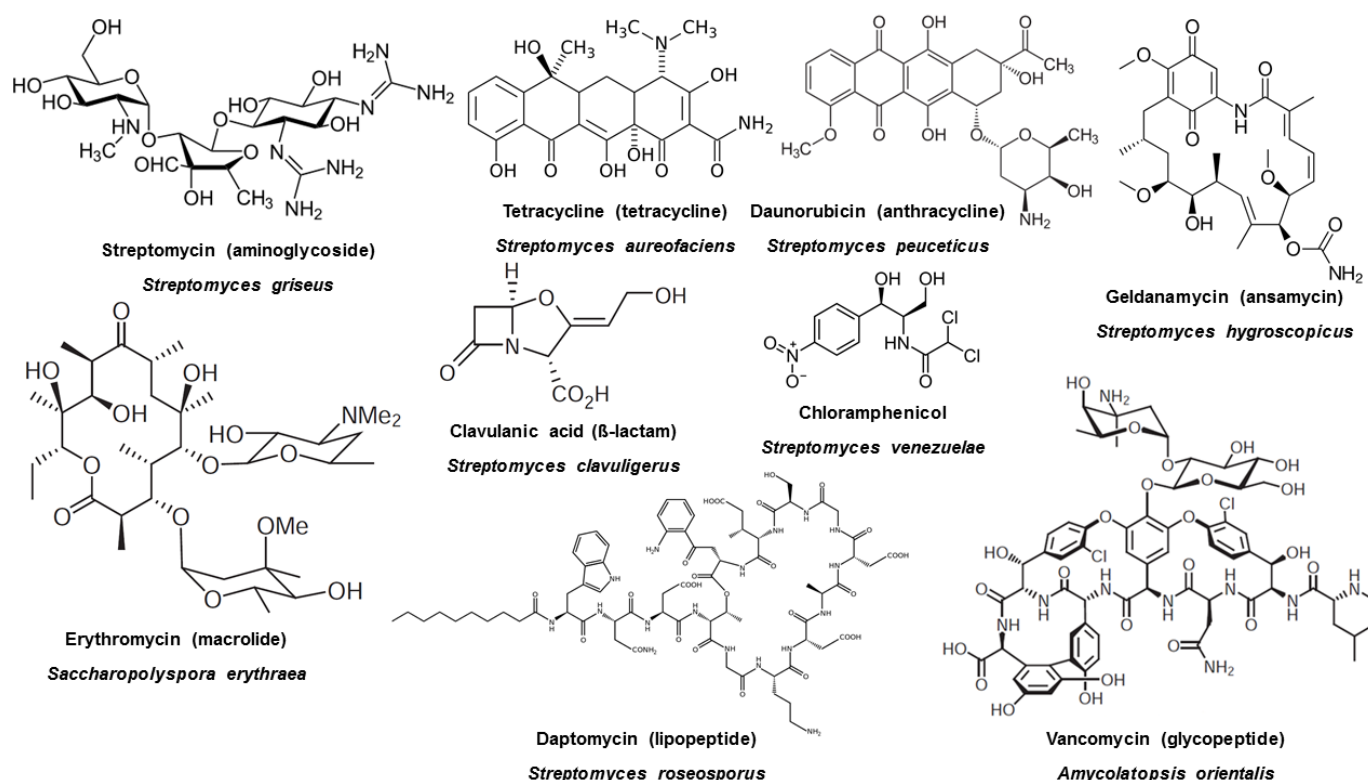


Figure 5: Examples of antibiotics from major classes produced by actinomycete bacteria.

3.3.1. Marine Actinomycetes

Since 1950s terrestrial actinomycetes were intensively explored, thereby finding many industrially useful natural products.²¹ Nevertheless, the re-discovery of bioactive compounds arose frequently, leading to a focused research in unique and unexplored or under-explored environmental habitats with extreme conditions, for example high pressure, low oxygen, toxic metals, no sunlight, high temperature, salinity, low pH or nutrient limitation.²²

Thus, marine environments became new interesting biological niches for bioprospecting, which means the process of the discovery of new products based on biological resources and their commercial use. Survival and proliferation of bacteria facing extreme conditions often depends on the ability to produce biologically active compounds.²³ The fact that 70% of the earth's surface is covered by oceans, which host approximately 87% of global biodiversity, it appears that marine habitats can be a rich source to find microorganisms, adapted to the marine environment, some of which represent rare and unknown taxa not found in terrestrial samples.²⁴

The first report of an isolation of actinomycetes from marine sources was published in 1969²⁵ and the first report of a bioactive compound isolated from *Chainia purpurogena*, a marine actinomycete, appeared in 1975.²⁶ In marine environments many actinomycetes, such as *Streptomyces*, *Nocardiopsis*, *Rhodococcus*, *Pseudonocardia* and *Micromonospora* have evolved to live in symbiosis with organisms like algae, seaweed and invertebrates most of which lack mobility like sponges, bryozoans, tunicates, sea-cucumbers and mollusks.²⁷ In sponges for example, the total biomass can consist up to 60% of the symbiotic microorganisms, which contributes to the strengthening of the sponges' structure.²⁸ The microbes might provide a chemical defense system to their hosts, because they lack the complex immune system of higher animals.²⁹ Natural products obtained from these organisms are suggested to be produced by their microbiome for the protection against pathogens, for growth promotion or for the degradation of complex natural polymers and thus, the supply of nutrients and the removal of waste products. On the other hand, microbes benefit from their hosts resources.¹⁴ In two *Streptomyces* species that interactions were shown to control the expression of biosynthetic gene clusters and that gene clusters were differentially acquired or lost through a divergent evolution in different sponge hosts.³⁰

3.3.2. *Streptomyces* spp.

Streptomyces, with several hundred (> 600) described species, represent the largest genus of the phylum Actinobacteria. They are Gram-positive, aerobic bacteria which form a network of branched filaments called a substrate mycelium.³¹ They characteristically reproduce by the formation of specialized aerial mycelium that gives rise to chains of uninucleoidal spores.³² They move from a vegetative phase to a reproductive phase, thereby releasing hydrolytic enzymes to break down polymers like chitin and cellulose often found in the environment, in order to provide nutrients.³³ Environmental conditions, like nutrient deprivation, and growth on the solid cultivation medium, induce sporulation.³ This process of differentiation (Figure 6) during their lifetime is controlled by a variety of regulatory systems. The onset of this differentiation is also involved in secondary metabolism, as a result, the production of secondary metabolites can be induced by changing environmental conditions like a lack of nutrients, a different temperature, addition of toxic chemicals like organic solvents³⁴, phage infection, co-cultivation with other microbes, etc.³⁵ The chromosomes of *Streptomyces* species have, like those of other bacteria of the phylum Actinobacteria, a high GC content in its DNA (usually around 70%) and ranges from 6 Mb to 12 Mb in size.³⁶ Approximately 7000 to 10000 genes and a large set of global, pleiotropic and pathway specific regulators control gene expression and product formation in these microorganisms.³⁷

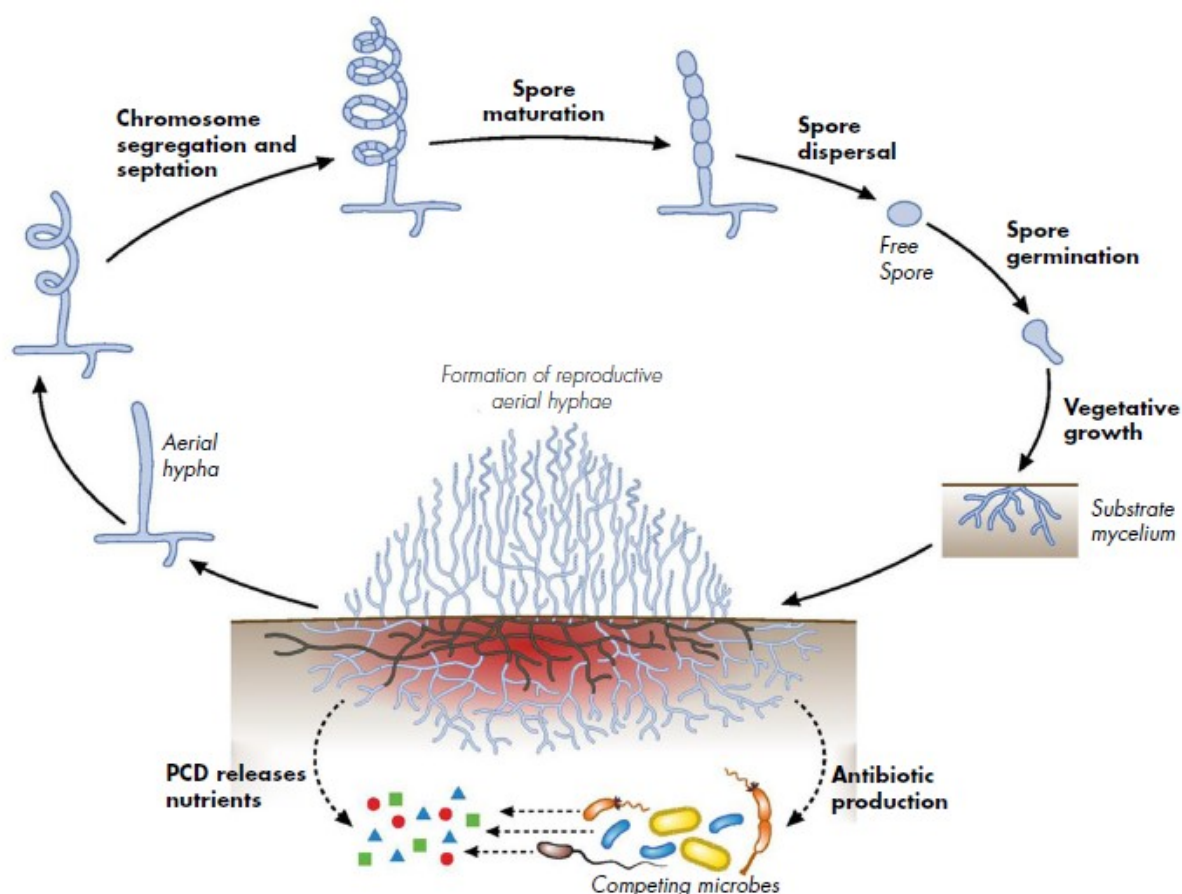


Figure 6: *Streptomyces* lifecycle.¹⁴

The variety of chemical structures of secondary metabolites from the genus *Streptomyces* include non-ribosomal peptides and polyketides synthesized by non-ribosomal peptide synthases (NRPS) and polyketide synthases (PKS),³⁸ and also lactams, lactones, terpenes, indoles and quinones.^{39–33} This chemical diversity features various biological activities. Substances isolated from *Streptomyces* species that are in clinical use include antifungals (e.g. amphotericin B, nystatin), antiparasitics (e.g. ivermectin), antitumorals (e.g. doxorubicin) and immunosuppressives (e.g. rapamycin), but mainly antibiotics (e.g. daptomycin, chloramphenicol).⁴⁰ A natural product known to be the main contributor to the smell of earth is called geosmin.⁴¹ Soil-dwelling bacteria like *Streptomyces* species among other actinobacteria and myxobacteria are able to produce this bicyclic terpene alcohol, which is responsible for an earthy smell.⁴²

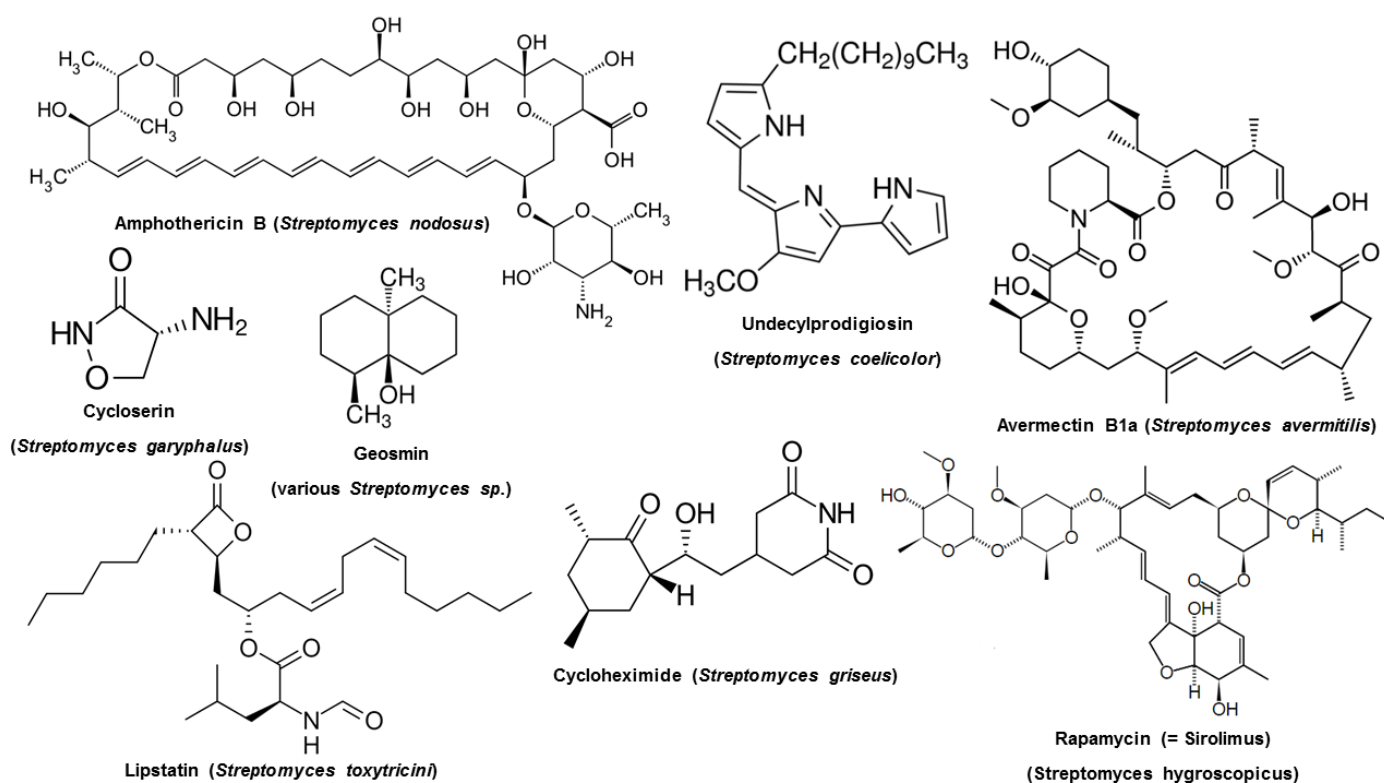


Figure 7: Examples for secondary metabolites produced by streptomycetes.

3.4. Biosynthesis of Secondary Metabolites in Actinomycetes

The biosynthesis of antibiotics and other secondary metabolites starts by obtaining building blocks, so called precursors, which are typically primary metabolites such as amino acids, acyl-CoAs, sugars, nucleotides, etc. Large, specific, multi modular core enzymes like polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) catalyze the assembly of precursors to scaffolds by condensation and other enzymatic modification reactions in a coordinated manner.³⁸ PKS use activated building blocks (acyl-CoAs) for assembly, done by acylation, and NRPS domains are able to activate amino acids for assembly.

The formed scaffolds are the molecular skeletons of the synthesized compounds, which are further modified by tailoring enzymes such as acyltransferases, oxidoreductases, glycosyltransferases, etc., thereby gaining their full biological activity.⁴³ (Figure 8)

A plethora of different chemical structures can be generated by PKS-based pathways because a difference in the selection of starter and extender units, carbon chain length, folding, degree of reduction, termination and tailoring can lead to various products.⁴⁴ In actinomycetes this complex process is typically governed by 10-30 genes, which are physically clustered together in their genomes, as shown in Figure 9, allowing an orderly expression of those genes involved in regulation, biosynthesis and secretion of secondary metabolites and resistance against the synthesized products.⁴³

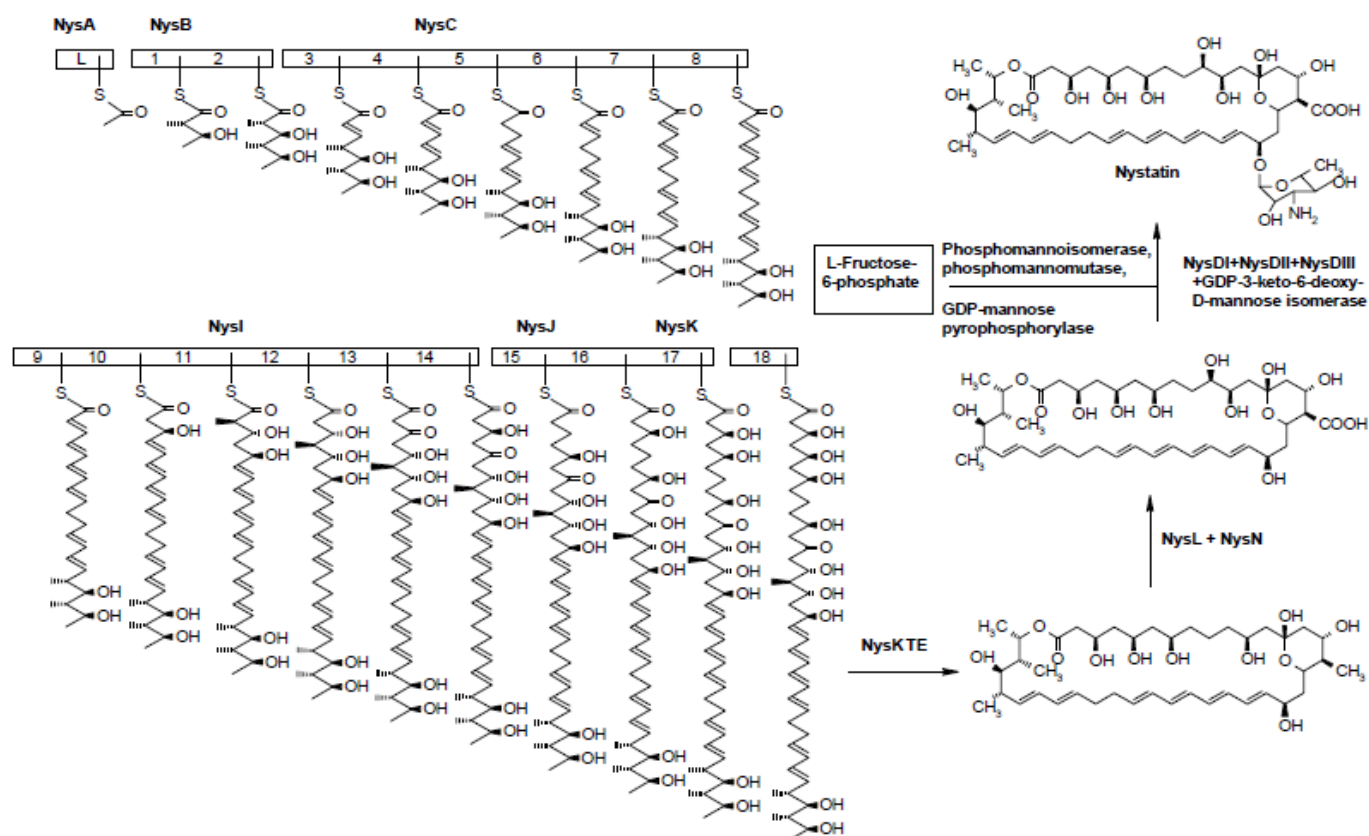


Figure 8: Antibiotic biosynthesis shown on the example of the polyene macrolide antibiotic nystatin in *Streptomyces noursei* ATCC 11455.⁴⁵

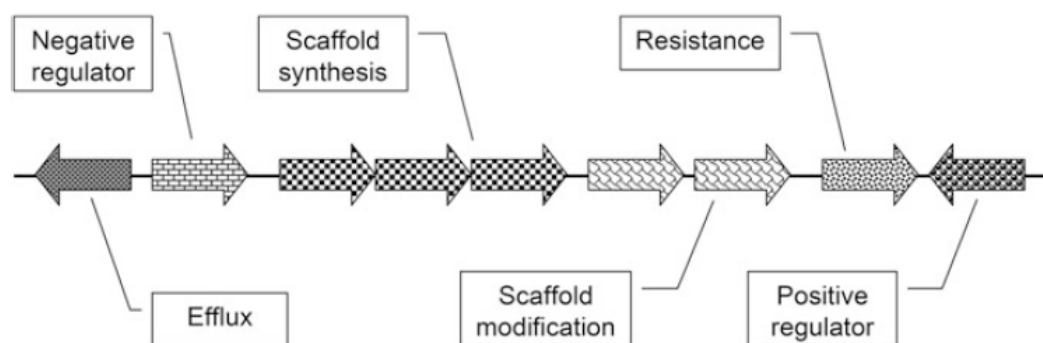


Figure 9: Typical setup of an antibiotic biosynthesis gene cluster. It includes genes for regulators, scaffold assembly and modification, secretion and resistance.⁴³

That orderly expression ensures coordinated enzymatic reactions in scaffold assembly and modification of the synthesized compounds. The production of antibiotics and other secondary metabolites is tightly controlled by pleiotropic and pathway specific regulation and it needs environmental signals for its activation.⁴⁶ Genes for pleiotropic regulators control different metabolic pathways, which are not linked to specific biosynthetic gene clusters.

It is possible that pleiotropic regulators activate the pathway specific regulators within biosynthetic gene clusters.³⁷ Microorganisms need these strict and complex regulation systems because secondary metabolite production is very energy consuming, posing a burden on the synthesizing organism. Precursor molecules (e.g. acetyl-CoA, amino acids, etc.) and reducing equivalents (e.g. NADPH) from primary metabolism are siphoned and because these metabolites are essential components for growth (e.g. acetyl-CoA for fatty acid formation), secondary metabolite biosynthesis inevitably competes with biomass formation, thus leading to a decreased cellular growth.³³ However, the onset of secondary metabolite production is correlated with growth, because on solid medium antibiotic production typically starts with morphological differentiation, therefore when the aerial mycelium is built and in liquid cultures the production mainly starts at the beginning of the stationary phase.⁴⁷

3.5. Identification and Activation of Silent Gene Clusters

3.5.1. Identification of BGCs by Genome Mining

Since DNA sequencing technologies have enabled multiple microbial genomes to be sequenced faster and cheaper, a large and increasing amount of sequenced microbial genomes in public databases appeared. Together with sophisticated bioinformatic tools, the strategy of genome mining was brought about, which revolutionized natural product discovery by the possibility to reveal an organism's true metabolic potential.²³ It has been shown that the number of biosynthetic gene clusters is higher than the number of natural products produced under standard laboratory conditions.³ Usually, 1-4 bioactive secondary metabolites are produced by *Streptomyces* species in conventional fermentation cultures, but their genomes typically harbor 20-50 biosynthetic gene clusters, showing a vast potential to produce many more compounds, some of which are unknown.⁴⁸ The first available genome sequences of actinomycete bacteria, that of *Streptomyces coelicolor*, showed that the number of biosynthetic gene clusters (>20) and thus its secondary metabolite coding capacity far exceeds the number of known compounds identified with standard screening methods.^{49 50} The revision of other well-characterized actinomycetes strains revealed a variety of hidden treasures as well.⁵¹ Gene clusters which are not or only poorly expressed, resulting in no production or an insufficient quantity of secondary metabolites for analytical detection are referred to as 'cryptic' or 'silent'.⁵² One of the most useful and freely available bioinformatic tool for genome mining is called antiSMASH (antibiotics and secondary metabolite analysis shell)⁵³, a software capable of identifying and annotating genes within biosynthetic gene clusters of known secondary metabolite classes like polyketides, non-ribosomal peptides, terpenes, butyrolactones, lantipeptides, lassopeptides, etc. in bacterial chromosomes.⁵⁴ antiSMASH sometimes also allows the prediction of a core chemical structure of the putative produced compound.

Even if it is possible to predict a chemical core structure of the encoded metabolite, the structure of the final product cannot be predicted due to the variety of possible modifications like tailoring reactions and cyclisation, which follow scaffold assembly.⁵⁴ Other tools capable to automatically identify gene clusters and certain biosynthetic pathways include ClustScan⁵⁵, CLUSEAN⁵⁶, NP.searcher⁵⁷, SMURF (secondary metabolite unknown region finder) for fungi⁵⁸ and others, which contribute to an easier application of genome mining.^{59 60}

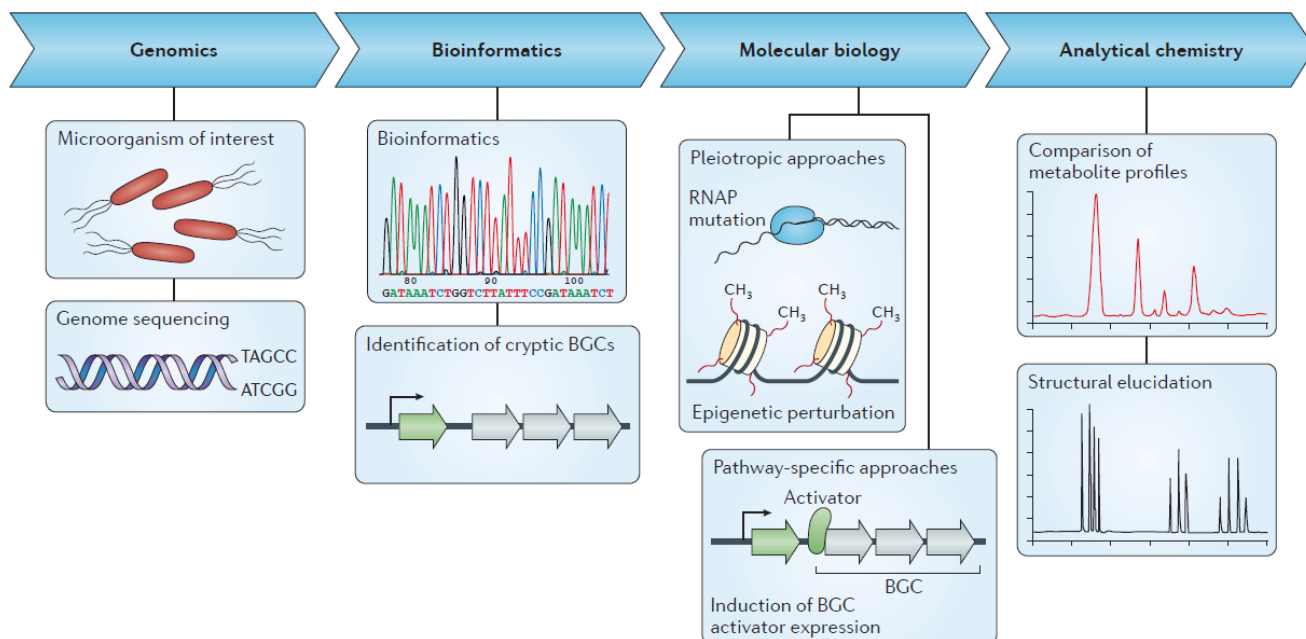


Figure 10: Bioprospecting of microbes for natural product discovery by genome mining.⁶¹

3.5.2. Methods for the Activation of Silent Gene Clusters

After identification of secondary metabolite biosynthetic gene clusters by genome mining, the hidden metabolic potential can be unleashed by several strategies. (Figure 10)

The tightly controlled secondary metabolism in microorganisms requires certain external and/or internal triggers, thus rational methods are needed, which mimic these signals for the onset of natural product synthesis. As already mentioned, the change of environmental factors such as different growth conditions (e.g. different nutrients in fermentation media, temperature, pH, etc.), co-cultivation with other microorganisms or chemical elicitors may lead to the production of previously undetected compounds.^{46 62} Next to those physiological triggers, metabolic engineering via genetic manipulation methods can be applied.²⁰

Recombinant DNA technology provides methods to assess the biosynthetic potential by activating cryptic clusters and by improving the titers of the encoded secondary metabolites. Rational reengineering of biosynthetic gene clusters gives the possibility to replace a native regulation system by a synthetic one. By manipulation of regulatory mechanisms, that control the expression of the biosynthetic gene cluster of interest, such as overexpression of positive regulators or deletion of repressors, novel metabolic product formation can be achieved.⁶³

For example, in *Streptomyces* a pathway specific positive regulator can be set under the control of a strong constitutive promoter like *ermE**p⁶⁴, the promoter in front of the erythromycin resistance gene in *Saccharopolyspora erythraea*, to activate the expression of a silent gene cluster.⁶⁵ Such a promoter replacement is called refactoring and can release the genes in a biosynthetic gene cluster from their tight environmental control, which can lead to the formation of the corresponding natural products.⁶¹

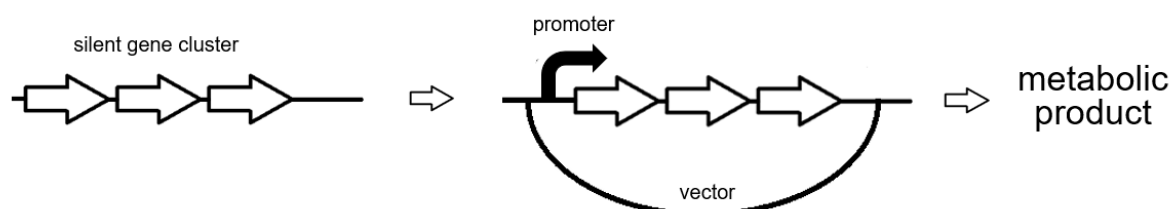


Figure 11: Construction of vectors including a constitutive or readily inducible promoter to activate silent biosynthetic gene clusters.

Other genetic modification approaches to activate silent clusters or to boost natural product synthesis include ribosomal engineering, metabolism remodeling, etc.⁶⁶

If a biosynthetic gene cluster is cloned into another host organism than the native one, this is referred to as heterologous expression. Therefore, it is possible to speed up the product formation process because of a rapidly growing and easy modifiable heterologous host.⁶⁷

After a novel compound has been produced due to the expression of a silent gene cluster, isolation and chemical structure elucidation follow. These tasks are often time consuming and depend on a sufficient yield of the generated secondary metabolites for analyses.

Therefore, reporter genes, which indicate a successful gene expression and compound formation due to the activation of a silent gene cluster, for example by a color change of the fermentation medium, can be very helpful.⁶⁸

3.5.3. The useful Reporter Gene *bpsA*

Reporter genes can be used to develop biosensor systems, which can detect metabolic activities in the host organism. For bacteria, mainly mentioned for actinomycetes herein, several reporter systems, such as those based on *gusA*⁶⁹, *xyIE*⁷⁰, *bpsA*⁷¹ etc. are available. The gene *bpsA* can be found in *Streptomyces* species such as *Streptomyces aureofaciens* CCM3239 and *Streptomyces lavendulae* ATCC 11924 and encodes a single module type NRPS, that is activated by a 4'-phosphopantetheinyl transferase and catalyzes the production of a stable, water insoluble extracellular blue pigment known as indigoidine (5,5'-diamino-4,4'-dihydroxy-3,3'-diazadiphenquinone-(2,2')), from only one amino acid (L-glutamine) as substrate.^{72 73 74} (Figure 12)

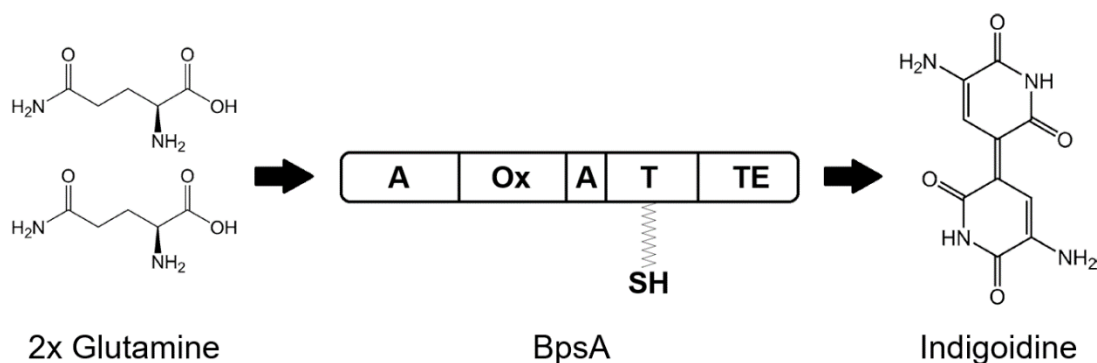


Figure 12: The enzyme BpsA, consisting of 5 domains (adenylation (A), oxidation (Ox), thiolation (T) and thioesterase (TE)) converts two L-glutamine molecules by a sequential catalytic process to the blue pigment indigoidine [(5,5'-diamino-4,4'-dihydroxy-3,3'-diazadiphenoquinone-(2,2'))]. ($C_{10}H_8N_4O_4$). (based on ⁷⁵)

Homologous genes, responsible to produce indigoidine, which even shows bioactive properties⁷⁶ were found in many other bacteria like *Erwinia chrysanthemi*, *Phaeobacter sp.* Y4I, *Pseudomonas indigofera*, *Corynebacterium insidiosum*, *Photorhabdus luminescens*, various *Arthrobacter sp.*, etc.^{77 78 79} The *bpsA* gene from streptomycetes can be used for the construction of a biosensor system, which is able to indicate promoter activity by the appearance of this blue pigment, which is secreted in the media during a presumably synchronous production of secondary metabolites.⁷¹ In a recent study, a reporter system based on this reporter gene was applied to indicate the production of coelimycin in *Streptomyces lividans* TK24⁸⁰ and can also be applied in other streptomycetes to detect activation of silent biosynthetic gene clusters. A reporter system based on *bpsA* can even be used in mammalian cells⁷⁵. The reporter gene *xylE*⁸¹ was used to detect a targeted activation of silent gene clusters in *Streptomyces* strains by reporter-guided mutant selection (RGMS) by Guo *et al.*⁶⁸

Genome-scale random mutagenesis (e.g. by UV-light) is often used to obtain mutants with increased production of secondary metabolites in microorganisms. However, screening for overproducing mutants after mutagenesis is very laborious, if no simple selection criterium is available. Therefore, combination of mutagenesis with a color-based promoter-reporter system may allow an easy selection of mutants, in which the transcription of the targeted gene cluster was activated. In the work by Guo et al., key promoter sequences from the gene cluster of interest were cloned in front of the *xylE-neo* reporter cassette to construct reporter plasmids. This cassette allows initial selection by kanamycin resistance due to the *neo* gene, followed by a color-based detection of *xylE* activity.⁷⁰ The reporter gene *xylE* makes it possible to detect the desired mutants and to differentiate between spontaneous kanamycin resistant and target-activated mutants, which show a yellow color after spraying an aqueous catechol solution.⁸²

With this RGMS method, target gene expression via genome-scale random mutagenesis can be detected. That experiment led to the overexpression of a biosynthetic gene, which is responsible for the production of jadomycin in *Streptomyces venezuelae* ISP5230 and brought about two new anthraquinone aminoglycosides, gaudimycin D and E, by an activation of a silent aromatic type II polyketide gene cluster in *Streptomyces* sp. PGA64.⁶⁸

3.6. This Project

Because Actinobacteria from underexplored environments (e.g. marine sponges) may become new sources for bioactive secondary metabolites, including compounds of clinical interest e.g. antibiotics, three marine sponge-derived *Streptomyces* bacteria were chosen for this project. Due to the initial absence of bioactive secondary metabolites production by these strains, approaches like genome mining and genetic manipulation of strains could help to reveal their true capacity in this regard. Typically, only a subset of gene clusters is expressed under standard laboratory conditions and specific environmental signals for an activation may be required for each individual pathway. Strategies based on the activation of silent clusters make it possible to access new chemical diversity that may lead to the development of new drugs. In this diploma project, a biosensor-based approach was used to detect novel secondary metabolite production in fermentation cultures of *Streptomyces* strains.

4. Results

4.1. Genome Analysis by antiSMASH

The genome sequences of three *Streptomyces* strains ADI 95-17, GBA 94-01 and PVA 91-01 were analyzed by antiSMASH 3.0.5⁸³ for the *in-silico* identification of secondary metabolite biosynthetic gene clusters (BGCs). For each *Streptomyces* strain, over 30 BGCs were detected (ADI 95-17 – 35 clusters, GBA 94-01 – 35 clusters, PVA 91-01 – 40 clusters), predicted to be involved in the production of non-ribosomal peptides, polyketides, terpenes, bacteriocins and lantipeptides, among others. Some of them, which have, according to the antiSMASH output and BLAST verification, a higher chance to produce an unknown compound, were chosen for this project (Results 4.3). Most of these unique BGCs show low gene similarity to any known gene cluster, indicating the potential novelty of their metabolic products. The tables 1, 2 and 3 show number, type and gene similarity of BGCs from *Streptomyces* strains analyzed by antiSMASH.

Table 1: List of secondary metabolites BGCs from the genome sequence of *Streptomyces* ADI95-17 identified using the antiSMASH platform.

Cluster number	Type of cluster	Most similar known cluster (gene similarity)
Cluster 1	Terpene	GE81112 (7%)
Cluster 2	Bacteriocin	-
Cluster 3	T1pks-Nrps	Avilamycin A (5%)
Cluster 4	Lantipeptide	-
Cluster 5	Lantipeptide	Akaeolide (8%)
Cluster 6	T2pks	R1128 (42%)
Cluster 7	Ectoine	Ectoine (75%)
Cluster 8	Terpene	Steffimycin (19%)
Cluster 9	Nrps	Friulimicin (27%)
Cluster 10	Nrps	Mirubactin (71%)
Cluster 11	Transatpks	Cycloheximide/actiphenol (61%)
Cluster 12	T3pks	Naringenin (100%)
Cluster 13	Bacteriocin	-
Cluster 14	Nrps	Friulimicin (9%)
Cluster 15	Nrps- T1pks	SGR PTMs (100%)
Cluster 16	Nrps	-
Cluster 17	Bacteriocin	Tautomycin (6%)
Cluster 18	Bacteriocin	-
Cluster 19	Terpene	Hopene (76%)
Cluster 20	Siderophore	-
Cluster 21	Nrps	Desotamide (36%)
Cluster 22	Bacteriocin	-
Cluster 23	Bacteriocin	Desotamide (18%)
Cluster 24	Nrps	Coelichelin (100%)
Cluster 25	T1pks	Meilingmycin (4%)
Cluster 26	Terpene- T2pks	Spore pigment (83%)
Cluster 27	Other	Stenothricin (13%)
Cluster 28	T1pks	Nystatin (36%)
Cluster 29	Ladderane-Nrps	Calcium-dependent antibiotic (25%)

Cluster number	Type of cluster	Most similar known cluster (gene similarity)
Cluster 30	Terpene	Salinilactam (8%)
Cluster 31	Butyrolactone	Merochlorin (7%)
Cluster 32	T2pks	Kinamycin (28%)
Cluster 33	T1pks	Chaxamycin A/ B/ C/ D (31%)
Cluster 34	T1pks	Cyclizidine (29%)
Cluster 35	T1pks-Otherks	-

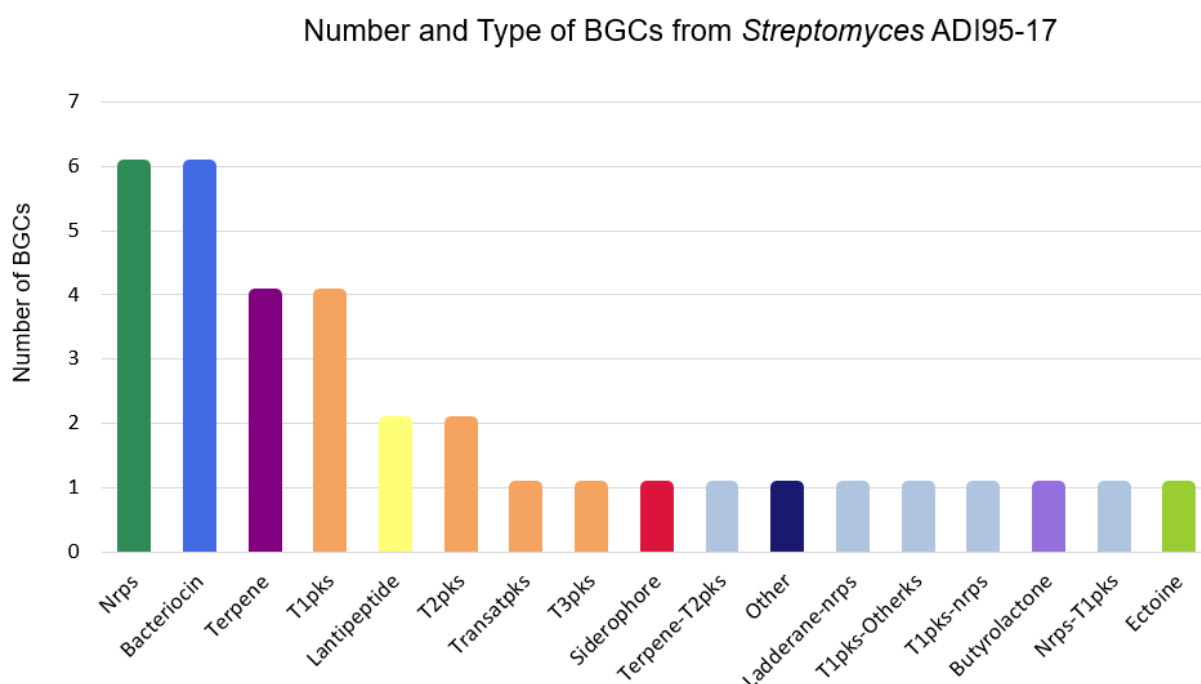


Figure 13: Overview of the presence and type of secondary metabolite BGCs from the genome sequence of *Streptomyces* ADI95-17 identified using the antiSMASH platform.

Table 2: List of secondary metabolites BGCs from the genome sequence of *Streptomyces* GBA94-01 identified using the antiSMASH platform.

Cluster number	Type of cluster	Most similar known cluster (gene similarity)
Cluster 1	Lantipeptide-Bacteriocin	-
Cluster 2	Nrp-Nucleoside	Pentalenolactone (11%)
Cluster 3	Butyrolactone-Nrp- T1pks	Polyoxypeptin (78%)
Cluster 4	Lasso peptide	SSV-2083 (50%)
Cluster 5	Bacteriocin	Informatipeptin (42%)
Cluster 6	Lantipeptide	-
Cluster 7	Nrp	Streptothricin 54%)
Cluster 8	Terpene	Hopene (92%)
Cluster 9	Siderophore	Grincamycin (5%)
Cluster 10	Other	Stenothricin (13%)
Cluster 11	Terpene	-
Cluster 12	Otherks- T1pks	-
Cluster 13	Bacteriocin	-
Cluster 14	Siderophore	-
Cluster 15	Terpene	Albaflavenone (100%)
Cluster 16	Nrp	Daptomycin (4%)
Cluster 17	Butyrolactone	Stenothricin (9%)

Cluster number	Type of cluster	Most similar known cluster (gene similarity)
Cluster 18	Other	Pactamycin (13%)
Cluster 19	Nrps	-
Cluster 20	Otherks- T1pks	Pyrrolomycin (8%)
Cluster 21	T1pks	Lasalocid (18%)
Cluster 22	T3pks	Alkylresorcinol (100%)
Cluster 23	T1pks	Stambomycin (20%)
Cluster 24	Lasso peptide	-
Cluster 25	Terpene	2-methylisoborneol (100%)
Cluster 26	T1pks	Kinamycin (5%)
Cluster 27	T3pks	Herboxidiene (8%)
Cluster 28	Nrps	Scabichelin (100%)
Cluster 29	Other	-
Cluster 30	Siderophore	Desferrioxamine B (83%)
Cluster 31	Melanin	Istamycin (4%)
Cluster 32	Nrps	SCO-2138 (57%)
Cluster 33	Ectoine	Ectoine (75%)
Cluster 34	T3pks	Merochlorin (56%)
Cluster 35	Butyrolactone	-

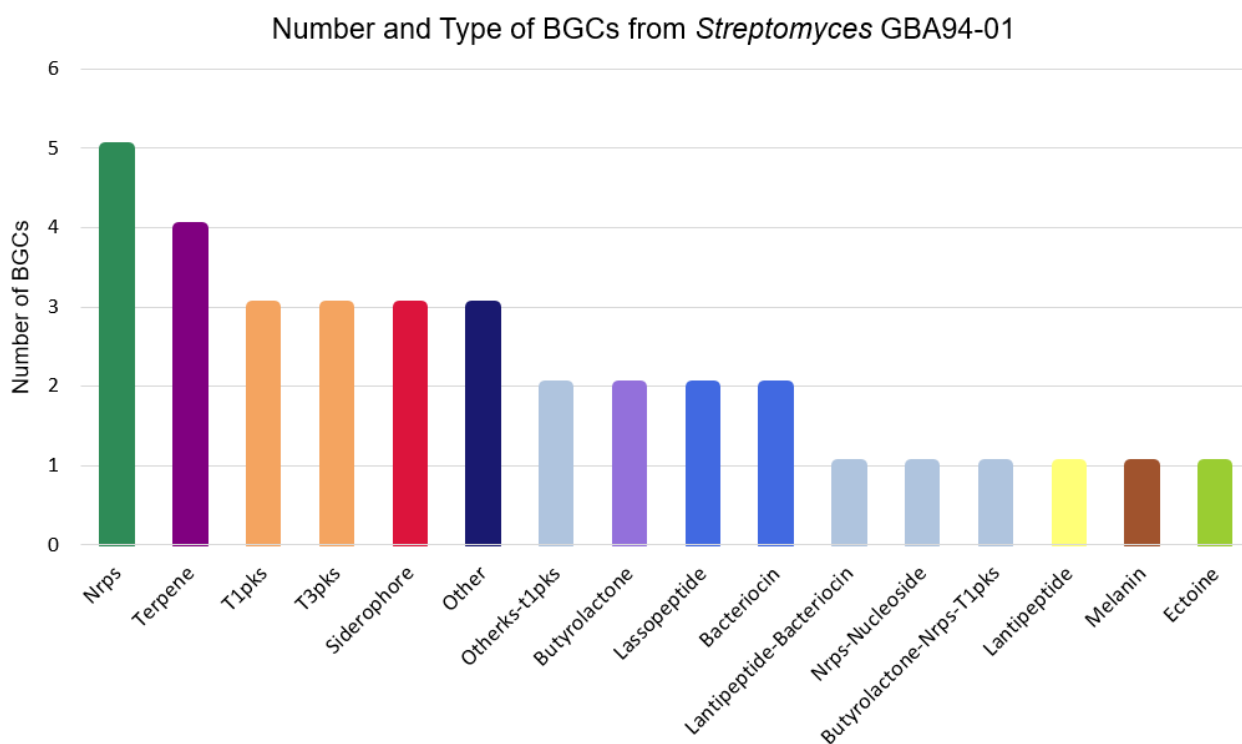


Figure 14: Overview of the presence and type of secondary metabolite BGCs from the genome sequence of *Streptomyces* GBA94-01 identified using the antiSMASH platform.

Table 3: List of secondary metabolites BGCs from the genome sequence of *Streptomyces* PVA91-01 identified using the antiSMASH platform.

Cluster number	Type of cluster	Most similar known cluster (gene similarity)
Cluster 1	Tt1pks	Coelimycin (25%)
Cluster 2	Lantipeptide	-
Cluster 3	Lasso peptide	SRO15-2005 (60%)
Cluster 4	Bacteriocin	-
Cluster 5	Lantipeptide	AmfS (100%)
Cluster 6	Terpene	-
Cluster 7	Nrps	-
Cluster 8	T1pks-Nrps- Thiopeptide-Lantipeptide	Elaiophyllin (45%)
Cluster 9	Terpene	Isorenieratene (100%)
Cluster 10	Nrps	Skyllamycin (4%)
Cluster 11	T3pks	Alkylresorcinol (100%)
Cluster 12	Melanin	Melanin (100%)
Cluster 13	Nrps	Valinomycin (22%)
Cluster 14	Terpene	2-methylisoborneol (100%)
Cluster 15	Bacteriocin	Tetronasin (3%)
Cluster 16	T1pks	SGR PTMs (100%)
Cluster 17	Lantipeptide	-
Cluster 18	Terpene	Hopene (69%)
Cluster 19	Butyrolactone	Kanamycin (5%)
Cluster 20	Nrps	Salinomycin (14%)
Cluster 21	Bacteriocin	-
Cluster 22	Lantipeptide	Kanamycin (1%)
Cluster 23	Siderophore	-
Cluster 24	Ectoine	Ectoine (100%)
Cluster 25	Nrps-Otherks	A47934 (8%)
Cluster 26	Lantipeptide	-
Cluster 27	Siderophore	Desferrioxamine B (80%)
Cluster 28	Thiopeptide-Lantipeptide	-
Cluster 29	Melanin	Grixazone (76%)
Cluster 30	Nrps	Phosphonoglycan (3%)
Cluster 31	Terpene	Steffimycin (19%)
Cluster 32	Other	Roseoflavin (100%)
Cluster 33	T3pks	Herboxidiene (6%)
Cluster 34	Nrps	Griseoviridin (10%)
Cluster 35	Melanin-Nrps	Coelichelin (81%)
Cluster 36	Nrps	Griseobactin (94%)
Cluster 37	Nrps-Transatpks- T1pks-Otherks	Cremimycin (5%)
Cluster 38	Terpene	Stenothricin (13%)
Cluster 39	Butyrolactone	gamma-butyrolactone (100%)
Cluster 40	Butyrolactone	-

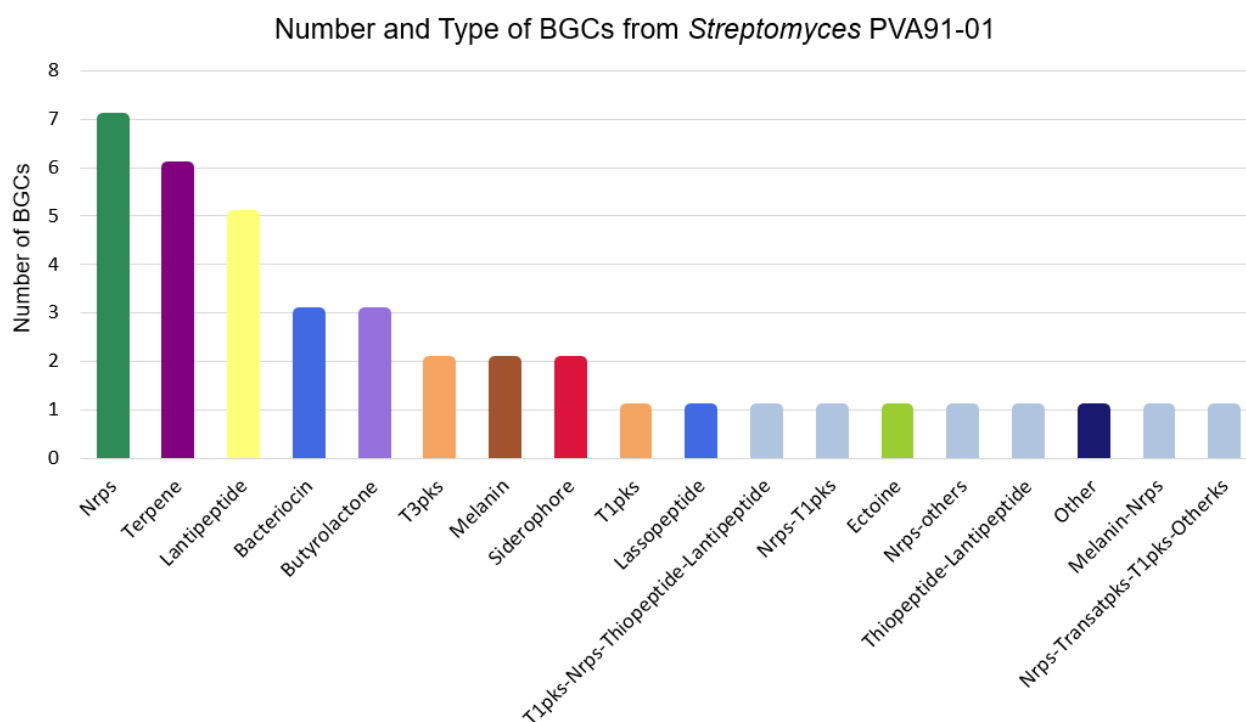


Figure 15: Overview of the presence and type of secondary metabolite BGCs from the genome sequence of *Streptomyces* PVA91-01 identified using the antiSMASH platform.

4.2. Design of the Biosensor System

First, promoter regions from gene specifying enzymes, which are responsible to synthesize natural product scaffold from a target gene cluster, were chosen. After checking the system by the construction of a positive and a negative control vector (Results 4.4), promoter probe vectors, in which the gene *bpsA* was under the control of the amplified promoters, were constructed. The resulting plasmids were introduced into the *Streptomyces* strains by conjugation from *E. coli*. Then, the recombinant strains were subjected to fermentation in different media to activate the target gene cluster. Because an expression of *bpsA* and the resulting blue color indicates target promoter activity and thus, a native promoter driving expression of scaffold biosynthesis gene, the production of the target metabolite can be expected.

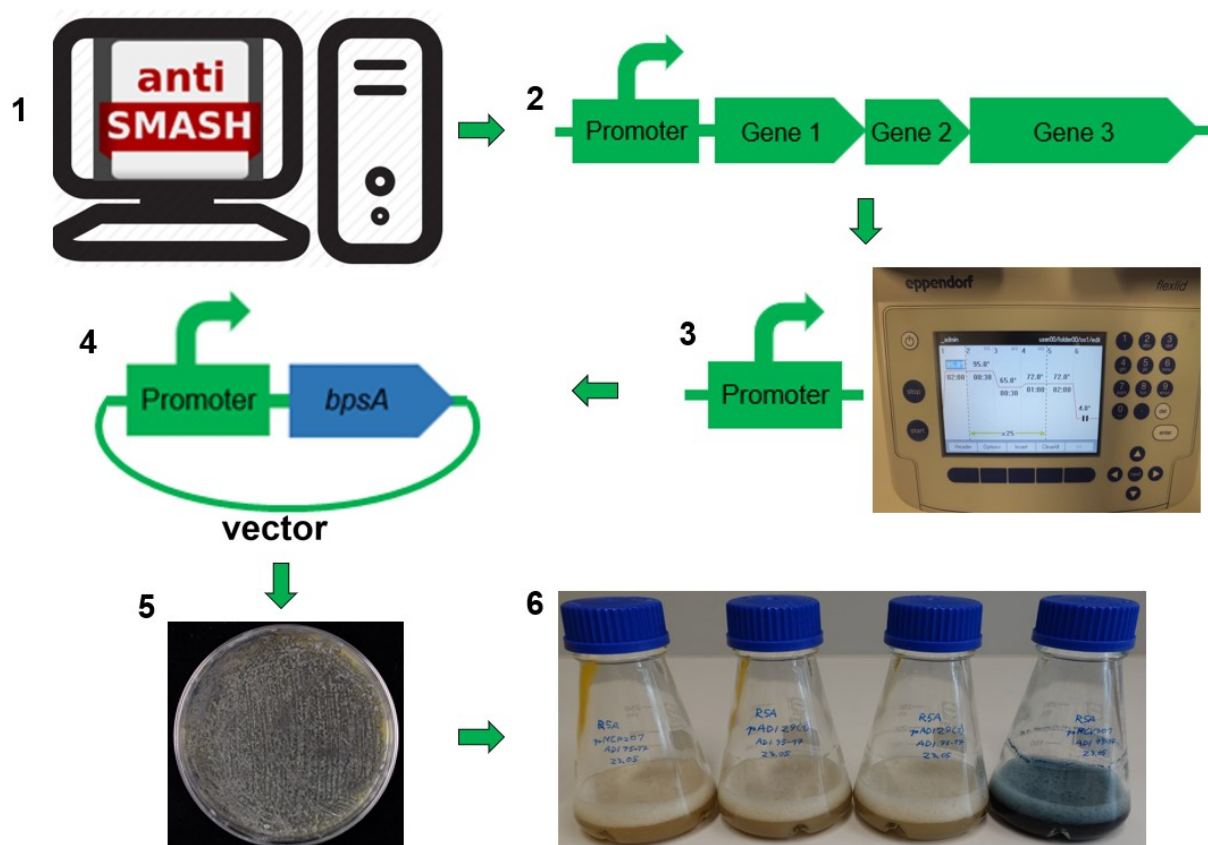


Figure 16: Scheme for the design of the biosensor system developed in this project. First, genome mining was applied on DNA sequences from 3 *Streptomyces* strains (1). Then, promoters from gene specifying enzymes needed to synthesize natural product scaffold from target gene clusters were chosen (2) and amplified by PCR (3). The promoter regions were further introduced into plasmids, in front of the reporter gene *bpsA* (4) and then transferred to *Streptomyces* via conjugation from *E. coli* (5). Afterwards, fermentations were performed in different media (6) to find and try to activate silent BGCs. A blue color of a culture indicates target promoter activity and thus, a native promoter driving expression of scaffold biosynthesis gene and the following production of the target metabolite can be expected.

4.3. Selected BGCs and Intergenic Regions

Following secondary metabolite biosynthetic gene clusters were chosen after antiSMASH analysis (Table 4). From all BGCs, except PVA 91-01 cluster 17, two intergenic regions, containing putative promoters in front of biosynthetic genes, were selected. (marked with (1) and (2) in Figures 17 to 23) These promoter regions were further amplified by PCR after primers for them had been designed.

Table 4: List of BGCs chosen for this project.

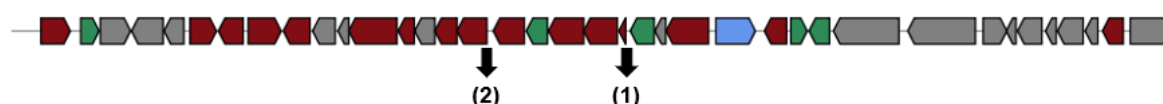
<i>Streptomyces</i> strain	Chosen clusters (type)
ADI 95-17	6 (T2pks), 29 (Ladderane-Nrps)
GBA 94-01	6 (Lantipeptide), 16 (Nrps), 24 (Lasso peptide)
PVA 91-01	8 (T1pks-Nrps-Thiopeptide-Lantipeptide), 17 (Lantipeptide)

- BCGs from *Streptomyces* ADI 95-17

c00002_str9118.. - Cluster 6 - T2pks

Gene cluster description

c00002_str9118.. - Gene Cluster 6. Type = t2pks. Location: 665779 - 708345 nt.



Legend:

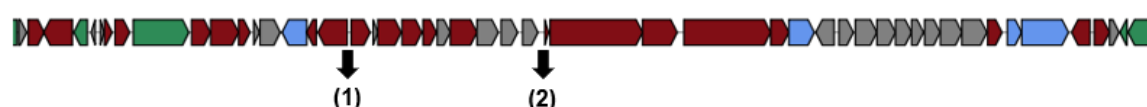
■ biosynthetic genes ■ transport-related genes ■ regulatory genes ■ other genes

Figure 17: ADI 95-17 Cluster 6, a T2pks gene cluster. The genes showed 42% similarity to R1128 biosynthetic gene cluster. Promoter regions of: (1) malonyl CoA-acyl carrier protein transacylase and (2) acyl-CoA dehydrogenase type 2.

c00010_str9118.. - Cluster 29 - Ladderane-nrps

Gene cluster description

c00010_str9118.. - Gene Cluster 29. Type = ladderane-nrps. Location: 122669 - 184217 nt.



Legend:

■ biosynthetic genes ■ transport-related genes ■ regulatory genes ■ other genes

Figure 18: ADI 95-17 Cluster 29, a Ladderane-Nrps gene cluster and its predicted core structure. The genes showed 25% similarity to the Calcium-dependent antibiotic biosynthetic gene cluster. Promoter regions of: (1) 2,3-diaminopropionate biosynthesis protein SbnA and (2) antibiotic synthesis protein MbtH.

- BCGs from *Streptomyces* GBA 94-01

c00001_str9401.. - Cluster 6 - Lantipeptide

Gene cluster description

c00001_str9401.. - Gene Cluster 6. Type = lantipeptide. Location: 1451534 - 1474308 nt.



Legend:

■ biosynthetic genes ■ transport-related genes ■ regulatory genes ■ other genes

Figure 19: GBA 94-01 Cluster 6, a Lantipeptide gene cluster. The genes showed no similarity to known clusters. Promoter regions of: (1) Lanthionine synthetase C-like protein/Protein kinase domain and (2) Calcineurin-like phosphoesterase.

c00001_str9401.. - Cluster 16 - Nrps

Gene cluster description

c00001_str9401.. - Gene Cluster 16. Type = nrps. Location: 4707740 - 4762324 nt.



Legend:

■ biosynthetic genes ■ transport-related genes ■ regulatory genes ■ other genes

Figure 20: GBA 94-01 Cluster 16, a Nrps gene cluster and its predicted core structure. The genes showed 4% similarity to the Daptomycin biosynthetic gene cluster. Promoter regions of: (1) MbtH-like protein and (2) 2-isopropylmalate synthase, yeast type.

c00002_str9401.. - Cluster 24 - Lasso peptide

Gene cluster description

c00002_str9401.. - Gene Cluster 24. Type = lasso peptide. Location: 695810 - 718601 nt.



Legend:

■ biosynthetic genes ■ transport-related genes ■ regulatory genes ■ other genes

Figure 21: GBA 94-01 Cluster 24, a Lasso peptide gene cluster. The genes showed no similarity to known clusters. Promoter regions of: (1) lasso RiPP family leader peptide-containing protein and (2) Transcriptional regulator/sugar kinase.

- BCGs from *Streptomyces* PVA 91-01

c00002_str9101.. - Cluster 8 - T1pks-nrps-thiopeptide-lantipeptide

Gene cluster description

c00002_str9101.. - Gene Cluster 8. Type = t1pks-nrps-thiopeptide-lantipeptide. Location: 1 - 84048 nt.



Legend:

■ biosynthetic genes ■ transport-related genes ■ regulatory genes ■ other genes

Figure 22: PVA 91-01 Cluster 8, a T1pks-Nrps-Thiopeptide-Lantipeptide gene cluster and its predicted core structure. The genes showed 45% similarity to the Elaiophylin biosynthetic gene cluster. Promoter regions of: (1) Polyketide synthase modules and related proteins and (2) Polyketide synthase modules and related proteins.

c00002_str9101.. - Cluster 17 - Lantipeptide

Gene cluster description

c00002_str9101.. - Gene Cluster 17. Type = lantipeptide. Location: 649492 - 674085 nt.



Legend:

■ biosynthetic genes ■ transport-related genes ■ regulatory genes ■ other genes

Figure 23: PVA 91-01 Cluster 17, a Lantipeptide gene cluster. The genes showed no similarity to known clusters. Promoter region of: (1) FxLD family lantipeptide.

4.4. Construction of Positive and Negative Control Vector

A positive and a negative control vector were constructed to check how the system based on *bpsA* functions in selected streptomycetes. Therefore, plasmids used for their construction were isolated from *E. coli* DH5 α , described in Methods 7.4.2. After isolation and analysis of the integrity of plasmid DNA, which was used for the construction of the positive and the negative control vector (Results 4.4.1 and 4.4.2) by gel electrophoresis (Methods 7.5), it was cut via restriction enzyme digestion to insert fragments (Methods 7.4.5).

After ligation (Methods 7.4.5) of vectors (pSOK804) and inserts (obtained fragments, part of pTSA105 for pos. control, part of pTSA105 and pGEB101 for neg. control), the ligation mixtures were transferred to the cloning host *E. coli* DH5 α by transformation (Methods 7.4.7) for amplification. Plasmid DNA was then isolated from clones picked from plates.

If the clones harbored the right plasmids, which was checked by gel electrophoresis after DNA isolation and following restriction digestion, the successful constructed vector molecules were transformed to *E. coli* ET12567 and further transferred to *Streptomyces* strains via conjugation (Methods 8.4.8 and Results 4.8). *Streptomyces* strains carrying the positive control vector, which is able to integrate into the chromosome, should develop a blue color on the plate and in liquid culture due to the transcription of *bpsA* forced by the strong constitutive promoter *ermE***p*. The transconjugants carrying the negative control vector should not develop a blue color, because *bpsA* gene is cloned there without any promoter and thus, no indigoidine should be produced.

4.4.1. Positive Control Vector

For the construction of the positive control vector, the plasmids pTSA105 and pSOK804 were used (Material 6.1.4). First, pTSA105 was digested with *HindIII*/*EcoRI* to get fragment C (4195 bps), which consists of the promoter region *ermE**p in front of the reporter gene *bpsA* (indigoidine-synthase) and a transcriptional terminator (*ter*). pSOK804 was digested with *HindIII*/*EcoRI*, so fragment C could be inserted in the vector pSOK804 by ligation (Methods 7.4.5). The resulting ligation product was the positive control vector pPCV (9637 bps). (Figure 23)

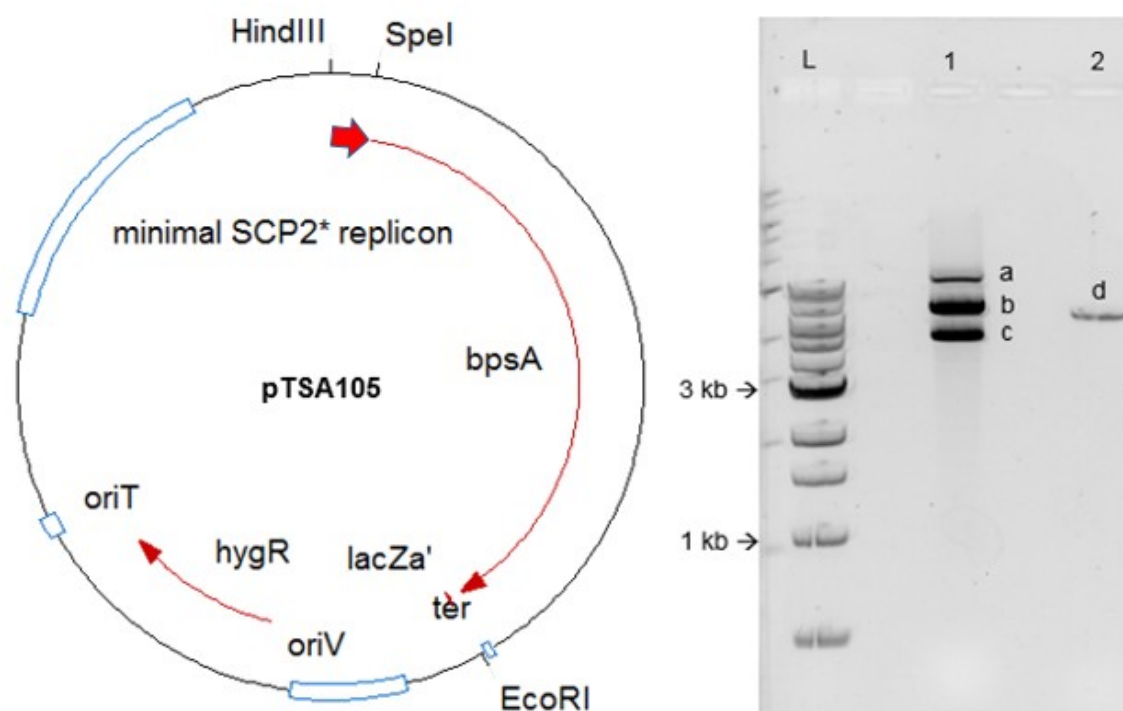


Figure 24: Plasmid map of pTSA105 (9989 bps) and picture of agarose gel after restriction digestion. Fragment C (1c) with a length of 4195 bps from the plasmid pTSA105 cut with *HindIII*/*EcoRI* consisted of *bpsA*, a transcriptional terminator (*ter*) and the promoter *ermE**p (thick red arrow) in front of *bpsA* between the restriction sites of *HindIII* and *SpeI*. The backbone (rest) of the plasmid pTSA105 (1b) is 5794 bps in size. The band 1a shows undigested vector molecules (9989 bps) and 2d shows pSOK804 (5493 bps) cut with *HindIII*/*EcoRI* to get the shown fragment with a length of 5442 bps and another small undetectable fragment (51 bps). L is the ladder (Quick load® 1kb ladder, Methods 7.5)

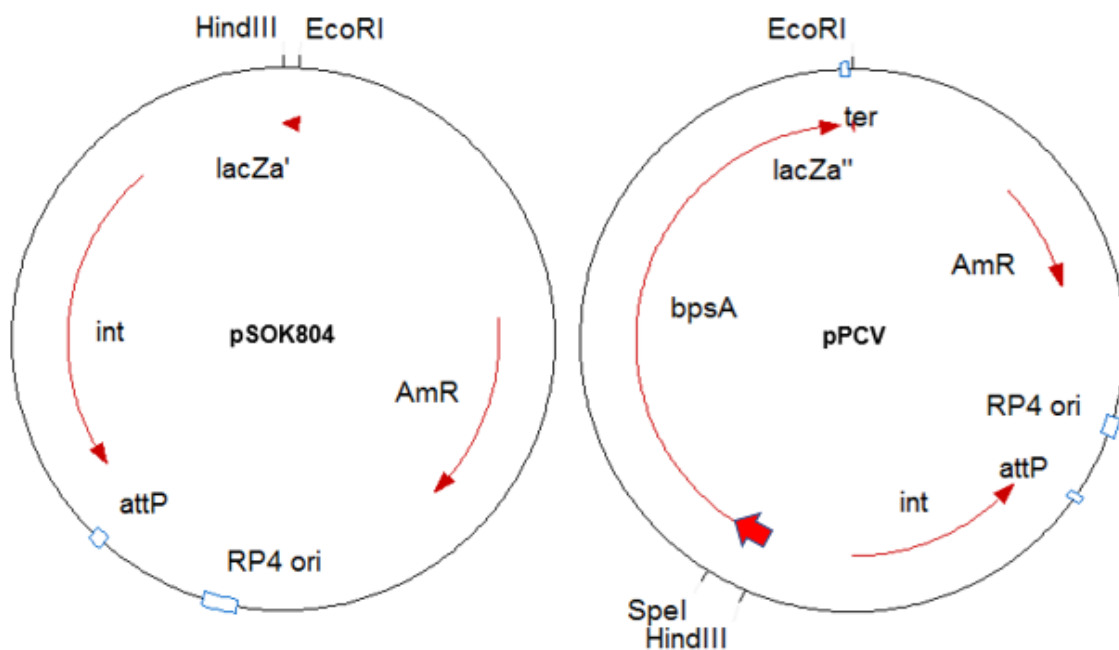


Figure 25: The vector maps of pSOK804 (left) and pPCV (right). Fragment C was inserted in pSOK804 by a ligation reaction, after cutting both DNA-molecules with the same restriction endonucleases (HindIII and EcoRI). The ligation product is the positive control vector pPCV (9637 bps), which has the strong promoter *ermE*p* (thick red arrow) in front of *bpsA*.

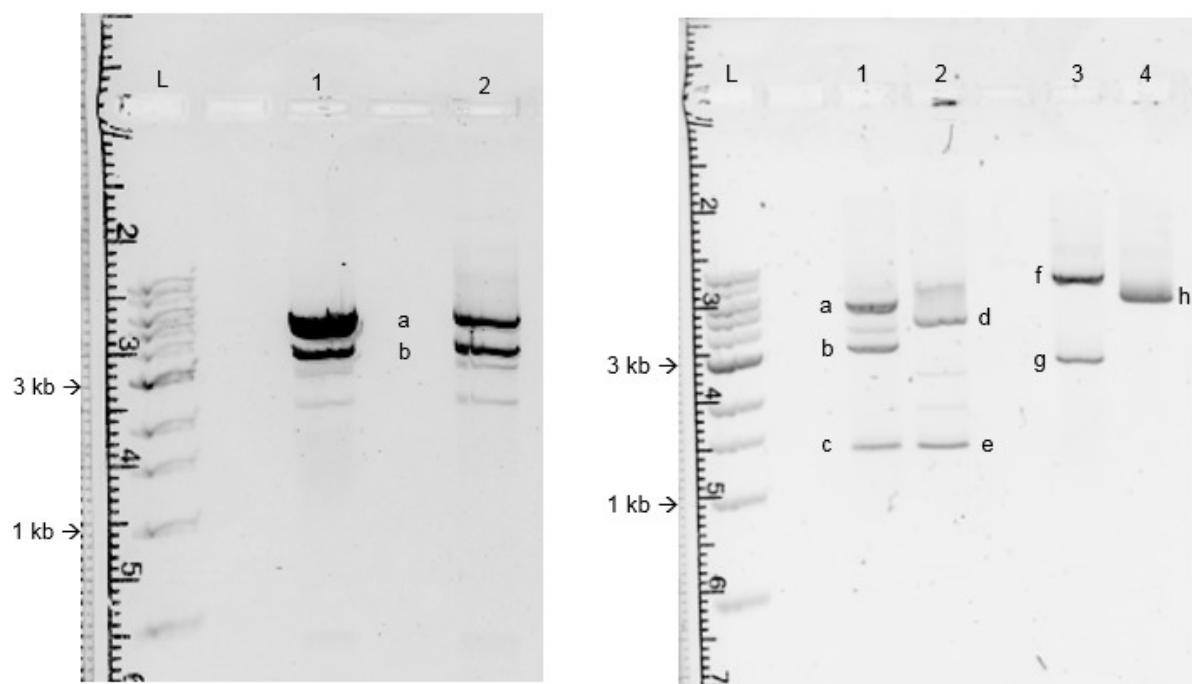


Figure 26: Left picture: Restriction analysis of pPCV with SpeI/EcoRI. 1a and 2a (6521 bps) and 1b and 2b (3116 bps) are expected fragments. L is the ladder (Quick load® 1kb ladder, Methods 7.5)

Right picture: Lane 1 shows pPCV digested with SphI/MluI. 3 expected fragments, 1a (5181 bps), 1b (3096 bps) and 1c (1340 bps) are shown. A fourth fragment with only 20 bps cannot be detected with this electrophoresis conditions. The fragments 2d (4133 bps) and 2e (1340 bps) are pieces from pSOK804 digested with SphI and MluI. On this lane, another fragment with only 20 bps cannot be detected as well. Lane 3 and 4 show the restriction pattern of pPCV cut with NotI and SpeI and pSOK804 as control. 3f (7041 bps) and 3g (2569 bps) are the fragments from pPCV and pSOK804 is 5493 bps in size.

4.4.2. Negative Control Vector

For the construction of the negative control vector, the plasmids pTSA105 and pGEM3Zf, isolated from *E. coli* DH5 α , were used (Material 6.1.4). First, both DNA molecules were digested with *SpeI*/*EcoRI*. pTSA105 (9989 bps) was cut to get fragment A (3960 bps), which consists of the reporter gene *bpsA* without the *ermE***p* promoter in front of it.

The ligation of fragment A into pGEM3Zf yielded plasmid pGEB101 (7132 bps), which was further digested with the same endonucleases (*EcoRI* and *HindIII*) as pSOK804, yielding fragment B. Fragment B was then inserted in pSOK804 by a ligation reaction. The product of this ligation was the negative control vector pNCV (9426 bps), which didn't include a strong constitutive promoter *ermE***p* in front of *bpsA*.

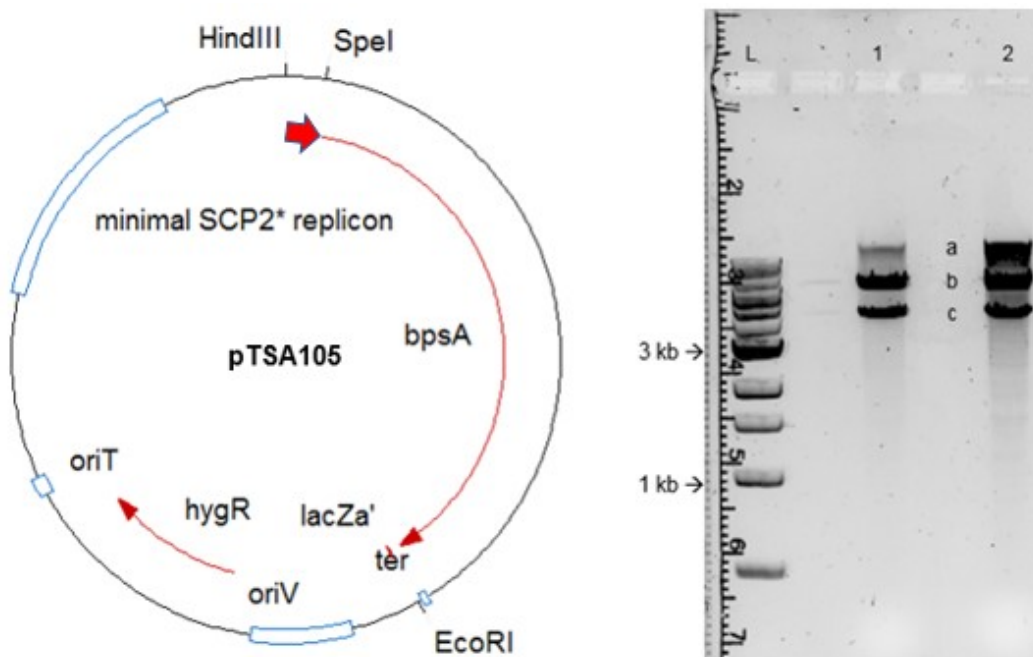


Figure 27: Plasmid map of pTSA105 (9989 bps) and picture of agarose gel after preparative electrophoresis. Fragment A (1c, 2c) with a length of 3960 bps from the plasmid pTSA105 after digestion with *SpeI* and *EcoRI* consisted of *bpsA* and a transcriptional terminator without the strong constitutive promoter *ermE***p* (not shown on the map) between the restriction sites of *HindIII* and *SpeI*. The backbone (rest) of the plasmid pTSA105 (1b, 2b) is 6029 bps in size. The lanes 1a and 2a show undigested vector molecules (9989 bps). L is the ladder (Quick load® 1kb ladder, Methods 7.5).

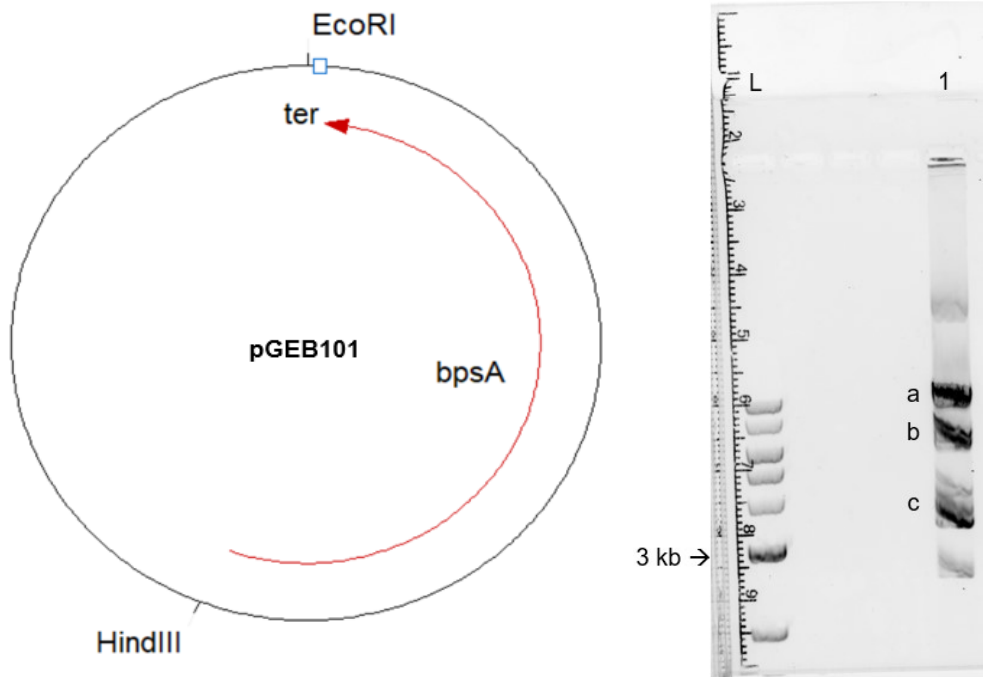


Figure 28: Vector map of pGEB101 (7132 bps) and preparative gel electrophoresis. Isolation of fragment B after cutting the ligation product from pTSA105 and pGEM3Zf, pGEB101 with EcoRI and HindIII. L is the ladder (Quick load® 1kb ladder. Lane 1 shows the fragments 1a, uncut pGEB101 (7132 bps), 1b (3984 bps) and 1c (3148 bps). 1c is the expected fragment B, which was isolated from the gel and further inserted into pSOK804.

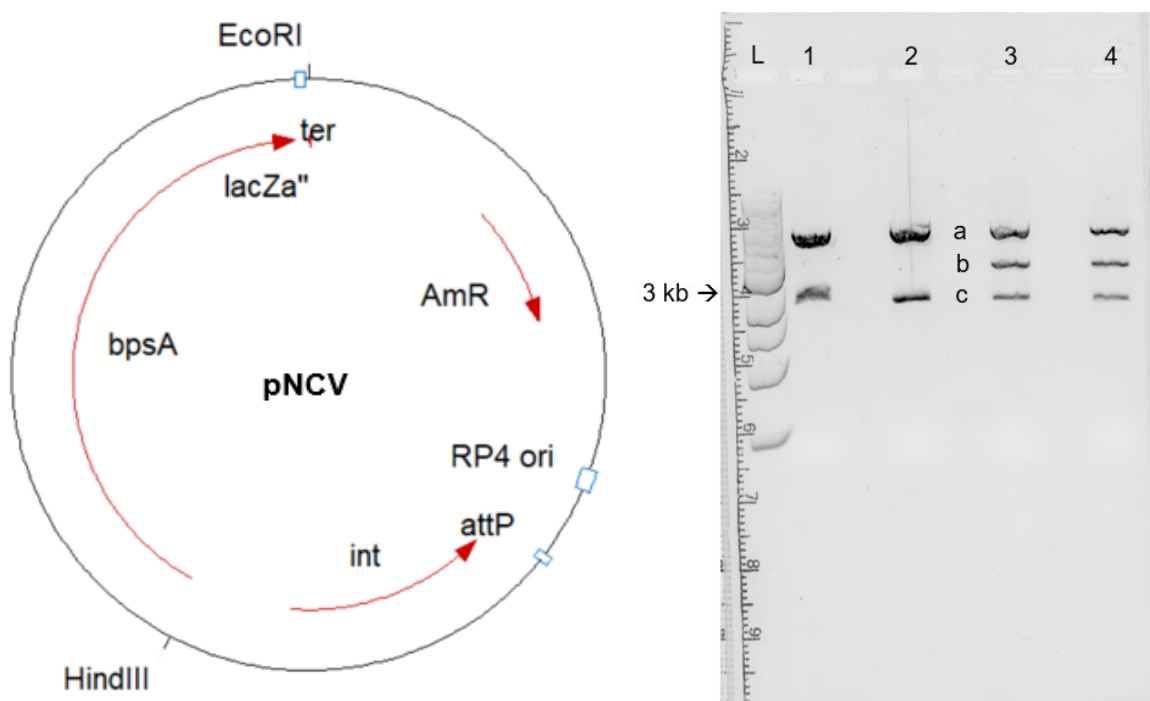


Figure 29: Plasmid map of the negative control vector pNCV (9426 bps) and gel electrophoresis to confirm successful cloning. All lanes show the restriction analysis of pNCV digested with EcoRI/HindIII/BamHI. The fragments 1a-4a are uncut vector molecules (9426 bps). The fragments 3b and 4b are both two unseparated DNA molecules. 3806 bps (cut with EcoRI and BamHI) and 3985 bps (cut with EcoRI and HindIII) are the expected sizes of digested plasmids. 1c-4c are the products of the digestion by BamHI and HindIII (1636 bps). Thus, restriction analysis showed proper cloning of pNCV in samples 3 and 4.

4.5. Primer design

All primers were designed as described in Methods 7.4.3. Following primers were designed to amplify intergenic regions (putative promoter regions) and biosynthetic genes by PCR, to get products (Results 4.6.2, Table 5), which were further used as inserts in plasmids.

These plasmids were used as promoter probe vectors or knock-out vectors. (Material 6.1.4, Results 4.6 and 4.7) A list of all designed primers and the length of the resulting PCR-product can be found in Appendix.

4.6. PCR and Construction of Promoter Probe Vectors

4.6.1. Isolation of Genomic DNA

For PCR-assisted amplification of promoter regions, genomic DNA from *Streptomyces* strains was isolated using Wizard® SV Genomic DNA Purification System (Promega, enhanced) (Methods 7.4.1). Figure 30 shows isolated DNA from *Streptomyces* ADI95-17, GBA94-01 and PVA91-01 on an agarose gel after electrophoresis.

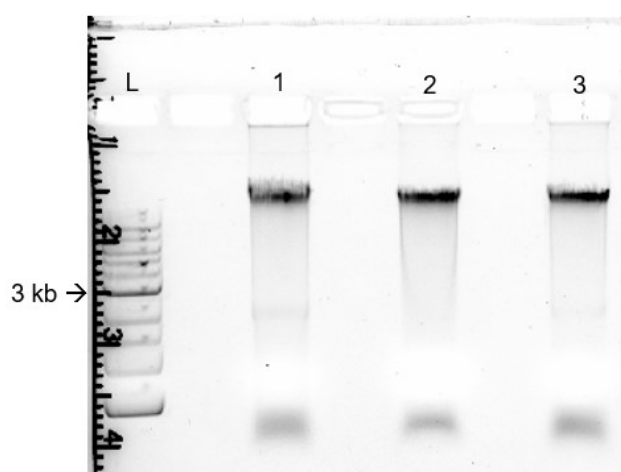


Figure 30: The bands in lanes 1-3 represent the genomic DNA from *Streptomyces* ADI95-17, GBA94-01 and PVA91-01. L is the ladder (Quick-Load® 1kb Ladder).

4.6.2. PCR-products for Promoter Probe Vectors

PCR was applied to amplify intergenic regions (promoter regions) from chosen clusters (Results 4.3). After performing PCR (Master Cycler Nexus X2 from Eppendorf®), using the designed primers, the integrity of products from all samples was analyzed by gel electrophoresis (Methods 7.5). Table 5 shows PCR-products, which were further inserted in the plasmid pNCV (cut with *HindIII*/*SfiI*) to generate promoter probe vectors.

Table 5: All generated PCR-products (2 putative promoter regions, except for PVA91-01 Cluster 17, of in total 8 BGCs of three *Streptomyces* strains) were used as inserts in pNCV for the construction of promoter probe vectors.

PCR-product (promoter region)	Size [bps]	Expected PCR-product confirmed
ADI95-17C6.1	177	+
ADI95-17C6.2	237	+
ADI95-17C29.1	210	+
ADI95-17C29.2	367	+
GBA94-01C6.1	184	+
GBA94-01C6.2	461	+
GBA94-01C16.1	207	+
GBA94-01C16.2	227	+
GBA94-01C24.1	396	+
GBA94-01C24.2	451	+
PVA91-01C8.1	269	+
PVA91-01C8.2	176	+
PVA91-01C17	215	+

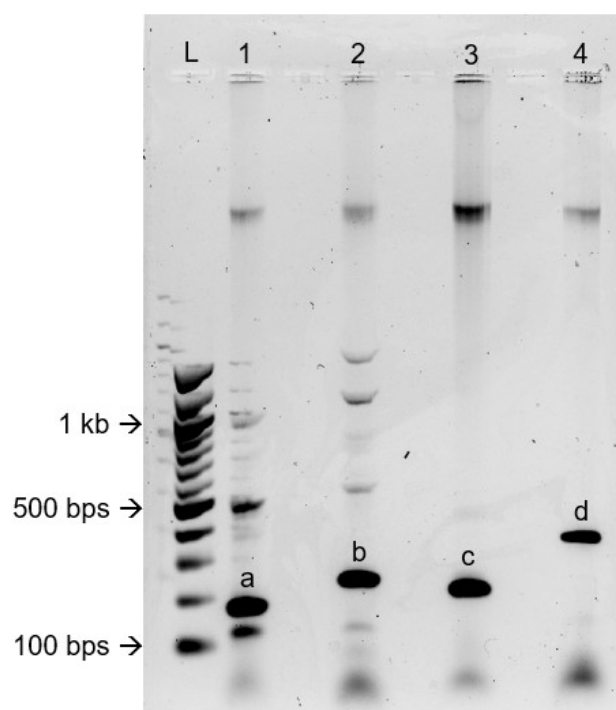


Figure 31: The synthesized PCR-products on this agarose gel picture are as follows: 1a is ADI95-17C6.1 (177 bps), 2b is ADI95-17C6.2 (237 bps), 3c is ADI95-17C29.1 (210 bps) and 4d is ADI95-17C29.2 (367 bps). L is the ladder (Quick load® 100bp ladder, Methods 7.5).

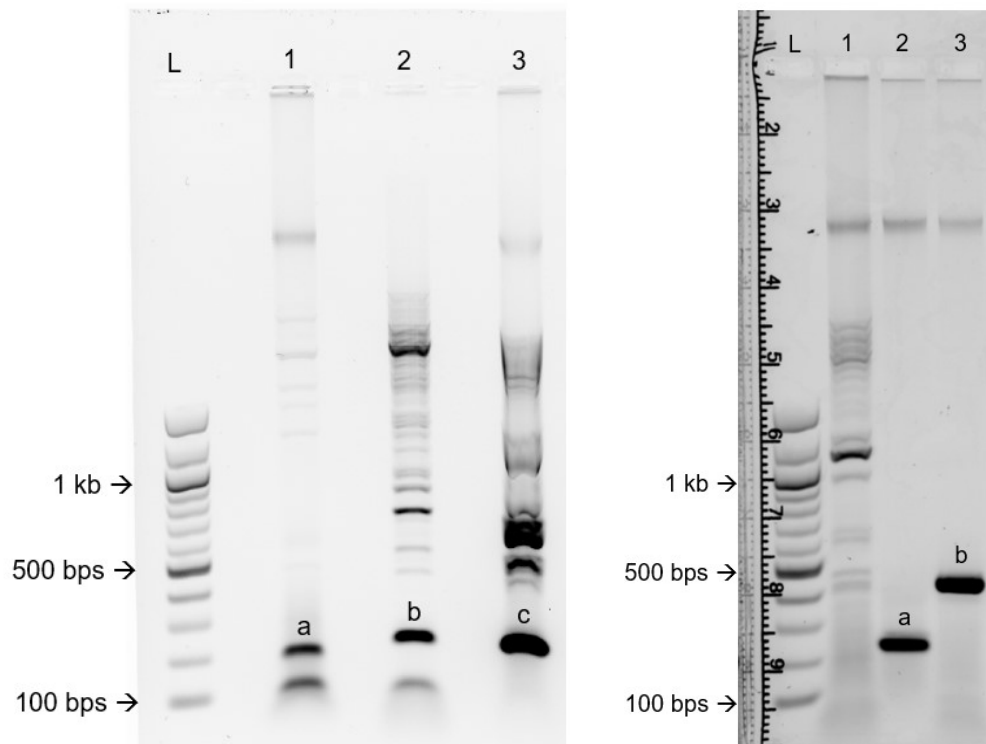


Figure 32: The PCR-products shown on the left picture were as follows: band 1a is GBA94-01C6.1 (184 bps), 2b and 3c are GBA94-01C16.1 (207 bps). L is the ladder (Quick load® 100bp ladder). The picture on the right side shows the products 1a, GBA94-01C16.2 (227 bps) and 2b, GBA94-01C24.2 (451 bps). On lane 1, the PCR-reaction didn't lead to the expected product GBA94-01C6.2 (461 bps).

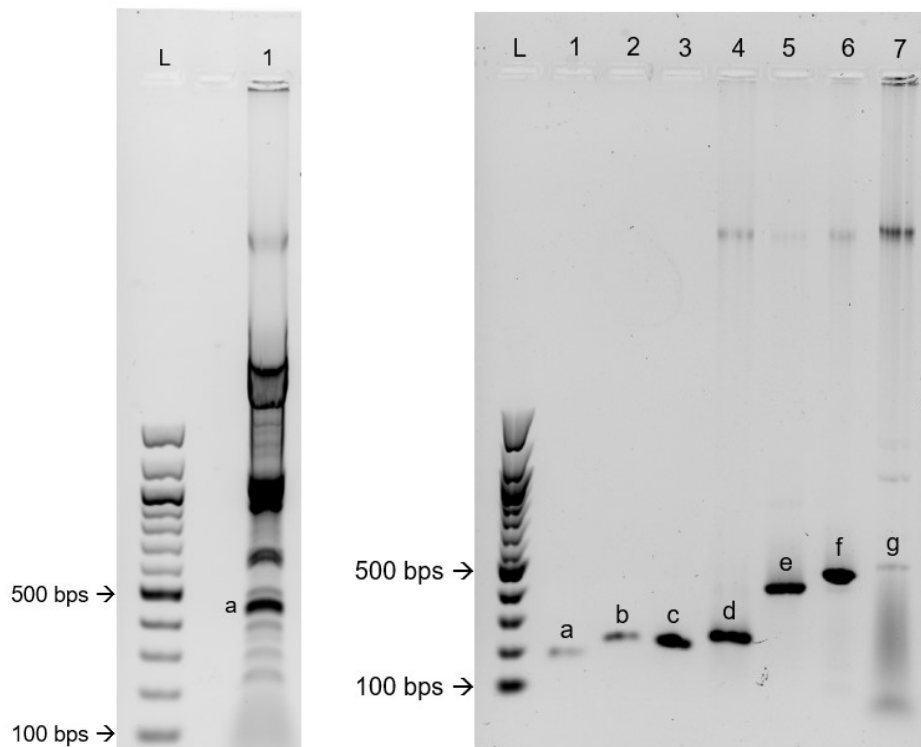


Figure 33: The PCR-product on the left picture was GBA94-01C6.2 (461 bps). L is the ladder (Quick load® 100bp ladder). The picture on the right side shows all PCR-products from the strain GBA 94-01. 1a is GBA94-01C6.1 (184 bps), 2b and 3c are GBA94-01C16.1 (207 bps), 4d is GBA94-01C16.2 (227 bps), 5e is GBA94-01C24.1 (396 bps), 6f is GBA94-01C24.2 (451 bps) and 7g is GBA94-01C6.2 (461 bps).

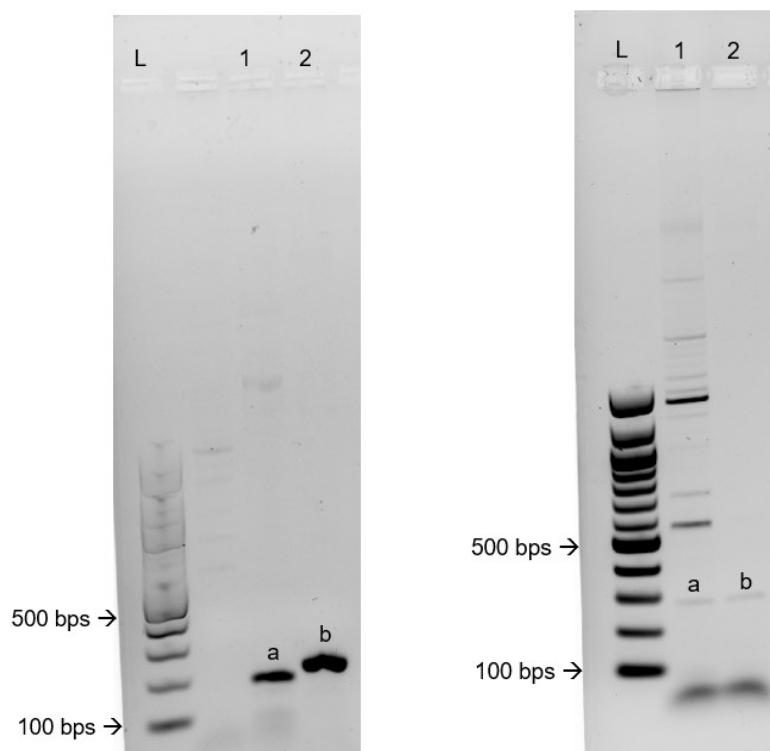


Figure 34: The PCR-products 1a, PVA91-01C8.2 (176 bps) and 2b, PVA91-01C817 (215 bps) are shown on the left picture of an agarose gel. The gel picture on the right side shows 1a and 2b, both PVA91-01C8.1 (269 bps). L is the ladder (Quick load® 100bp ladder).

4.6.3. Construction of Promoter Probe Vectors

All promoter regions amplified by PCR (Results 4.6.2, Table 5) were inserted into the negative control vector pNCV to generate promoter probe vectors. Therefore, pNCV and PCR-products were cut with *HindIII*/*SfiI* to combine vector molecule with each promoter fragment by ligation. (Methods 7.4.5) Resulting promoter probe vectors were analyzed by gel electrophoresis (Methods 7.5) after restriction digestion. (Methods 7.4.5) Figure 34 shows an example of a constructed promoter probe vector. All vector constructs can be found in Material 6.1.4.

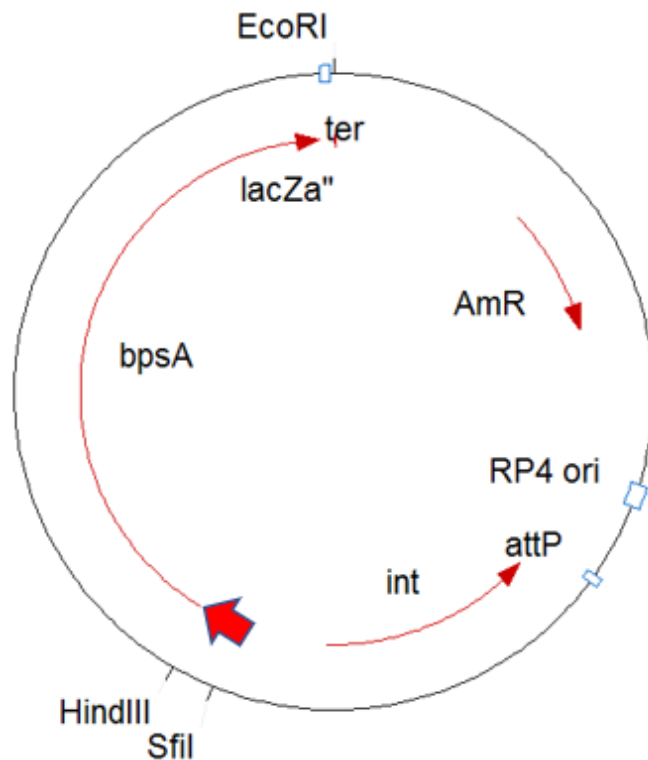


Figure 35: Map of a promoter probe vector. The thick red arrow between the restriction sites of HindIII and SfiI represents the promoter region, which was inserted in the vector pNCV.

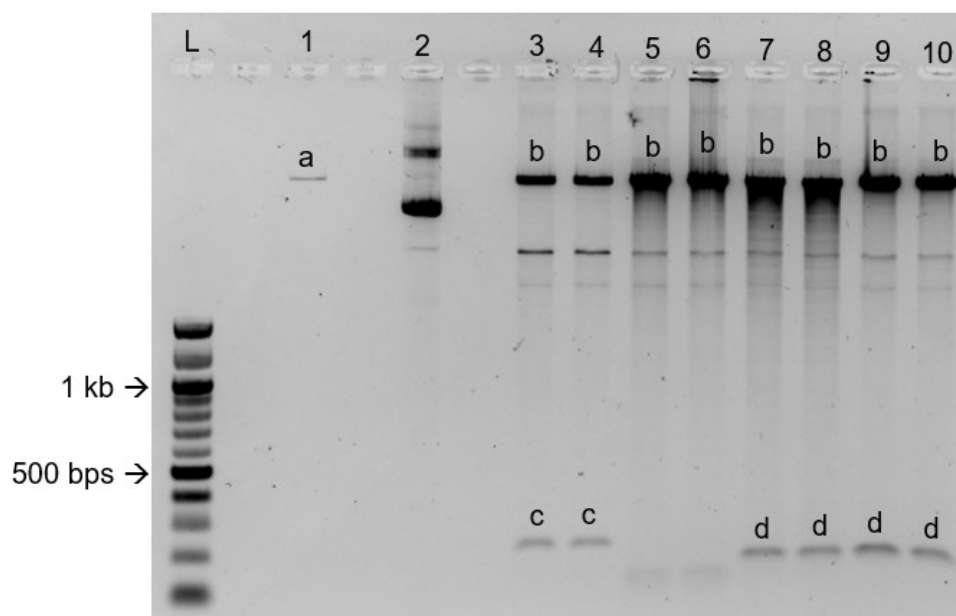


Figure 36: The agarose gel picture depicts pADI95-17C6.2 (9523 bps) on lanes 3 and 4, and pADI95-17C6.1 (9583 bps) on lanes 5-10. 3b-10b is the linear plasmid pNCV (9426 bps) cut with HindIII/SfiI. The band 1a shows pNCV cut with HindIII/SfiI, used as a control. Lane 2 is pNCV uncut. 3c and 4c (216 bps) and 7d-10d (156 bps) are the inserts ADI95-17C6.2 and ADI95-17C6.1. On lanes 5 and 6 the restriction analysis could not confirm right cloning. L is the ladder (Quick load® 100bp ladder).

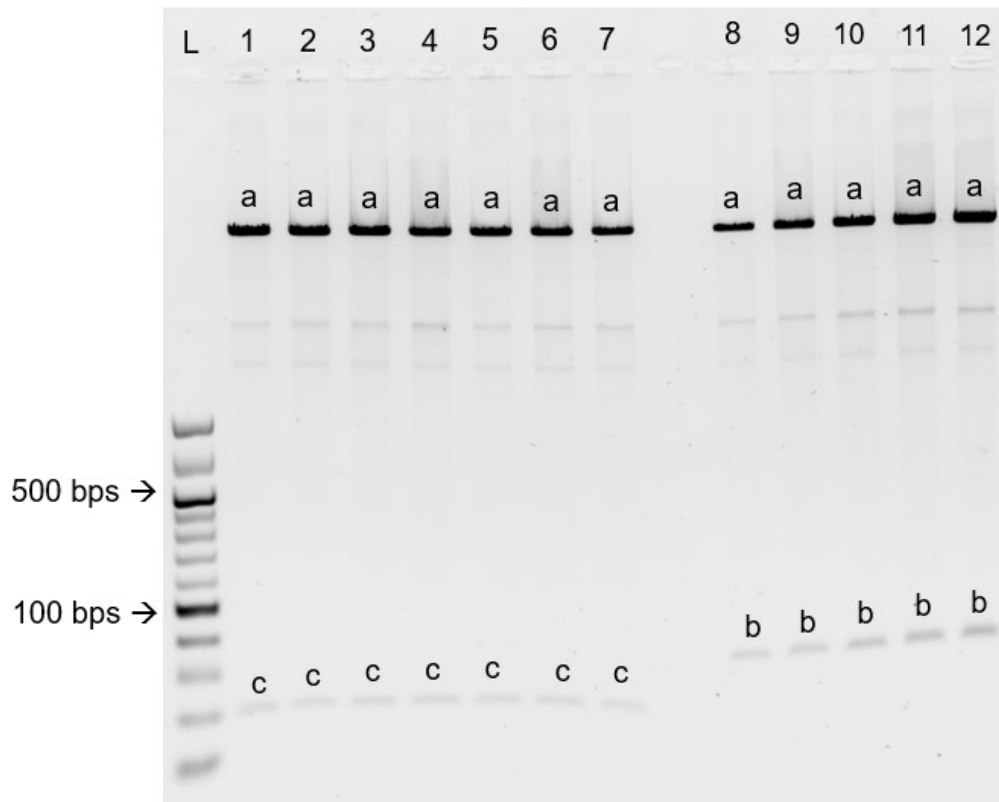


Figure 37: The agarose gel picture shows *pADI95-17C29.1* (9556 bps) on lanes 1 and 7 and *pADI95-17C29.2* (9713 bps) on lanes 8-12. 1a-12a is the linear plasmid *pNCV* (9426 bps) cut with *HindIII/SfiI*. 1c-7c and 8b-12b are the inserts *ADI95-17C29.1* and *ADI95-17C29.2*. L is the ladder (Quick load® 100bp ladder).

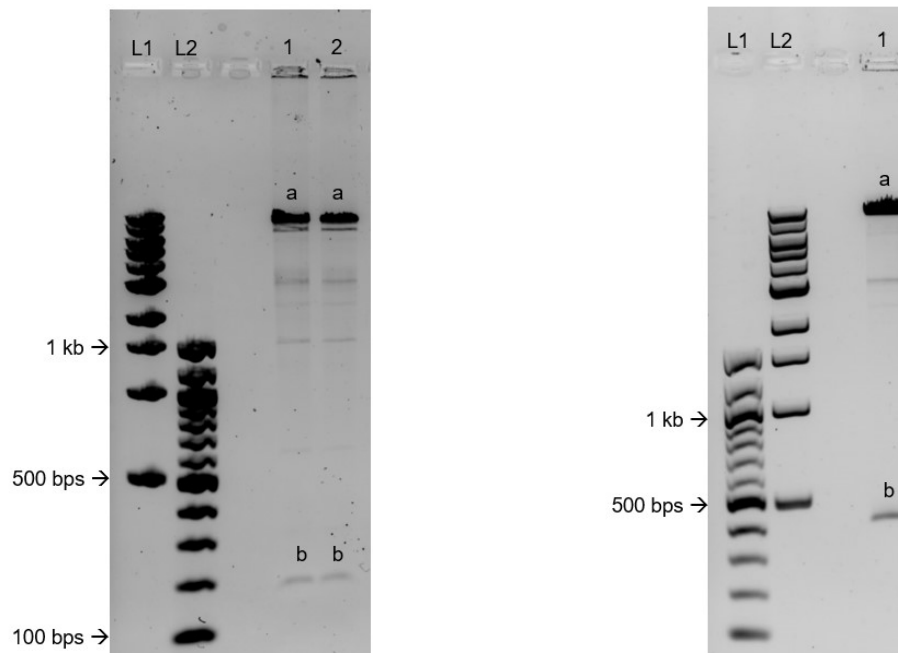


Figure 38: The agarose gel picture on the left side shows *pGBA94-01C6.1* (9530 bps) digested with *HindIII/SfiI*. The bands 1a and 2a are *pNCV* cut (9367 bps) and 1b and 2b are the inserts *GBA94-01C6.1* (163 bps). Restriction analysis of *pGBA94-01C6.2* (9807 bps) is shown on the picture on the right side. 1a is *pNCV* cut (9367 bps) and 1b is the fragment *GBA94-01C6.2* (461 bps). L1 and L2 are the ladders (Quick load® 1kb ladder and Quick load® 100bp ladder).

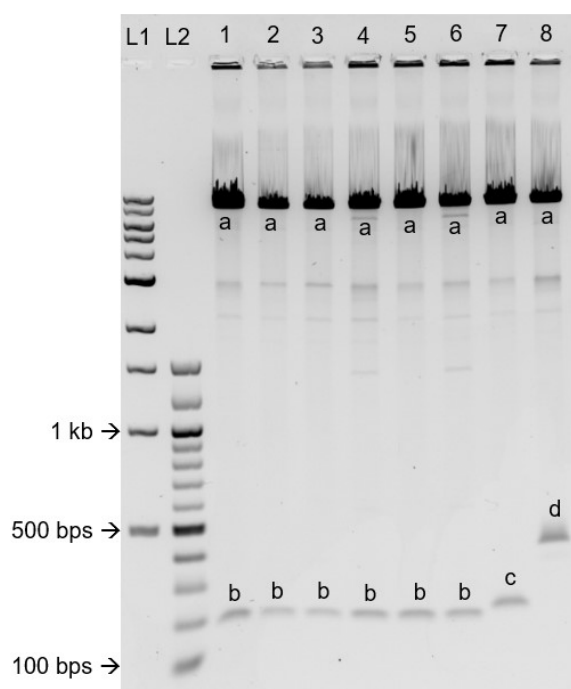


Figure 39: This gel picture depicts the restriction analysis of the promoter probe vectors pGBA94-01C16.1 (9553 bps), pGBA94-01C16.2 (9573 bps) and pGBA94-01C24.2 (9797 bps). In lane 1-8, band a represents pNCV cut with HindIII/SfiI (9367 bps). The fragments 1b-6b are GBA94-01C16.1 (207 bps), 7c is GBA94-01C16.2 (227 bps) and 8d GBA94-01C24.2 (451 bps). L1 and L2 are the ladders (Quick load® 1kb ladder and Quick load® 100bp ladder).

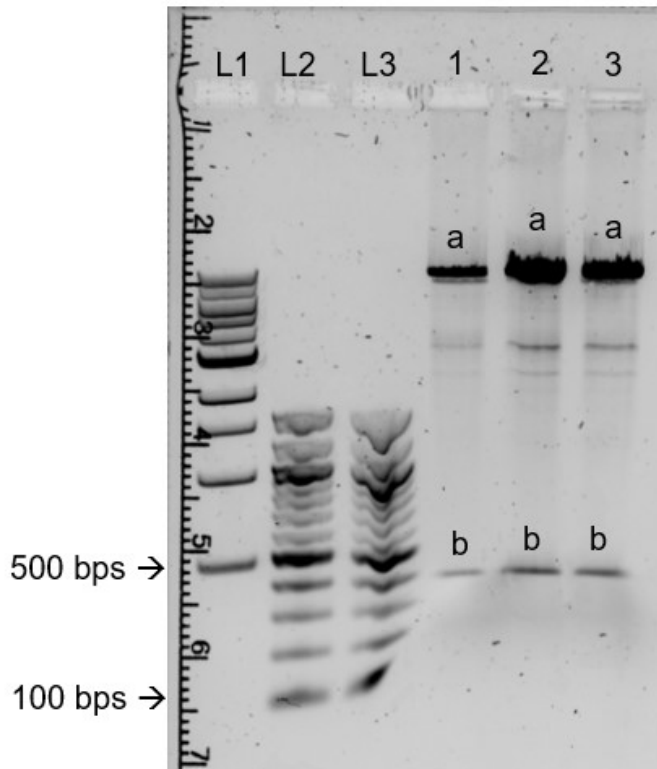


Figure 40: Performed restriction analysis of pGBA94-01C24.1 (9742 bps) is shown here. Bands 1a-3a show the vector pNCV cut with HindIII/SfiI (9367 bps) and 1b-3b are the inserts GBA94-01C24.1 (375 bps). L1, L2 and L3 are the ladders (Quick load® 1kb ladder and 2x Quick load® 100bp ladder).

Due to a lack of time, no promoter probe vectors for *Streptomyces* PVA91-01 were constructed.

4.7. PCR and Construction of Knock-out Vectors

Internal parts of biosynthetic genes from chosen clusters were amplified by PCR with the designed KO-primers to gain products, which were further introduced in the vector molecule pKC1218 to create knock-out vectors. These vectors were constructed to knock-out the corresponding biosynthetic gene via homologous recombination to stop the production of a secondary metabolite scaffold molecule. If the SM is therefore not produced anymore, the corresponding peak of this compound in the chromatogram should disappear.

With comparative HPLC analysis of promoter probe vectors and knock-out vectors it is possible to identify this peak. Thus, if a peak disappears through a gene knock-out, you can expect the knocked-out gene to be responsible for the production of the secondary metabolite associated to this peak.

4.7.1. PCR-products for Knock-out Vectors

PCR was carried out due to the procedure described in Methods 7.4.4.

Table 6: Following PCR-products were used as inserts for knock-out vectors.

PCR-product	Size [bps]	Expected PCR-product confirmed
ADI95-17C6KO	757	+
ADI95-17C29KO	866	+
GBA94-01C6KO	585	+
GBA94-01C16KO	574	+
GBA94-01C24KO	845	+

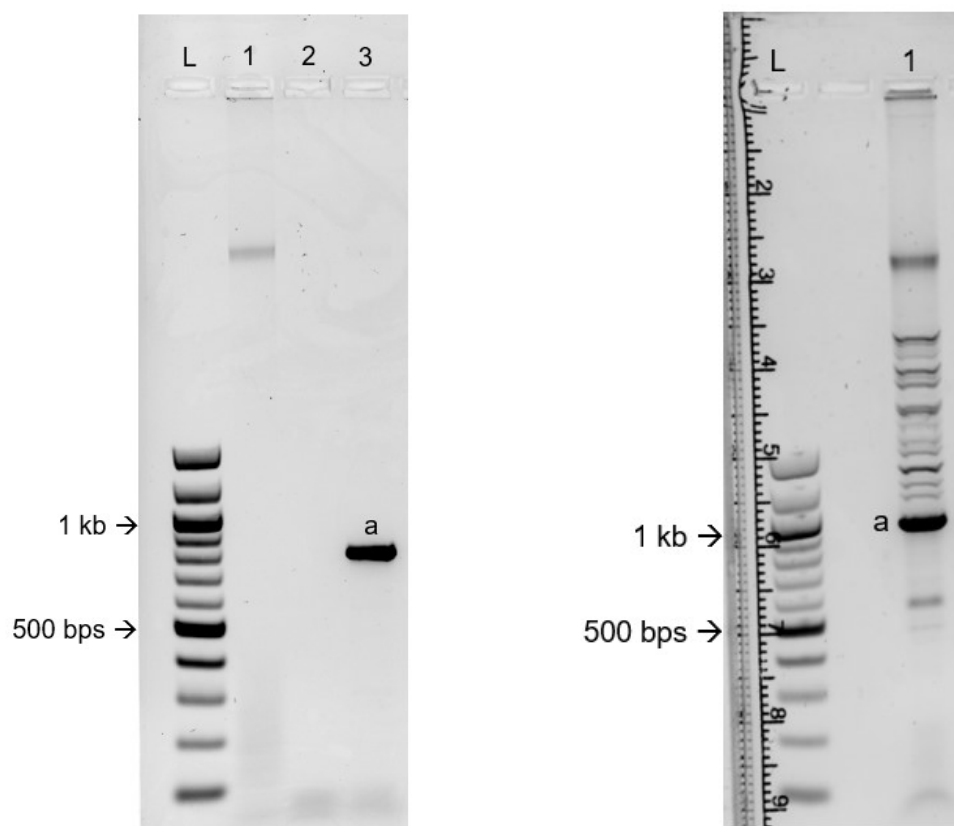


Figure 41: On the left gel picture, the PCR-product ADI95-17C6KO (743 bps) is shown on lane 3. The picture on the right side represents the analysis of ADI95-17C29KO (1a, 866 bps) on lane 1 next to by-products from PCR. L is the ladder (Quick load® 100bp ladder).

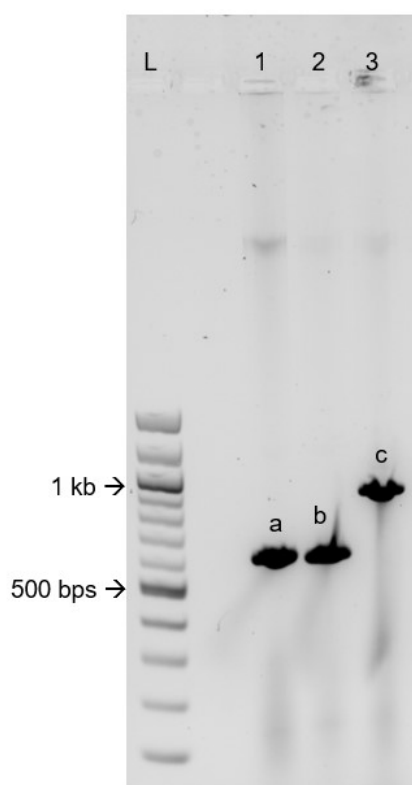


Figure 42: On this gel picture, the PCR-products GBA94-01C6KO (1a, 585 bps), GBA94-01C16KO (2b, 574 bps) and GBA94-01C24KO (3c, 845 bps) are shown. L is the ladder (Quick load® 100bp ladder).

4.7.2. Construction of Knock-out Vectors

After the successful production of inserts for a knock-out by PCR and the isolation of pKC1218 from *E. coli* DH5 α , all fragments were ligated in these vector molecules cut with *AgeI/EcoRI*. Thus, the minimal SCP2 replicon is replaced by a knock-out PCR-product (Figure 43). pKC1218 and knock-out PCR-products were cut with *AgeI/EcoRI* and joined together, what resulted in knock-out vector constructs. After transferring these plasmids into *Streptomyces* strains by conjugation from *E. coli*, the insert should knock-out the homologous biosynthetic gene by homologous recombination.

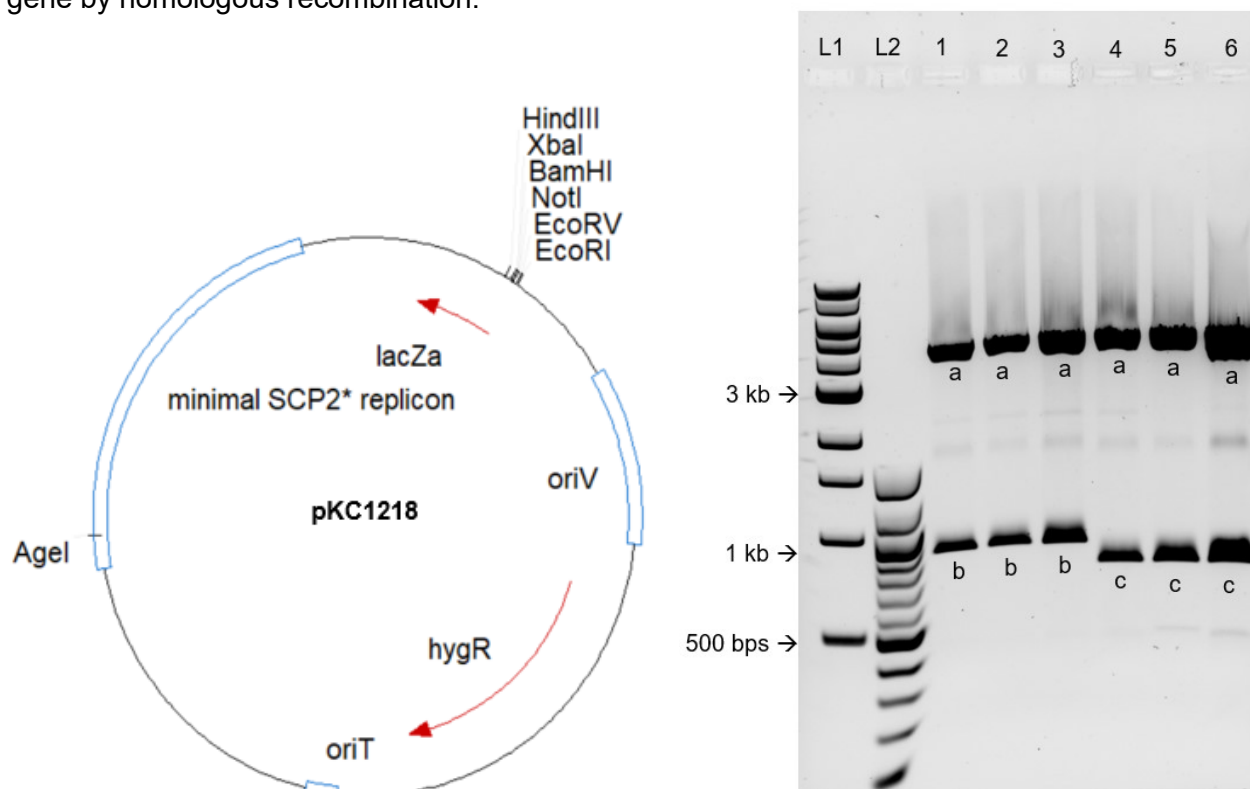


Figure 43: The plasmid pKC1218 (5854 bps) was used as knock-out vector after the insertion of fragments gained by PCR. The restriction with *AgeI/EcoRI* of the knock-out vectors pADIC6KO (4529 bps) and pADIC29KO (4624 bps) was analyzed by agarose gel electrophoresis. The bands 1a-6a show the vector pKC1218 cut, 1b-3b show the insert ADIC6KO (743 bps) and 4c-6c show ADIC29KO (845 bps). L1 and L2 are the ladders (Quick load® 1kb ladder and Quick load® 100bp ladder).

Due to a lack of time, no knock-out vectors for *Streptomyces* GBA94-01 were constructed.

4.8. Conjugation

After the successful construction of the positive control vector, the negative control vector, promoter probe vectors and knock-out vectors, they were transferred to *Streptomyces* strains by conjugation from *E. coli* ET12567 (Methods 7.4.8). All recombinant strains, in which a certain vector was introduced, can be found in Material 6.1.2, Table 13. The conjugation of knock-out vectors (pADI95-17C6KO and pADI95-17C29KO) failed.

4.9. Fermentation Cultures

Recombinant and wild-type *Streptomyces* ADI95-17 and GBA94-01 strains (Material 6.1.1 Table 12) were grown in different fermentation media (Material 6.2.1) to monitor *bpsA* gene expression by a color-change of the culture (Table 7). All fermentations were started with a seeding culture (Material 7.3.3). *Streptomyces* strains carrying the positive control vector (ADI95-17PCV, GBA94-01PCV and PVA91-01PCV (not shown)) developed a blue color in all of the used media (Figure 44). After incubation in fermentation media at cultures, which developed a different color compared to the strain containing the negative control vector, were harvested and stored at -20°C until processing. One problem, which appeared during fermentations, was that in most media, the cultivated strains developed a dark brown color by producing compounds like melanin, which made a distinction whether indigoidine is produced or not more difficult. Figure 45 shows *Streptomyces* ADI95-17C29.1 grown in all used fermentation media.

Table 7: List of strains grown in fermentation media. The positive control vector developed a blue color in every used media after one day of growth. The sign + represents all recombinant strains, which had a different color compared to the negative control strain (ADI95-17NCV, GBA94-01NCV) and the wild-type (ADI95-17WT, GBA94-01WT), thus indicating gene expression of *bpsA* driven by inserted promoter regions in front of *bpsA*. The sign - means that no color-change could be detected (silent promoters). The sign ++ means the development of a blue color due to a high production of indigoidine.

Strain	Medium						
	TSB	SM3	SM10	SM12	SM17	Simocyclinone	R5A
ADI95-17PCV	++	++	++	++	++	++	++
ADI95-17NCV	-	-	-	-	-	-	-
ADI95-17C6.1	-	-	-	-	-	-	+
ADI95-17C6.2	-	-	-	-	-	-	+
ADI95-17C29.1	-	-	-	-	-	-	+
ADI95-17C29.2	-	-	-	-	-	+	+
ADI95-17WT	-	-	-	-	-	-	-
GBA94-01PCV	++	++	++	++	++	++	++
GBA94-01NCV	-	-	-	-	-	-	-
GBA94-01C6.1	-	-	-	-	-	-	-
GBA94-01C6.2	-	-	-	-	-	-	-
GBA94-01C16.1	-	-	-	-	-	+	-
GBA94-01C16.2	-	-	-	-	-	-	-
GBA94-01C24.1	-	-	-	-	-	-	-
GBA94-01C24.2	-	-	-	-	-	-	-
GBA94-01WT	-	-	-	-	-	-	-

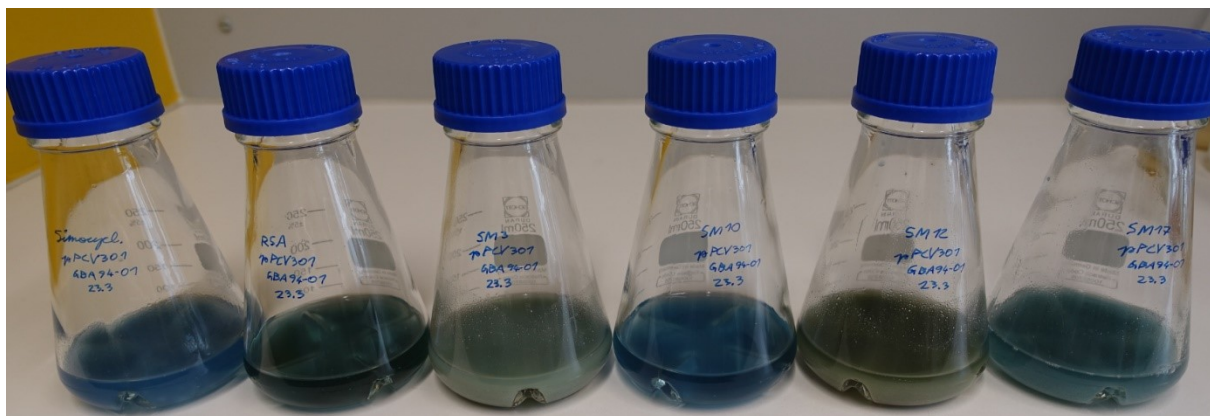


Figure 44: Indigoidine production in *Streptomyces* GBA94-01PCV in all used fermentation media (Simocyclinone fermentation medium, R5A, SM3, SM10, SM12 and SM17). In TSB media, which was used to grow seeding cultures, all strains harboring the positive control vector pPCV developed a blue color as well (not shown).



Figure 45: *Streptomyces* ADI95-17C29.1 grown in following media (from left to right) after 3 days: TSB, R5A, Simocyclinone fermentation medium, SM3, SM10, SM12 and SM17.

Streptomyces ADI95-17C6.1, ADI95-17C6.2, ADI95-17C29.1 and ADI95-17C29.2 developed a brown color in the fermentation medium R5A, which was darker than that of ADI95-17NCV, which showed a yellow color in the same medium (Figure 46). ADI95-17C29.2 showed a color difference to ADI95-17NCV in Simocyclinone fermentation medium (Figure 47).

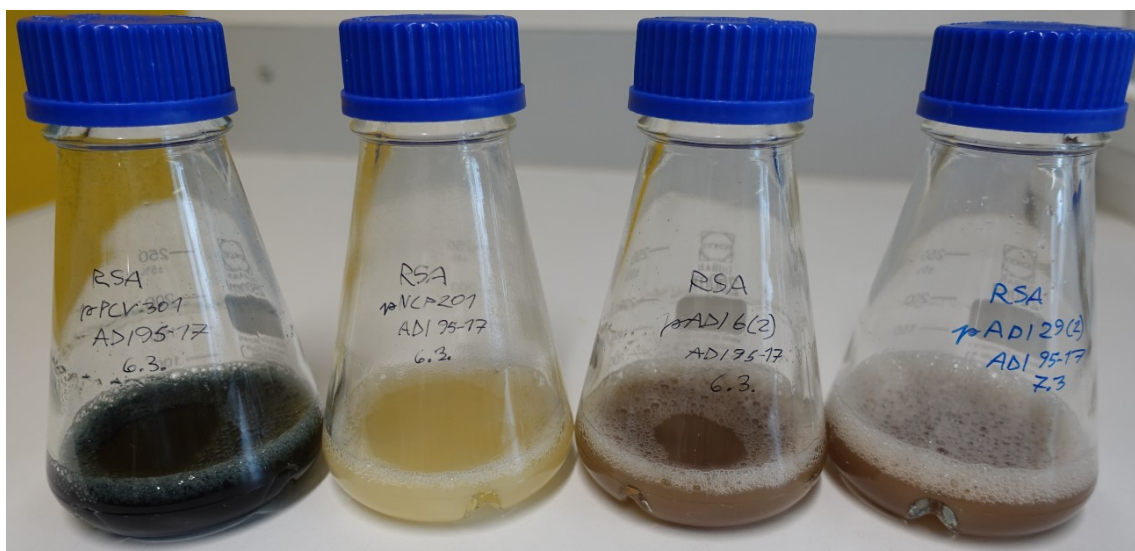


Figure 46: Fermentations of recombinant *Streptomyces* strains shown on the picture are as follows (from left to right): ADI95-17PCV, ADI95-17NCV, ADI95-17C6.2 and ADI95-17C29.2 grown for 7 days in RSA.

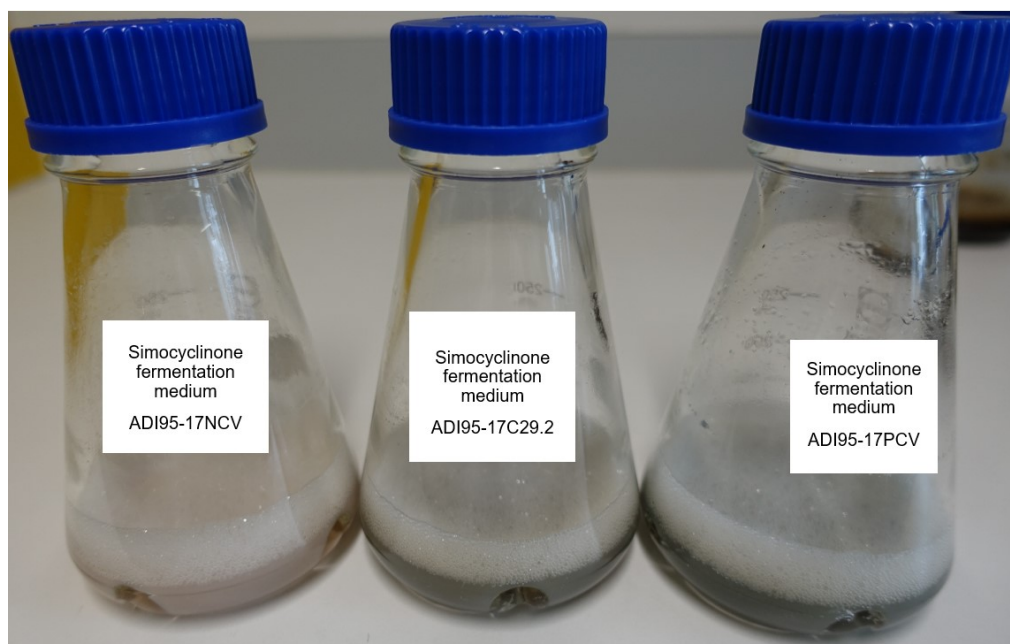


Figure 47: Fermentations of recombinant *Streptomyces* strains shown on the picture are as follows (from left to right): ADI95-17PCV, ADI95-17NCV, ADI95-17C6.2 and ADI95-17C29.2 grown for 7 days in RSA.

GBA94-01C16.1 showed a color difference compared to GBA94-01NCV in Simocyclinone fermentation medium. The culture showed a darker color than GBA94-01 carrying pNCV (Figure 48).



Figure 48: *Streptomyces* GBA94-01PCV and GBA94-01NCV compared to GBA94-01C16.1.

4.9.1. Ethanol-shock

In order to trigger gene expression in strains, which didn't lead to indigoidine production through expression of *bpsA* or to a visible color difference of cultures, they were treated with an ethanol-shock (Methods 7.3.3). Unfortunately, this experiment didn't lead to an activation of silent promoters, which didn't show a color difference without ethanol-shock. (Results 4.8, Table 7). Ethanol-shock didn't activate silent promoters. The cultures of ADI95-17C6.1, ADI95-17C6.2, ADI95-17C29.1 and ADI95-17C29.2 showed a color-change compared to ADI95-17NCV with and without ethanol-shock. Thus, the results in color development were the same with and without ethanol-shock.

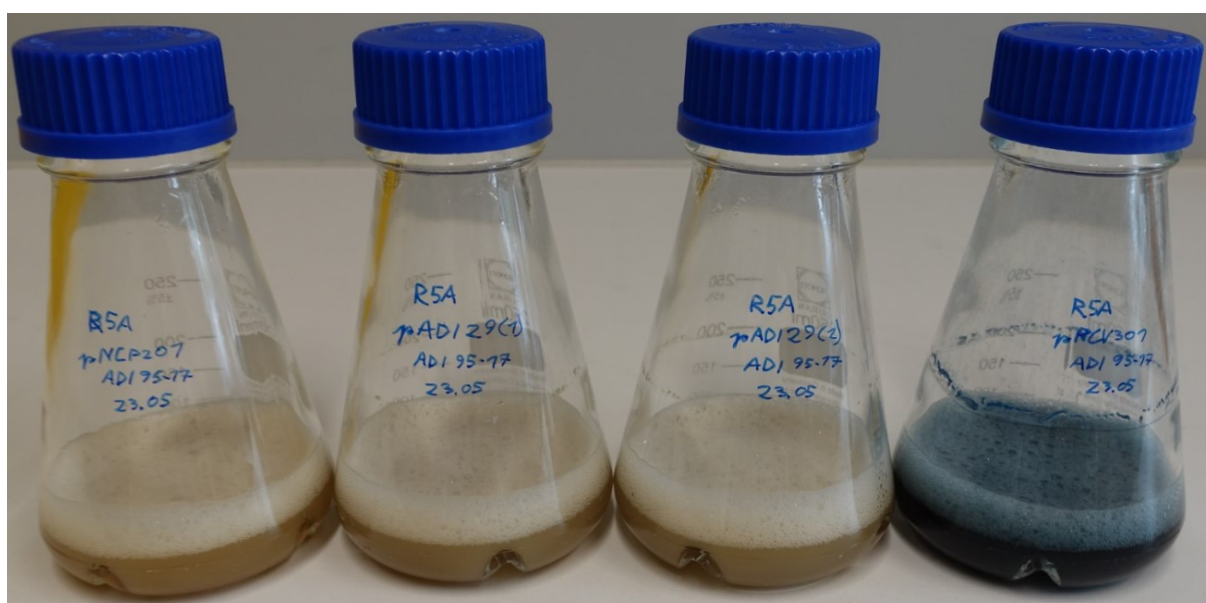


Figure 49: *Streptomyces* ADI95-17C29.1 and ADI95-17C29.2 after ethanol-shock compared to ADI95-17NCV and ADI95-17PCV.

4.10. Extraction of SMs from Fermentation Cultures

Extraction of SMs from fermentation cultures was carried out as described in Methods 7.6.1.

Methanolic extracts shown in Table 8 were obtained from *Streptomyces* strains.

All extracts were tested for growth inhibition via disc diffusion assay (Methods 7.6.2).

Table 8: Following extracts were obtained from fermentation cultures in a certain media from recombinant strains of *Streptomyces* ADI95-17 and GBA94-01. The medium R5A and Simocyclinone fermentation medium were extracted to serve as control.

Extract name	Days of fermentation	Fermentation medium
ADI95-17C6.2EX	6	R5A
ADI95-17C6.2ESEX	6	R5A
ADI95-17C29.2EX	6	R5A
ADI95-17C29.2ESEX	6	R5A
ADI95-17NCVEX	6	R5A
ADI95-17NCVEX1	6	R5A
ADI95-17NCVESEX	6	R5A
ADI95-17WTEX	6	R5A
R5A medium control	-	-
GBA94-01C16.1EX	5	Simocyclinone
GBA94-01NCVEX	5	Simocyclinone
GBA94-01NCVESEX	5	Simocyclinone
GBA94-01WTEX	5	Simocyclinone
GBA94-01WTESEX	5	Simocyclinone
Simocyclinone medium only control	-	-

4.11. Growth Inhibition Assays

Bioactivity of all extracts was tested via disc diffusion assay (Methods 7.6.2).

Growth inhibition assays were performed with different test organisms (Material 6.1.3) and growth inhibition of *Saccharomyces cerevisiae* was found in some extracts (Table 9 and Figure 50), which were further analyzed for bioactive substances. Table 9 depicts the result from growth inhibition assay of *Streptomyces* ADI95-17 extracts against the test organisms used in this project. For extracts from *Streptomyces* GBA94-01 strains, no growth inhibition could be detected.

Table 9: Bioactivity of extracts obtained from fermentation cultures of *Streptomyces* ADI95-17 and GBA94-01.

Extract	Test organism and size of inhibition zone [mm]					
	<i>Pseudomonas fluorescens</i>	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i>	<i>Fusarium graminearum</i>
ADI95-17C6.2EX	-	-	-	+ (2.5)	-	-
ADI95-17C29.2EX	-	-	-	+ (2)	-	-
ADI95-17NCVEX	-	-	-	+ (3.5)	-	-
ADI95-17NCVEX1	-	-	-	+ (2.5)	-	-
ADI95-17WTEX	-	-	-	+ (5.5)	-	-
GBA94-01C16.1EX	-	-	-	-	-	-
GBA94-01NCVEX	-	-	-	-	-	-
GBA94-01NCVEX	-	-	-	-	-	-
GBA94-01WTEX	-	-	-	-	-	-
GBA94-01WTESEX	-	-	-	-	-	-



Figure 50: Disc diffusion assay of 60µl of each extract from *Streptomyces* ADI95-17 strains against *Saccharomyces cerevisiae*. Extracts on discs are as follows: 1 is ADI95-17NCVEX1, 2 is ADI95-17C6.2EX, 3 is ADI95-17C29.2EX, 4 is ADI95-17NCVEX, 5 is R5A medium control, 6 is ADI95-17WTEX and 7 is solvent only (methanol) control.

4.12. Comparative HPLC Analysis of Crude Extracts

All crude extracts (Results 4.10, Table 8) were analyzed by HPLC (Methods 7.6.3) to compare chromatograms of recombinant strains to that of the strain carrying the negative control vector with and without ethanol-shock. Media only controls (R5A for ADI95-17 and Simocyclinone fermentation medium for GBA94-01) were also analyzed to identify peaks originating from the media. Figure 50 shows the chromatogram of extracts from recombinant *Streptomyces* ADI95-17 strains. Other extracts showed the same peak pattern as ADI95-17NCVEX in the chromatogram, but two peaks, which appear at minute 24 and 25.5 (marked with arrows), were bigger compared to the same two peaks in other extracts. Figure 51 shows the chromatogram of ADI95-17NCVEX compared to the wild-type strain, to see if the peaks at minute 24 and 25.5 in Figure 50 are also present in the extract of ADI95-17 wild-type (ADI95-17WTEX).

These peaks, which appeared at minute 27.5 and 29.5, when another comparative HPLC analysis was performed, were also smaller compared to those of ADI95-17NCVEX.

If these peaks are considered to be the substances responsible for bioactivity, the peaks of ADI95-17WTEX should have been bigger in size than those of ADI95-17NCVEX according to the growth inhibition assay (Figure 50), where the activity of the wild-type strain was the highest. Thus, and because the extract ADI95-17NCVEX had the biggest growth inhibition zone of extracts from recombinant strains against *Saccharomyces cerevisiae* this extract was further analyzed to identify its bioactive substances. For *Streptomyces* GBA94-01 strains, the chromatograms didn't show a differential peak pattern as well. (Figure 53) Even the size of the peaks was nearly the same for all samples. For GBA94-01 extracts, which didn't show bioactivity at growth inhibition assays, no more analysis was done.

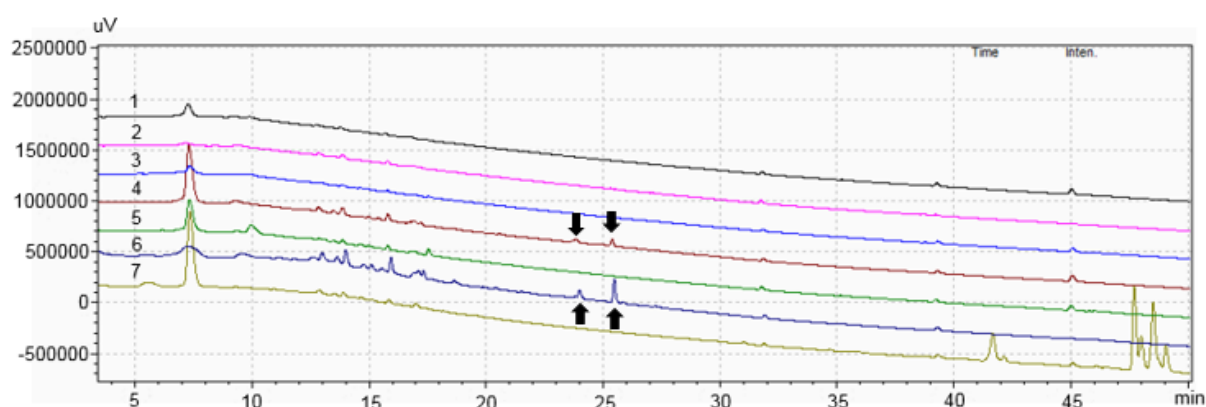


Figure 51: HPLC- PDA chromatogram of extracts from *Streptomyces* ADI95-17 mutants. The chromatogram shows ADI95-17C29.2ESEX (1, black), ADI95-17C29.2EX (2, pink), ADI95-17C6.2ESEX (3, blue), ADI95-17C6.2EX (4, brown), ADI95-17NCVESEX (5, green), ADI95-17NCVEX (6, dark blue) and R5A medium control (7, olive green).

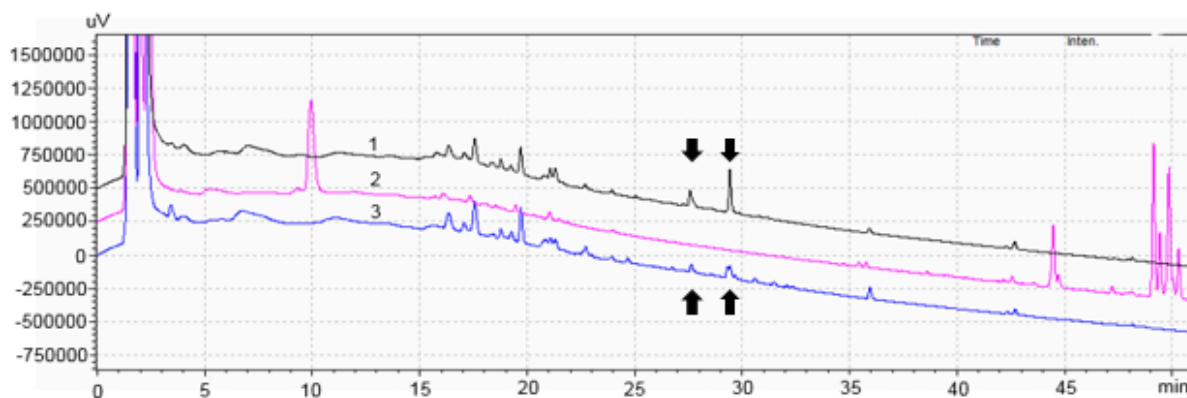


Figure 52: Comparative HPLC- PDA analysis of extracts ADI95-17NCVESEX (1, black), ADI95-17WTEX (3, blue) and R5A medium control (2, pink).

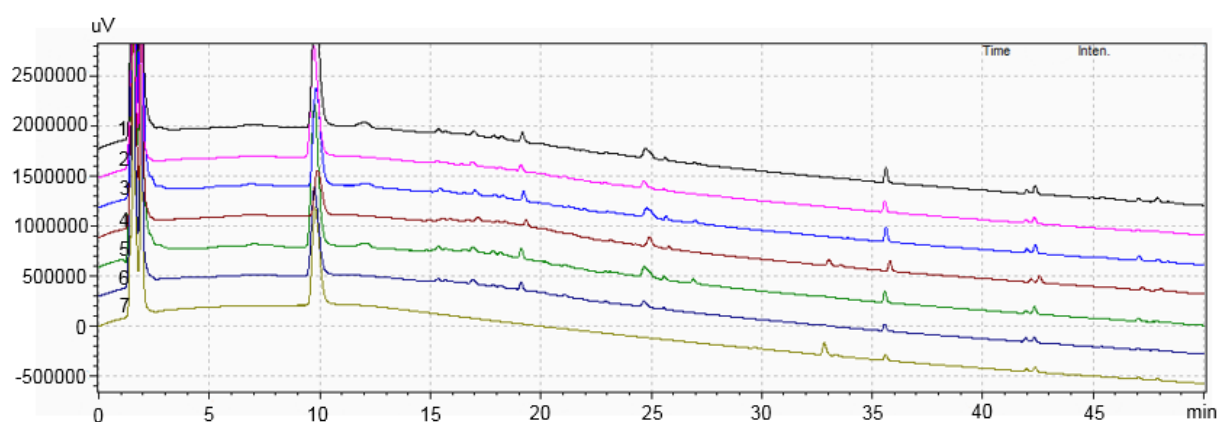


Figure 53: Comparative HPLC of extracts GBA94-01WTESEX (1, black), GBA94-01WTEX (1, pink), GBA94-01C16.1ESEX (3, blue), GBA94-01C16.1EX (4, brown), GBA94-01NCVESEX (5, green), GBA94-01NCVEX (6, dark blue), and Simocyclinone medium only control (7, olive green).

4.13. HRMS Analysis

HPLC coupled with high resolution mass-spectrometry (Methods 7.6.4) measured in the positive and the negative ion mode was performed to identify the masses and to suggest possible sum formulas of the compounds in the crude extract ADI95-17NCVEX (Table 10). The reference substances $C_{16}H_{22}O_4$, the dibutyl phthalate ion with an exact m/z of 279.1598 for the positive mode and $C_{18}H_{36}O_2$, the ion of a fatty acid, the palmitate anion with an exact m/z of 255.4329 for the negative mode, were used to evaluate the error of the measurements. Formulas with the most minimal error value compared to the corresponding reference were chosen.

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.8 Bar
Focus	Active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	7.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Source
		Set Corona	0 nA	Set APCI Heater	0 °C

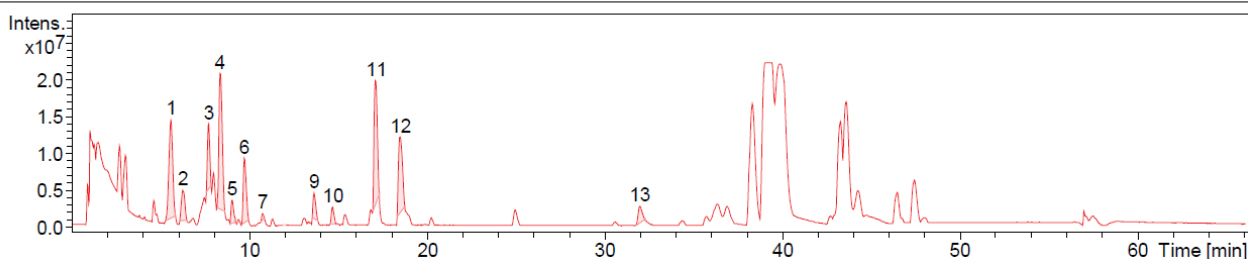


Figure 54: Resulting chromatogram of ADI95-17NCVEX measured in the positive ion mode. Peak 13 is the reference substance $C_{16}H_{22}O_4$, the dibutyl phthalate ion with an exact m/z of 279.1598.

Acquisition Parameter

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.8 Bar
Focus	Active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	7.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Source
		Set Corona	0 nA	Set APCI Heater	0 °C

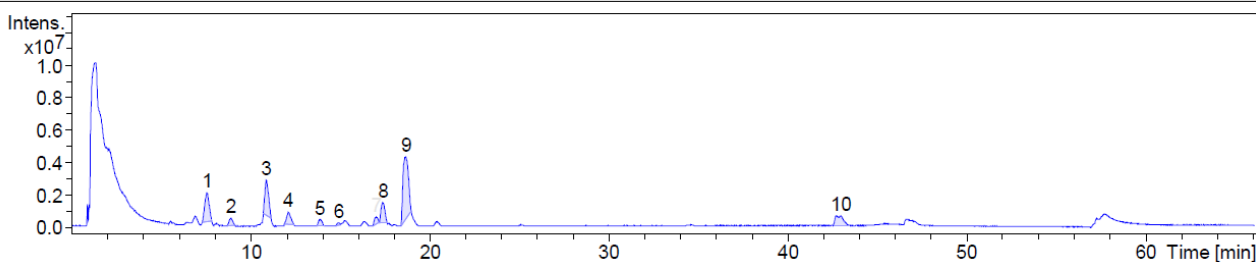


Figure 55: Chromatogram of ADI95-17NCVEX measured in the negative ion mode. Peak 10 is the reference substance $C_{18}H_{36}O_2$, the palmitate anion with an exact m/z of 255.4329.

Table 10: High-resolution mass spectrometry of compounds and comparison of peaks measured in the positive and the negative ion mode. Some peaks couldn't be detected in both modes, but 5 peaks could be correlated to each other due to their same m/z , their same RT= retention time and the same suggested sum formulas. (Ref)= reference.

Positive Mode					Negative Mode				
Peak #	RT	m/z	Error [ppm]	Ion Formula	Peak #	RT	m/z	Error [ppm]	Ion Formula
1	5.6	261.1239	-2.1	$C_{14}H_{17}N_2O_3$	-	-	-	-	-
2	6.3	180.1023	-1.9	$C_{10}H_{14}NO_2$	-	-	-	-	-
3	7.7	211.1445	-2.1	$C_{11}H_{19}N_2O_2$	-	-	-	-	-
4	8.3	211.1445	-1.9	$C_{11}H_{19}N_2O_2$	-	-	-	-	-
5	9.0	245.1290	-2.1	$C_{14}H_{17}N_2O_2$	-	-	-	-	-
6	9.7	245.1290	-2.4	$C_{14}H_{17}N_2O_2$	-	-	-	-	-
7	10.8	353.1074	-3.6	$C_{16}H_{17}O_9$	3	10.9	351.0737	-4.3	$C_{16}H_{15}O_9$
9	13.6	617.3307	-2.2	$C_{30}H_{45}N_6O_8$	5	13.9	615.0153	-5.5	$C_{30}H_{43}N_6O_8$
10	14.7	631.3463	-2.1	$C_{31}H_{47}N_6O_8$	6	14.9	629.3339	-5.5	$C_{31}H_{45}N_6O_8$
11	17.1	670.4049	-2.1	$C_{33}H_{52}N_9O_6$	8	17.4	668.3916	-4.0	$C_{33}H_{50}N_9O_6$
12	18.5	599.3204	-2.7	$C_{30}H_{43}N_6O_7$	9	18.7	597.3072	-5.0	$C_{30}H_{41}N_6O_7$
13 (Ref)	32.0	279.1598	-2.6	$C_{16}H_{23}O_4$	-	-	-	-	-
-	-	-	-	-	1	7.6	241.1204	-4.4	$C_{11}H_{17}N_2O_4$
-	-	-	-	-	2	8.9	275.1049	-4.2	$C_{14}H_{15}N_2O_4$
-	-	-	-	-	4	12.1	205.0153	-4.9	$C_{10}H_5O_5$

Positive Mode					Negative Mode				
-	-	-	-	-	7	17.0	583.2913	-4.8	C ₂₉ H ₃₉ N ₆ O ₇
-	-	-	-	-	10 (Ref)	43.0	255.2349	-7.7	C ₁₆ H ₃₁ O ₂

The accurate masses of the uncharged molecules and the sum formulas were searched for in the database Dictionary of Natural Products (DNP) (Table 11).

Table 11: Comparison of the masses and sum formulas to the DNP to identify compounds in the extract ADI95-17NCVEX. The symbol (+) in the column peak number represents peaks measured in the positive mode, (-) stands for peaks measured in the negative mode. 5 peaks could be correlated to each other and 3 of them are putatively unknown natural products. (Ref)= reference substance.

Peak #	Mass [g/mol]	Sum Formula [DNP hits]	Putative Compound
1(+)	260	C ₁₄ H ₁₆ N ₂ O ₃ (20)	cyclic dipeptide
2(+)	179	C ₁₀ H ₁₃ NO ₂ (90)	1,7a-Dihydro-7-hydroxy Abikoviromycin
3(+)	210	C ₁₁ H ₁₈ N ₂ O ₂ (20)	cyclic dipeptide
4(+)	210	C ₁₁ H ₁₈ N ₂ O ₂ (20)	cyclic dipeptide
5(+)	244	C ₁₄ H ₁₆ N ₂ O ₂ (24)	isopropyl pyrazine
6(+)	244	C ₁₄ H ₁₆ N ₂ O ₂ (24)	isopropyl pyrazine
7(+), 3(-)	352	C ₁₆ H ₁₆ O ₉ (6)	8β-Hydroxy Bostrycin
9(+), 5(-)	616	C ₃₀ H ₄₄ N ₆ O ₈ (no hit)	potentially new
10(+), 6(-)	630	C ₃₁ H ₄₆ N ₆ O ₈ (no hit)	potentially new
11(+), 8(-)	669	C ₃₃ H ₅₁ N ₉ O ₆ (no hit)	potentially new
12(+), 9(-)	598	C ₃₀ H ₄₂ N ₆ O ₇ (3)	Antibiotic WS 7338 A
13(+)	278	C ₁₆ H ₂₂ O ₄ (Ref)	dibutyl phthalate (Ref)
1(-)	242	C ₁₁ H ₁₈ N ₂ O ₄ (11)	cyclic dipeptide
2(-)	276	C ₁₄ H ₁₆ N ₂ O ₄ (13)	cyclic dipeptide
4(-)	206	C ₁₀ H ₆ O ₅ (17)	...-benzodioxin-6-carboxylic acid
7(-)	584	C ₂₉ H ₄₀ N ₆ O ₇ (3)	Antibiotic WS 7338 D
10(-)	256	C ₁₆ H ₃₂ O ₂ (Ref)	palmitic acid (Ref)

Most compounds searched in the DNP resulted in hits, suggesting known microbial compounds (Table 11). For 3 compounds [Peak number: (9(+), 5(-)); (10(+), 6(-)); (11(+), 8(-))], no hits could be found. If a substance couldn't be matched to a known natural product in a database, the associated products were considered as potentially new.

Most of the suggested microbial compounds with a lower mass (< 300 g/mol) had more hits and were considered to be cyclic dipeptides, which probably originate from the fermentation media R5A. Other peaks were identified as isopropyl pyrazines.

The molecular formula C₁₀H₁₃NO₂ resulted in 90 hits and 1,7a-Dihydro-7-hydroxy Abikoviromycin, the first entry in the list when searched in the DNP, can be found in *Streptomyces* species^{84 85}. Therefore, it is most probable a compound in the extract ADI95-17NCVEX.

The compound 8 β -Hydroxy Bostrycin, an Anthraquinone, could be found in the DNP next to 5 other hits (1: 3-*O*-Caffeoylquinic acid; (*E*)-form, 3',4'-Quinone; 2: 3,3',4',5,5',7'-Hexahydroxy-8,9-dinor-4,8'-oxylignan-7-oic acid; 3: 3,3',4',5,5',7'-Hexahydroxy-8,9-dinor-4,8'-oxylignan-7-oic acid; (7'*S*,8'*R*)-form; 4: 7-Hydroxy-4-methyl-2*H*-1-benzopyran-2-one; β -D-Glucuronopyranoside and 5: 2,3',4',5'-Tetrahydroxyacetophenone; Tetra-Ac).

8 β -Hydroxy Bostrycin was the only database entry of a natural product in the Dictionary of Marine Natural Products with the molecular formula C₁₆H₁₆O₉.

For the sum formula C₃₀H₄₂N₆O₇ the compound Antibiotic WS 7338 A, a cyclic pentapeptide was a hit next to two others, Penaeustatin 17 and Spumigin C, which were not considered to be compounds in the extract ADI95-17NCVEX, because the Antibiotics WS 7338 A-D are known to be produced by *Streptomyces* species and Penaeustatin 17 is only known to be produced by animals such as *Penaeus monodon* (tiger prawns), where these substances serve as neuropeptides.⁸⁶ Spumigin C is known to be produced by cyanobacteria (*Nodularia spumigena*)⁸⁶

The sum formula C₂₉H₄₀N₆O₇, which also resulted in only 3 hits in the DNP, was suggested to be Antibiotic WS 7338 D, next to Carcinustatin 14 and Cleromyrin II, which were excluded to be produced by *Streptomyces* ADI95-17NCV as well, because Carcinostatins are known to act as neuropeptides in animals such as the shore crab *Carcinus maenas*⁸⁶ and Cleromyrin II is a cyclohexapeptide isolated from the plant *Clerodendrum myricoides*.⁸⁷

Further fractionation, purification and following analyzation of the compounds by NMR would be necessary to verify these suggestions.

5. Discussion

In this project, the potential of three marine *Streptomyces* species (ADI95-17, GBA94-01 and PVA91-01) to produce secondary metabolites by a reporter-guided activation of gene clusters was explored. The bioinformatic tool antiSMASH⁸³ together with manual verification of secondary metabolite biosynthetic genes from promising gene clusters by BLAST⁸⁸ was used for genome mining. Since the founders of this platform improve and develop the software, the results and predictions are improving all the time. Next to these enhanced bioinformatic methods, the sequencing of DNA is becoming more accurate and cheaper, making application of genome mining a very useful tool in drug discovery. Following genome mining, putative promoter regions of scaffold molecule building biosynthetic genes were searched.

For this purpose, a bacterial promoter region detecting software called BPROM from Softberry⁸⁹, was used in this project. An improved version of this tool, which should be able to also detect unconventional promoters in *Streptomyces* species would be helpful.

PCR was performed to amplify these putative promoter regions (Results 4.6.2) from chosen clusters (Results 4.3). The enzymes encoded in these clusters are polyketide synthases, non-ribosomal peptide synthases and lantipeptide- and lassopeptide synthases.

Polyketide synthases are known to be responsible for the production of bioactive secondary metabolites such as macrolides like amphotericin, erythromycin and nystatin (PKS type I) and aromatic polyketides such as actinorhodin, doxorubicin and tetracyclines (PKS type II).³³

For example, Cluster 6 from *Streptomyces* ADI95-17 is a PKS type II. Thus, the production of an aromatic polyketide can be expected if the genes of this cluster are expressed.

Other clusters encoding for peptide producing enzymes (NRPS, lanti- and lassopeptide synthases) were chosen because their products, as those of PKS, are also often bioactive (daptomycin, ciclosporin A, vancomycin).⁹⁰ The promoter regions amplified by PCR were further introduced into the shuttle vector carrying a reporter gene, thus yielding biosensors that could detect expression of target gene clusters. Recombinant strains carrying such biosensors should produce indigoidine, an easily detectable blue-colored pigment, when the promoter is active. Because none of the biosensors-carrying strains of *Streptomyces* ADI95-17 and GBA94-01 showed indigoidine production when subjected to fermentation, the promoters were considered to be silent or too weak for an expression of the reporter. The attempt to trigger the activity of silent promoters by ethanol shock did not lead to the desired result when compared to the non-shocked cultures. It is possible that other chemicals would have been able to trigger promoter activity for a stronger expression of *bpsA*. Such chemical elicitors of secondary metabolism as histone deacetylase inhibitors⁹¹ could be used in further studies on recombinant strains generated in this work. Another problem was that in most media, the cultivated strains developed a dark brown color by producing compounds like melanin, which made a distinction whether indigoidine was produced or not more difficult.

Especially in the fermentation media SM 3,10,12 and 17 the brown color prevailed. Nevertheless, secondary metabolites of some cultures grown in R5A or Simocyclinone fermentation medium, which showed a color difference to the negative control vector, were extracted (Results 4.10). The attempt to knock-out the biosynthetic genes from the clusters of the corresponding promoter region, which was considered as activated due to a color difference, failed. HPLC analysis was performed to compare the chromatograms of recombinant strains carrying biosensors to the strains harboring the negative control vector and to the wild-type strain. Because extracts of *Streptomyces* ADI95-17 were bioactive against *Saccharomyces cerevisiae* (Results 4.11, Figure 50), further analyzes was done to identify the bioactive compounds. The masses and the molecular formulas of compounds in the crude extract ADI95-17NCVEX were determined by high-resolution mass spectrometry (Results 4.13, Table 10). After comparing the masses and the sum formulas to the DNP, a distinction between potentially known and unknown natural products was possible (Results 4.13, Table 11). For some compounds the hits in the DNP could be identified as most likely true hits because they could be linked to a certain BGC of *Streptomyces* ADI95-17.

For the sum formula $C_{10}H_{13}NO_2$, a small compound with a molecular weight of 179 g/mol, was tentatively identified as 1,7a-Dihydro-7-hydroxy Abikoviromycin, a substance that exhibits antiviral activity known to be produced by *Streptomyces* sp. SANK 65986⁸⁵. Most likely, its production is specified by PKS type I cluster number 25 (Figure 56).

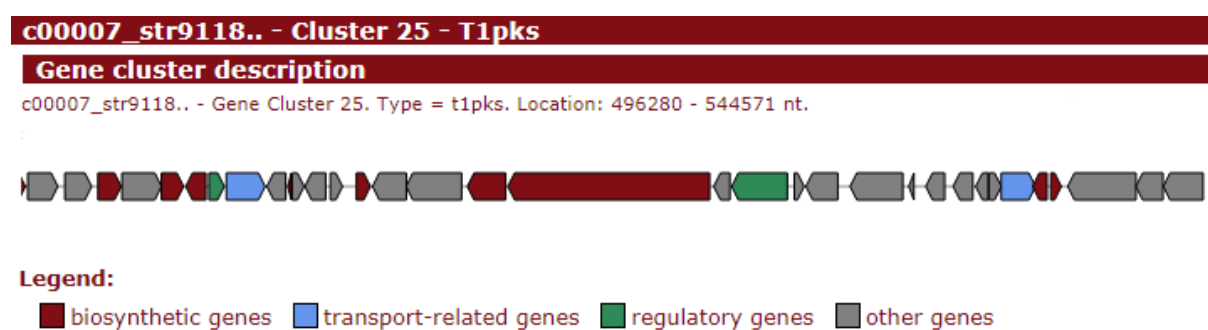


Figure 56: Cluster 25 from *Streptomyces* ADI95-17, a PKS I is most probable responsible for the production of 1,7-Dihydro-7-hydroxy Abikoviromycin, an antimicrobial substance produced by *Streptomyces* sp. SANK 65986⁸⁵.

8β-Hydroxy Bostrycin was one of the putative compounds, which could be identified by searching for its sum formula ($C_{16}H_{16}O_9$) in the DNP. This substance is not known to be produced by *Streptomyces* species, however, Bostrycin is known to inhibit the growth of *Saccharomyces cerevisiae*.⁹² Thus, it is possible that this compound or its analogue is also produced by *Streptomyces* ADI95-17 wild-type and mutants, which were shown to inhibit the growth of the test organism *Saccharomyces cerevisiae* in this project (Results 4.11, Figure 50).

The biosynthetic gene cluster number 6, a T2pks from *Streptomyces* ADI95-17, from which 2 promoter regions were chosen for this project, is most likely the one expected to be responsible for the production of 8 β -Hydroxy Bostrycin. This cluster shows a 42% similarity to the R1128 BGC, responsible for the production of R1128, an 1,3,6-trihydroxy-8-n-butylantraquinone with structural similarities to Bostrycin ((1S)-1,2,3,4-Tetrahydro-1,2 β ,3 β ,5,8-pentahydroxy-6-methoxy-3-methyl-9,10-anthraquinone) (Figure 57).

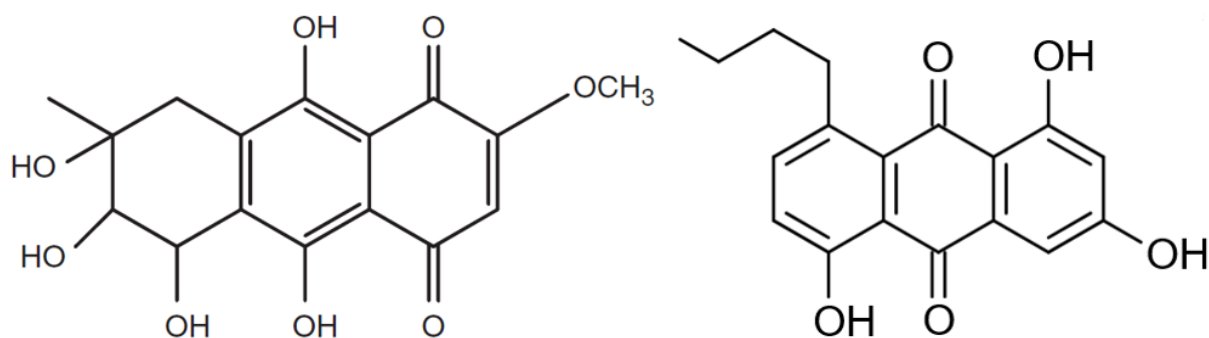


Figure 57: Comparison of the structures 8 β -Hydroxy Bostrycin (left side), probably produced by BGC number 6, a T2pks, from *Streptomyces* ADI95-17. R1128 A (right side) is an anthraquinone produced by the R1128 BGC, which shows 42% gene similarity compared to cluster 6 from *Streptomyces* ADI95-17.

Another explanation for the bioactivity of *Streptomyces* ADI95-17 extracts against *Saccharomyces cerevisiae* could be the compound cycloheximide, a protein synthesis inhibitor of eukaryotic cells, known to be produced by *Streptomyces griseus*.⁹³ The antiSMASH analyzes identified cluster 11, a Transatpks, as responsible for the production of cycloheximide, but somehow this compound could not be identified by mass spectrometry.

Two compounds, Antibiotic WS 7338-A and Antibiotic WS 7338-D, cyclic pentapeptides known as endothelin receptor antagonists isolated from *Streptomyces* sp. No. 7338⁹⁴, could be found in the DNP. Because the amino acid sequence of both peptides is known, the BGC responsible for their production could tentatively be identified. The amino acids of which Antibiotic WS 7338-A and D are composed are -D-Val-L-Leu-D-Trp-D-Glu-L-Ala- and -D-Val-L-Val-D-Trp-D-Glu-L-Ala-, respectively. BGC number 24, a NRPS cluster from *Streptomyces* ADI95-17 (Figure 58), is most likely the one responsible for the production of these pentapeptides, because it is the only cluster with five predicted adenylation domains. Due to a poor sequence quality and a low accuracy of the specificity prediction for the A-domains, it was difficult to be sure about this link. A more accurate DNA sequence of this strain and a more precise prediction of gene clusters by enhanced versions of antiSMASH could lead to an optimized result. A further identification, purification and analyzes of the compounds in the extract from ADI95-17NCVEX would be necessary to verify these suggestions.

The other compounds masses of which were determined by MS could not be matched with any known compounds in the DNP database. Therefore, they may be novel and thus worth investigating further by purification and structure elucidation.

c00006_str9118.. - Cluster 24 - Nrps

Gene cluster description

c00006_str9118.. - Gene Cluster 24. Type = nrps. Location: 684575 - 735479 nt. Click on genes for more information.

Show pHMM detection rules used



Legend:

■ biosynthetic genes ■ transport-related genes ■ regulatory genes ■ other genes

Detailed annotation

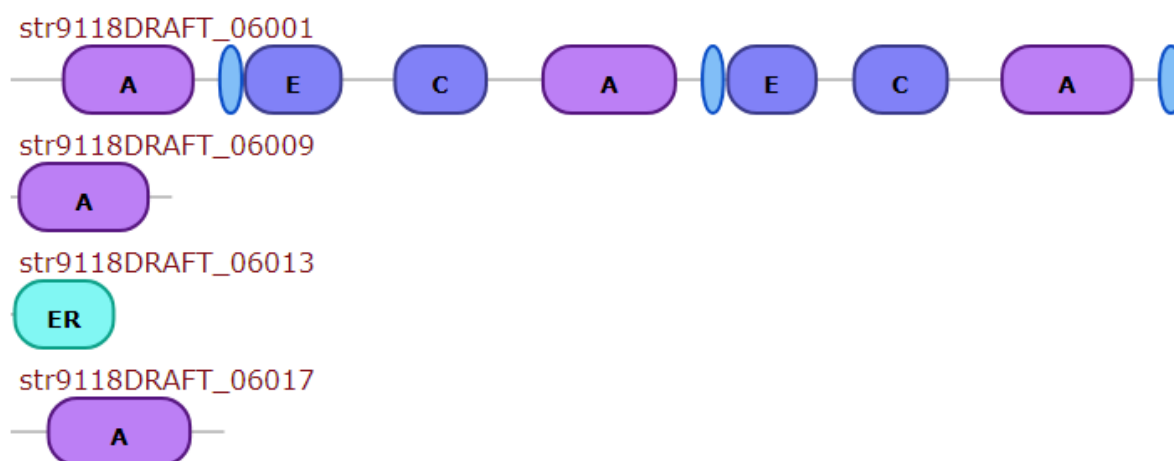


Figure 58: *Streptomyces* ADI95-17 BGC number 24, a NRPS cluster. This NRPS is most probable responsible for the production of the pentapeptides Antibiotic WS 7338-A and D, known endothelin receptor antagonists. Because the sequence quality of this cluster is very poor and the prediction of the adenylation domains specificity is not very accurate, it is hard to say for sure if this cluster is responsible for the production of the compounds Antibiotic WS 7338-A and D.

6. Material

6.1. Organisms and Vectors

6.1.1. Streptomyces

The following marine *Streptomyces* strains were used and genetically manipulated for this project.

Table 12: *Streptomyces* strains and mutants used and created during this project.

Strain	Properties	Source
ADI95-17	WT	provided by the lab from marine sponge: <i>Antho dichotoma</i>
ADI95-17PCV	ADI95-17 harboring pPCV	this project
ADI95-17NCV	ADI95-17 harboring pNCV	
ADI95-17C6.1	ADI95-17 harboring pADIC6.1	
ADI95-17C6.2	ADI95-17 harboring pADIC6.2	
ADI95-17C29.1	ADI95-17 harboring pADIC29.1	
ADI95-17C29.2	ADI95-17 harboring pADIC29.2	
GBA94-01	WT	provided by the lab from marine sponge: <i>Geodia barretti</i>
GBA94-01PCV	GBA94-01 harboring pPCV	this project
GBA94-01NCV	GBA94-01 harboring pNCV	
GBA94-01C6.1	GBA94-01 harboring pGBAC6.1	
GBA94-01C6.2	GBA94-01 harboring pGBAC6.2	
GBA94-01C16.1	GBA94-01 harboring pGBAC16.1	
GBA94-01C16.2	GBA94-01 harboring pGBAC16.2	
GBA94-01C24.1	GBA94-01 harboring pGBAC24.1	
GBA94-01C24.2	GBA94-01 harboring pGBAC24.2	
PVA91-01	WT	provided by the lab from marine sponge: <i>Phakellia ventilabrum</i>
PVA91-01PCV	PVA91-01 harboring pPCV	this project
PVA91-01NCV	PVA91-01 harboring pNCV	

6.1.2. Escherichia coli

Following *E. coli* strains were used for cloning and conjugation:

- *E. coli* DH5 α , able for blue/white color selection (F- Φ 80 lacZ Δ M15 Δ (lacZYA-argF)), general recombination deficient (*recA1*) and endonuclease I deficient (*endA1*), other features: U169 *deoR*, *hsdR17* (rk-, mk+) *phoA supE44* λ -*thi-1* *gyrA96 relA1*⁹⁵
- *E. coli* ET12567 (pUZ8002), methylation deficient (*dam*-, *dcm*-, *hsdM*-), mediates conjugative DNA transfer from RP4 *oriT* with helper plasmid pUZ8002 (Kan^R, Cml^R)⁹⁶

Table 13: *E. coli* strains and the vectors they carry, which were used and created in this project.

E. coli Strain	Vector	Source
DH5α	pTSA105	provided by the lab
DH5α	pGEM3Zf	
DH5α	pSOK804	
ET12567	pSOK804	
DH5α	pKC1218	this project
DH5α	pGEB101	
DH5α	pPCV	
ET12567	pPCV	
DH5α	pNCV	
ET12567	pNCV	
DH5α	pADI95-17C6.1	
ET12567	pADI95-17C6.1	
DH5α	pADI95-17C6.2	
ET12567	pADI95-17C6.2	
DH5α	pADI95-17C29.1	
ET12567	pADI95-17C29.1	
DH5α	pADI95-17C29.2	
ET12567	pADI95-17C29.2	
DH5α	pGBA94-01C6.1	
ET12567	pGBA94-01C6.1	
DH5α	pGBA94-01C6.2	
ET12567	pGBA94-01C6.2	
DH5α	pGBA94-01C16.1	
ET12567	pGBA94-01C16.1	
DH5α	pGBA94-01C16.2	
ET12567	pGBA94-01C16.2	
DH5α	pGBA94-01C24.1	
ET12567	pGBA94-01C24.1	
DH5α	pGBA94-01C24.2	
ET12567	pGBA94-01C24.2	
DH5α	pADI95-17C6KO	
ET12567	pADI95-17C6KO	
DH5α	pADI95-17C29KO	
ET12567	pADI95-17C29KO	

6.1.3. Bioassay Test Organisms

Microorganisms shown in table were used for bioactivity testing.

Table 14: Strains of microorganisms used for bioactivity testing.

Microorganism	Test against	Source
<i>Pseudomonas fluorescens</i>	gram negative bacteria	provided by the lab
<i>Escherichia coli</i>	gram negative bacteria	
<i>Bacillus subtilis</i>	gram positive bacteria	
<i>Saccharomyces cerevisiae</i>	fungi	
<i>Aspergillus niger</i>	filamentous fungi	
<i>Fusarium graminearum</i>	filamentous fungi	

6.1.4. Vectors

Table 15: Vectors used in this project.

Vector (Plasmid)	Genotype	Function	Length	Source
pTSA105	<i>bpsA, hygR, lacZa`, minimal SCP2* replicon, oriT, oriV, ter</i>	used for subcloning (to gain <i>bpsA</i> fragment)	9989bps	provided by the lab
pGEM3Zf	<i>AmpR</i>	used for subcloning	3199bps	
pGEB101	<i>AmpR, bpsA, ter</i>	used for cloning	7132bps	
pSOK804	<i>AmR, attP, ColEI ori, int, lacZa`, RP4 oriT</i>	used for cloning of promoter probe vectors	5300bps	
pKC1218	<i>hygR, lacZa, minimal SCP2* replicon, oriT, oriV</i>	used for cloning of knock-out vectors	5845bps	
pPCV	<i>AmR, attP, bpsA, ColEI ori, ermE p*, int, lacZa``, RP4 oriT, ter</i>	positive control vector	9426bps	this project
pNCV	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, ter</i>	negative control vector	9637bps	
pADI95-17C6.1	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, promoter ADIC6.1, ter</i>	promoter probe vector	9523bps	
pADI95-17C6.2	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, promoter ADIC6.2, ter</i>	promoter probe vector	9583bps	
pADI95-17C29.1	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, promoter ADIC29.1, ter</i>	promoter probe vector	9556bps	
pADI95-17C29.2	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, promoter ADIC29.1, ter</i>	promoter probe vector	9713bps	
pGBAC6.1	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, promoter GBAC6.1, ter</i>	promoter probe vector	9530bps	
pGBAC6.2	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, promoter GBAC6.2, ter</i>	promoter probe vector	9807bps	
pGBAC16.1	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, promoter GBAC16.1, ter</i>	promoter probe vector	9553bps	
pGBAC16.2	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, promoter GBAC16.2, ter</i>	promoter probe vector	9573bps	
pGBAC24.1	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, promoter GBAC24.1, ter</i>	promoter probe vector	9742bps	
pGBAC24.2	<i>AmR, attP, bpsA, ColEI ori, int, lacZa``, RP4 oriT, promoter GBAC24.2, ter</i>	promoter probe vector	9797bps	
pADI95-17C6KO	<i>hygR, knock-out ADIC6.2, lacZa, minimal SCP2* replicon, oriT, oriV</i>	knock-out vector	4529bps	
pADI95-17C29KO	<i>hygR, knock-out ADIC29.2, lacZa, minimal SCP2* replicon, oriT, oriV</i>	knock-out vector	4624bps	

6.2. Media, Solutions and Buffers

6.2.1. Media used for *Streptomyces*

Preparation of media can be found at 7.2.1.

- Solid media used for growth:

SFM medium⁸²

Mannitol	20g
Soy flour	20g
Agar	20g
H ₂ O	up to 1000ml

ISP4 medium⁸²

Soluble starch	10g
Dipotassium phosphate	1g
Magnesium sulfate	1g
Sodium chloride	1g
Ammonium sulfate	2g
Calcium carbonate	2g
Ferrous sulfate	1g
Manganese chloride	1g
Zinc sulfate	1g
Agar	20g
dH ₂ O	up to 1000ml

- Liquid media used for growth:

TSB medium⁸²

CASO-Bouillon	30g
dH ₂ O	up to 1000ml

2XYT medium⁸²

Difco bacto tryptone	16g
Difco bacto yeast extract	10g
Sodium chloride	5g
dH ₂ O	up to 1000ml

- Fermentation media:

R5A medium⁹⁷

Sucrose	100g
K ₂ SO ₄	0.25g
MgCl ₂	10.2g
Glucose	10g
Casamino acids	0.1g
Yeast extract	5g
MOPS	21g
Trace Elements Solution	2ml
dH ₂ O	up to 1000ml
pH: approximately 6.8 before autoclaving	

Simocyclinone fermentation medium⁹⁷

Glutamine	1.46g
Starch	25g
NaCl	1g
KH ₂ PO ₄	1g
MgSO ₄	0.5g
Trace Elements Solution	2ml
dH ₂ O	up to 1000ml
pH: approximately 5.6-5.7 before autoclaving	

SM3 medium⁸²

Glucose	5g
Maltodextrin	50g
Soy flour	25g
Molasses (beet)	3g
KH ₂ PO ₄	0.25g
CaCO ₃	2.5g
dH ₂ O	up to 1000ml
pH: approximately 7.0 before autoclaving	

SM10 medium⁸²

MOPS	20.9g
L-Proline	10.5g
Glycerol	23g
NaCl	0.5g
KH ₂ PO ₄	2.1g
Na ₂ SO ₄	0.28g
0,02M MgSO ₄	10ml (0.49g/100ml)
0,02M CaCl ₂	10ml (0.29g/100ml)
Trace Elements Solution	5ml
dH ₂ O	up to 1000ml
pH: approximately 6.5 before autoclaving	

SM 12 medium⁸²

Soy flour	10g
Glucose	50g
Peptone (Difco)	4g
LAB- Lemco (Oxoid)	4g
Yeast extract	1g
NaCl	2.5g
CaCO ₃	5g
dH ₂ O	up to 1000ml
pH: approximately 7.6 before autoclaving	

SM17 medium⁸²

Glucose	2g
Glycerol	40g
Soluble starch	2g
Soy flour	5g
Peptone	5g
Yeast extract	5g
Sodium chloride	5g
Calcium carbonate	2g
H ₂ O	up to 1000ml
pH: approximately 6.4 before autoclaving	

6.2.2. Media for *E. coli* and Bioassay Test Organisms

Preparation of media can be found at 7.2.1.

L Agar

Agar	10g
Difco bacto tryptone	10g
Difco yeast extract	5g
Sodium chloride	5g
dH ₂ O	up to 1000ml

L Broth

Difco bacto tryptone	10g
Difco yeast extract	5g
Sodium chloride	5g
dH ₂ O	up to 1000ml

Potato Glucose Agar (PDA)

PDA powder (Sigma Aldrich®)	39g
dH ₂ O	up to 1000ml

YPD Medium

Yeast extract	10g
Peptone	20g
Glucose	20g
Agar	15g
dH ₂ O	up to 1000ml

6.2.3. Stock Solutions and Buffers

- Antibiotic stock solutions

The antibiotic stock solutions, except chloramphenicol, were filter sterilized using a 0.22µm filter and stored as 500µl aliquots at -20°C.

Table 16: Antibiotic stock solutions.

Antibiotic	Concentration (stock) [mg/ml]	Concentration (medium) [µg/ml]	Solvent
Apramycin (AM)	100	50-100	dsH ₂ O
Chloramphenicol (CML)	30	30	ethanol (96%)
Hygromycin (HYG)	100	50-100	dsH ₂ O
Kanamycin (KAN)	40	20	dsH ₂ O
Nalidixic acid (NAL)	30	30	NaOH (0,1M)

- Solution for preparation of cell suspensions:

Glycerol (20%) stock

Glycerol	20ml
dH ₂ O	80ml

- Solutions for conjugation:

MgCl₂ (1M) stock

Magnesium chloride	95.2g
dH ₂ O	up to 1000ml

CaCl₂ (0.6M) stock

Calcium chloride	66.6g
dH ₂ O	up to 1000ml

- Solution for preparation of media:

Trace elements solution

FeSO ₄ .7H ₂ O	5000mg
CuSO ₄ .5H ₂ O	390mg
ZnSO ₄ .7H ₂ O	440mg
MnSO ₄ .H ₂ O	150mg
Na ₂ MoO ₄ .2H ₂ O	10mg
CoCl ₂ .6H ₂ O	20mg
1M HCl	50ml
dH ₂ O	up to 1000ml

- Solution for preparation of competent cells:

TSS buffer

PEG 8000	5g
1M Magnesium chloride	1.5ml
DMSO	2.5ml
LB medium	up to 50ml

- Solutions used for gel electrophoresis:

TBE buffer (10x)

Tris base	108g
Boric acid	55g
0.5M EDTA (pH 8.0)	51ml
dH ₂ O	up to 1000ml

Agarose gel (0.8%) for electrophoresis

TBE buffer (1x)	400ml
Agarose	3.2g
Gel Red (10.000x)	20µl

The gel was stored at 60°C to stay liquid until the gel was cast.

6.3. Primers

How primers were designed can be found at 7.4.3 and a detailed list of all primers can be found in Results 4.4.

6.4. Kits

Genomic DNA extraction

Wizard® SV Genomic DNA Purification System (Promega, enhanced)

Isolation of plasmid DNA

Wizard® Plus SV Minipreps DNA Purification System (Promega)

Purification of DNA

DNA Clean & Concentrator™-5 (Zymo Research)

DNA recovery from gel

Zymoclean™ Gel DNA Recovery Kit (Zymo Research)

PCR kits

Q5® High-Fidelity 2X Master Mix (NEB)

GC-rich Kit (Roche)

7. Methods

7.1. *In Silico* Methods

7.1.1. Bioinformatic Genome Analysis by antiSMASH

AntiSMASH⁵⁴ is a bioinformatic tool, which can predict bacterial gene clusters in genome sequences. The draft genome sequences of *Streptomyces* strains used in this project were uploaded to the antiSMASH server and analyzed for gene clusters by this software.

The online version is free available at <https://antismash.secondarymetabolites.org/>.

The output is a list of all detected clusters and the functions of the single genes, thus the type of the encoded enzymes. Predicted biosynthetic genes and mainly core genes were verified by BLAST⁹⁸ searches on the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

7.1.2. Software used in this Project

Software	Usage
BPROM	With this bacterial promoter recognition software, the uploaded genomic regions were analyzed for promoter regions. The free online version is available at http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb
Clone Manager	Clone Manager is a program used in this project for <i>in-silico</i> construction of vector maps, for cloning simulations of vectors with restriction enzymes and for primer design.
Image Lab™	The software Image Lab™ was used to check and process images of gels after gel electrophoresis (restriction analyses, PCR-products, quality of genomic DNA).
Lab Solutions	In this project, the software Lab Solutions was used to analyze HPLC- chromatograms.
Bruker Compass Data Analysis 4.2	This software was used to analyze mass spectra to calculate masses and the chemical sum formulas based on mass accuracy and isotopic pattern matching (SmartFormula algorithm).

Table 17: Software used in this project.

7.2. Preparation of Media and Antibiotic Stock Solutions

7.2.1. Preparation of Media

All media were autoclaved (121°C, 20min) and stored at room temperature after preparation. Prepared plates were stored at +4°C. Media, which contained agar was melt before usage by using a microwave oven. Media recipes can be found at Material 6.2.

7.2.2. Preparation of Antibiotic Stock Solutions

Antibiotics were weighed and dissolved by vortexing in a certain liquid (dH₂O, ethanol or 0,1M NaOH). Then the solution was sterile filtered and stored as aliquots in a freezer at -20°C.

For concentrations of antibiotic stock solutions see Material 6.2.3.

7.3. Cultivation and Storage of *Streptomyces* and *E. coli*

7.3.1. Cultivation and Storage of *Streptomyces*

For growth of *Streptomyces* strains, 100-200µl of spore suspension were spread on 10cm plates containing 30ml of a certain medium (Table 18). To grow recombinant strains, carrying an antibiotic resistance gene, the medium was supplemented with 0.5µl Am stock solution per milliliter. Plates were incubated at 28°C for 4-7 days and the spores were gained by preparing a spore suspension.

Table 18: Media used for sporulation of *Streptomyces*.

Strain	Medium used for growth
ADI95-17	ISP4
GBA94-01	ISP4
PVA91-01	SFM

- Preparation of *Streptomyces* spore suspensions:

5ml glycerol (20%) were pipetted onto a plate containing sporulated *Streptomyces*.

The surface of the plate was gently scratched with the tip of a pipette to suspend the spores in glycerol. The suspension was filtered through a sterile syringe containing cotton wool into a sterile tube to get rid of the mycelium. Aliquots of the resulting spore suspension were stored in Cryo tubes at -80°C.

7.3.2. Cultivation of *Streptomyces* Strains for total DNA Isolation

To cultivate *Streptomyces* for total DNA isolation, a 100ml flask, containing 10ml TSB medium, was inoculated with 50-100µl of spore suspension and then incubated at 200rpm and 28°C for 24h. 1ml of the culture was used for DNA isolation.

7.3.3. Fermentation Cultures of *Streptomyces* Strains

To start a seeding culture, 50-100µl spore suspension were transferred to a 250ml flask, containing 25 or 50ml of TSB Medium, and were incubated at 200rpm and 28°C for 24h. (incubator: Infors HT Multitron Pro, Infors AG) A 250ml flask, containing 50ml of a certain fermentation medium, was inoculated with 3ml of the resulting culture and incubated for 200rpm and 28°C for 5 to 14 days. During growth, the color of the cultures had been observed. If a color-change appeared and the amount of biomaterial was high enough, the fermentation cultures were harvested and stored at -20°C or immediately extracted according to the procedure described in 7.6.1.

- Ethanol-shock

After one day of growth in fermentation media, ethanol-shock was performed to try to trigger promoter activity and the resulting secondary metabolite production. 6% ethanol (96%) was added to 50ml of fermentation medium. (= 3ml)

7.3.4. Cultivation and Storage of *E. coli*

For cultivation of *E. coli*, 100µl of *E. coli* cell suspension were spread on a 10cm plate containing 25ml of LA medium and appropriate antibiotics if needed (Table 19).

After 24h of incubation at 37°C, a single colony was picked using a toothpick and transferred to a culture vessel containing 2ml of LB medium and appropriate antibiotics if needed for propagation. After 24h of incubation at 200rpm and 37°C, the culture was either used to prepare a cell suspension or for plasmid DNA isolation.

- **Preparation of *E. coli* Cell Suspension:**

After 24h of propagation at 200rpm and 37°C, 1.5ml of LB medium, containing cells of a single clone, was centrifuged at 13.400rpm for 5minutes to gain a cell pellet.

The supernatant was removed, and the pellet was resuspended in 1500µl glycerol (20%) and stored in Cryo tubes at -80°C.

Table 19: Volumes of antibiotic stock solutions used to grow *E. coli*.

Wild type strains	Vol. of antibiotics stock solution per 1ml of medium
<i>E. coli</i> DH5α	no antibiotics
<i>E. coli</i> ET12567	1µl CML, 0.5µl KAN
Transformed strains	Vol. of antibiotics stock solution per 1ml of medium
<i>E. coli</i> DH5α	1µl AM for cells harboring promoter probe vectors
<i>E. coli</i> DH5α	1µl HYG for cells harboring knock-out vectors
<i>E. coli</i> ET12567	1µl AM, 1µl CML, 0.5µl KAN for cells harboring promoter probe vectors
<i>E. coli</i> ET12567	1µl HYG, 1µl CML, 0.5µl KAN for cells harboring knock-out vectors

7.3.5. Cultivation and Storage of Bioassay Test Organisms

Test organisms were grown over night in 10-20ml media at a certain temperature (Table 20), except *Aspergillus* and *Fusarium*, which were only grown on solid media (PDA) at room temperature. For preparing stocks, cells were pelleted at 13.400rpm for 5min, resuspended in 20% glycerol and stored at -80°C after separation in aliquots in Cryo tubes to 1ml.

The filamentous fungi were scratched from the plate and suspended in glycerol before freezing.

Table 20: Media and incubation temperature of microbial test organisms.

Test organism	Media	T°C
<i>Pseudomonas fluorescens</i>	LA	28°C
<i>Escherichia coli</i>	LA	28°C
<i>Bacillus subtilis</i>	LA	37°C
<i>Saccharomyces cerevisiae</i>	YPD	37°C
<i>Aspergillus niger</i>	PDA	room temperature
<i>Fusarium graminearum</i>	PDA	room temperature

7.4. Molecular Biological Methods

7.4.1. Genomic DNA Isolation from *Streptomyces* Strains

Genomic DNA was isolated with Wizard® SV Genomic DNA Purification System (Promega, enhanced) and used as template DNA for PCR.

- 1) 1ml overnight culture was pelleted, resuspended in 800µl dsH₂O and divided into two.
- 2) The two 400µl solutions were then pelleted again. One pellet was used for DNA isolation, the other one was kept as backup at -20°C to use it if DNA extraction was not successful.
- 3) The pellet was resuspended in 275µl of digestion solution master mix and incubated at 55°C for 60min.

Table 21: Recipe of digestion solution master mix for the isolation of genomic DNA.

Component	Volume [µl]
Nuclei Lysis Solution	200
0.5M EDTA (pH 8.0)	50
Alkaline Protease Solution*	20
RNase A Solution, 4mg/ml	5
Total volume	275

*from the Wizard® Plus SV Minipreps Purification System

- 4) 250µl Wizard® SV Lysis Buffer were added to each sample and the warm lysate was processed as soon as possible.
- 5) Lysates were transferred to a collection tube containing a minicolumn and spun down at 13.400rpm for 3min. The liquid in the collection tubes was discarded and the columns were washed with 650µl Column Wash Solution. This step was repeated once.
- 6) The liquid in the collection tube was discarded, the assembly was centrifuged at 13.400rpm for 2min to dry the binding matrix and then the minicolumn was transferred to a 1.5ml Eppendorf tube.
- 7) After adding 100µl Nuclease-free water, the tube-column assembly was incubated at room temperature for 2min and finally eluted by centrifugation at 13.400rpm for 1min. The resulting genomic DNA was stored at -20°C.

7.4.2. Plasmid DNA Isolation from *E. coli*

For isolation of plasmid DNA from *E. coli*, 2ml overnight culture were pelleted. The pellet was resuspended, lysed and neutralized before washing and the final elution step. After isolation, plasmid DNA was stored at -20°C. For details see protocol Wizard® Plus SV Minipreps Purification System.

7.4.3. Primer Design

The software Clone Manager was used to design primers and all of them were ordered from Eurofins Genomics. Primers were designed to generate PCR-products (promoter regions and genes used for a knock-out), which were inserted in integrative plasmids e.g. pSOK804, which can be used as vectors. Primers for amplification of promoter regions by PCR and of genes to knock-out were designed with an average length of 17-20bps. All primers were adjusted to a melting temperature (T_m) between 60-75°C and primer pairs to a maximum difference of T_m of 5°C. Certain restriction sites for endonucleases (*Sfi*I in forward primers for promoter regions, *Eco*RI in reverse primers for promoter regions, *Age*I in forward primers for knock-out PCR-products and *Eco*RI in reverse primers for knock-out PCR-products) were added at the 5' end to enable molecular cloning of vector molecules, which were cut by the same restriction enzymes as the PCR-products, so that they could be joined together by their compatible ends. Additionally, a docking site consisting of 4 base pairs (GATC) was added at the 5' end to each primer for an easier attachment of restriction endonucleases to the resulting PCR-products. Therefore, the total length of forward primers ranged from 26bps - 37bps and for reverse primers from 28bps - 33bps. PCR-products of promoter regions ranged from 176bps to 461bps in size. Knock-out PCR-products, which covered over 75% of the target genes sequence, without the start codon and with the shortest possible distance to it, were about 800bps in size. A detailed list of all primers can be found in Results 4.4.

7.4.4. PCR

2 Kits (GC-rich PCR System (Roche) and Q5® High-Fidelity 2x Master Mix (NEB)) were used to perform PCR-reactions. Table 22 and 23 show the composition and Table 24 the program used for PCR-reactions, carried out with a Master Cycler Nexus X2 from Eppendorf®.

During the addition of the single components, the reaction tubes were kept on an Eppendorf® PCR Cooler to stay cold to prevent unspecific binding, which can appear at room temperature leading to wrong products. All PCR reactions were stored at -20°C.

Table 22: Setup of PCR-reactions when GC-rich PCR System (Roche) was used.

Component	Volume [μl]
Template DNA	1
Forward primer (10pm/μl)	0.5
Reverse primer (10pm/μl)	0.5
Resolution solution (5x)	8
GC-rich buffer (5x)	8
dNTP's (10mM)	1
Polymerase (expand high-fidelity)	0.7
dsH ₂ O	20.3
Total reaction volume	40

Table 23: Setup of PCR-reactions when Q5® High-Fidelity 2x Master Mix (NEB) was used.

Component	Volume [μl]
Template DNA	1
Forward primer (10pm/μl)	1
Reverse primer (10pm/μl)	1
Master mix (2x)	12.5
Nuclease free water	9.5
Total reaction volume	25

Table 24: Program used for PCR-reactions. The steps denaturation, annealing and elongation ran in cycles to allow the steps to be repeated 25-30 times.

Stage	Temperature [°C]	Time [sec]
Initial denaturation	98	10
Denaturation	95	30
Annealing	60	30
Elongation	72	30
Final extension	72	120
Hold	4	∞

7.4.5. PCR-product Purification

Before an insertion of a fragment, cut from a plasmid, or of a PCR-product in a vector molecule by ligation, the products needed to be purified whether directly from the reaction tube or from the gel after electrophoresis. All PCR-products were stored at -20°C.

- Purification from total reaction volume:
PCR products were purified after analyzing 1-2μl of the total reaction volume (40μl) on a gel. For detailed procedure see protocol of DNA Clean & Concentrator™ (Zymo Research).
- Purification from the gel:
If a DNA fragment was cut from the gel after restriction digestion, the fragment was purified with Zymoclean™ Gel DNA Recovery Kit (Zymo Research). See protocol for detailed procedure.

7.4.6. Cloning

- Restriction digestion

Restriction digestions were performed with restriction endonucleases whether to cut the vector molecules and DNA fragments to be compatible for ligation or for restriction analysis of constructed vector molecules after cloning steps.

Isolated plasmids and purified PCR products were incubated at 37°C in a water bath or in the PCR device with certain restriction enzymes. After cutting, enzymes were inactivated by heating to a certain temperature (e.g. *EcoRI* 65°C, *HindIII* 80°C) for 20min.

Table 25: Recipes for restriction digestions.

Substance	Plasmid DNA [µl]	PCR-product [µl]
DNA	2-8	5-10
enzyme 1	1	1
enzyme 2	1	1
Cut Smart buffer (10x)	3	3
dsH ₂ O	15-23	10-15
total volume	30	30

- Ligation of vector and PCR-product

After a finished restriction digestion, the vector molecule and the fragment for insertion could be joined together by a ligation reaction. First, the concentration of vector and fragment were examined by gel electrophoresis. Then vector and fragment were used in a concentration ratio of about 1:3-5 for the reaction. The ligation reactions took place in 1.5ml Eppendorf® tubes in a rack, which was incubated on an ice box overnight.

Table 26: Recipe for ligation of vector and insert fragment.

Substance	Volume [µl]
plasmid DNA (cut)	1-3
PCR- product (cut)	5-8
T4-DNA ligase	1
T4-DNA ligase buffer	2
dsH ₂ O	6-11
total volume	20

7.4.7. Preparation of Competent Cells

Competent *E. coli* cells were prepared to introduce the constructed plasmids into those *E. coli* strains by transformation. The cells were either used for cloning (DH5α) or conjugation purposes (ET12567).

- 1) 0.5ml of 10ml overnight culture were transferred to a flask containing 50ml LB medium (1:100 dilution).
- 2) After 1-2h the OD₆₀₀ of the cultures was measured by UV-Vis spectrophotometry (LB medium as BLANK) and ranged between 0.4 – 0.6. After reaching this density, the culture was divided and transferred to 50ml falcon tubes.
- 3) The tubes were kept on ice for 10min and centrifuged at 3000rpm for 10min at 4°C.
- 4) The supernatant was removed, and the cell pellet was resuspended in 2.5ml ice cold TSS buffer each.
- 5) 100µl aliquots of the resulting suspension were pipetted fast into ice cold 1.5ml Eppendorf® tubes and immediately frozen in liquid nitrogen before storing in -80°C.⁹⁹

7.4.8. Transformation of *E. coli*

- 1) 1µl of plasmid DNA or 10µl of ligation product were added to slowly on ice melted competent *E. coli* cells in Eppendorf® tubes and softly stirred with the tip of the pipette.
- 2) After keeping the tube on ice for 15min, the cells were heat shocked for 1min at 42°C on a heater (AccuBlock™).
- 3) Then they were instantly transferred back on ice to recover for 5min and incubated at 37°C for 1-2h after adding 800µl LB media.
- 4) Twice, 100-200µl of LB media were pipetted on a dish containing LA media and the corresponding antibiotics to grow transformed *E. coli* cells. (see 7.3.4 for cultivation of *E. coli* cells)
- 5) The plates were incubated overnight at 37°C and clones could be picked the next day.

7.4.9. Conjugation to *Streptomyces*

E. coli ET12567 cells harboring a correct vector construct (Material 6.1.2) were used to transfer plasmid DNA to *Streptomyces* strains via conjugation. This strain allows mobilization of any plasmid carrying RK2 (RP4) *oriT* into the recipient. *E. coli* ET12567 is a methylation deficient host and harbors the plasmid pUZ8002 containing a function for intergeneric transfer, thus able to introduce foreign DNA into *Streptomyces*.

- Conjugation

- 1) For conjugation approximately 2cm² of the surface of a plate containing LA medium and the corresponding antibiotics (Methods 7.3.4) was scratched to get *E. coli* cells, which were suspended in 500µl 2XYT medium.
- 2) 50-80µl of *Streptomyces* spore suspension (Methods 7.3.1) was added to 350µl 2XYT medium, vortexed and heat shocked at 50°C for 5min to germinate the spores.

- 3) After allowing the mixture to cool down at room temperature, 100µl of *E. coli* ET12567 cell suspension in 2XYT was transferred to the heat shocked spore suspension.
- 4) The mixture was then pelleted at 13.400rpm for 1min and 250µl of the supernatant were removed.
- 5) The pellet was resuspended in the remaining 2XYT medium and stroke out on a plate containing 30ml SFM medium supplemented with 1M MgCl₂.
- 6) The plate was incubated at 28°C for about 24h and then antibiotic selection was performed.
- 7) 1ml dH₂O containing AM (selection for transconjugants) and NAL (selection against *E. coli*) (0.5µl AM stock solution and 1µl NAL stock solution per 1ml of SFM medium) was pipetted on the plate and carefully spread on its surface.
- 8) After allowing the plate to dry for about 20min, the incubation at 28°C was continued for about 4-7 days until transconjugants appeared.
- 9) Single transconjugants were then transferred to a SFM plate containing AM and NAL (0.5µl AM stock solution and 1µl NAL stock solution per 1ml of SFM medium) using a toothpick.
- 10) After this second selection, spores from a single clone grown on the plate were spread on a plate containing SFM medium supplemented with AM (0.5µl AM stock solution per 1ml of medium) and incubated at 28°C for 4-7 days.
- 11) When the number of spores was sufficient on a plate, they were harvested by preparing a spore suspension.

7.5. Analytical and Preparative Gel Electrophoresis

Gel electrophoresis was performed to do restriction analysis, to check the integrity of isolated DNA and to isolate PCR-products or vector fragments after restriction digestions from the gel. Preparation of agarose gels (0,8%) can be found at Material 6.2.3. After a gel was cast, it was allowed to cool down to get solid for some minutes. Then the comb was removed, and the samples could be applied. First, the reference ladder (Figure 57) was loaded in the left chamber, which gives the possibility to identify the sizes of the separated DNA molecules. Before the samples were applied, they were staggered with blue or purple gel loading dye (6x) from Thermo Scientific™, which made it possible to see how far the samples have already run during an electrophoresis. The gels were run for 30- 180min at 80- 100V in TBE buffer (1x). Because GelRed®, a dye which interferes with DNA was added directly to the agarose gels at preparation, the UV light of the GelDoc™, a device for the analyzation of gels, made it possible to view DNA. The pictures of the gels, taken by a camera in this device, could be processed directly on the GelDoc™ or on a computer by the software Image Lab™.

Table 27: Sample volumes used for gel electrophoresis.

Sample	Volume applied for analysis [μl] (total volume [μl])	Volume applied for preparation [μl] (total volume [μl])
Genomic DNA	2 (75-100)	-
Plasmid DNA	1-2 (75-100)	-
PCR-products	2,5-4 (25-40)	20 (25-40)
Vector fragments	1-2 (20)	20 (20)

Quick-Load® 1kb Ladder **Quick-Load® 100bp Ladder**

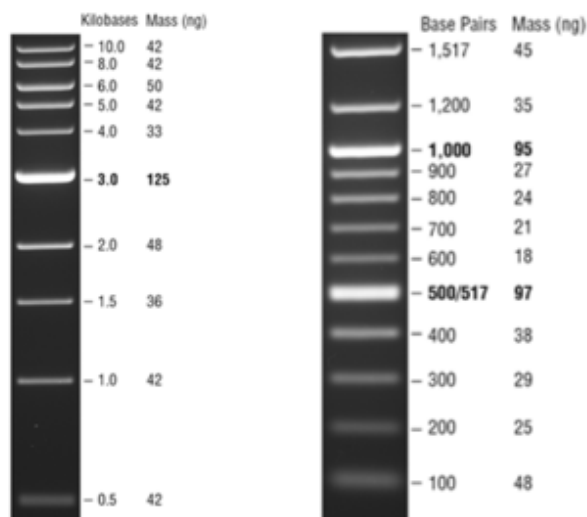


Figure 59: Reference ladders used for gel electrophoresis.

7.6. Extraction and Analysis of Secondary Metabolites

7.6.1. Extraction of SMs from Fermentation Cultures

After finishing fermentation (7.3.3), the cultures were extracted to concentrate secondary metabolites under reduced pressure.

- 1) The whole culture (50ml) was transferred to a 100ml round- bottom flask and frozen whether in the -20°C freezer overnight or on the ethanol bath.
- 2) After freezing, the flask was connected to the freeze-drier. The material was completely freeze-dried after about 24h. Half the amount of the volume of the fermentation medium (= 25ml) of solvent was used for extraction.
- 3) A mixture of methanol: dichloromethane 1:2 was used as solvent. The solvent was added to the 100ml round flask containing the freeze- dried material and incubated for 1h, shaking the flask from time to time.
- 4) The solution was then transferred to a 50ml falcon tube, briefly vortexed and centrifuged for 10min at 4.000rpm.
- 5) The supernatant was depleted to a 50ml pear- shaped flask and the solvent was evaporated on a rotary evaporator.
- 6) The residue was dissolved in 1-3ml methanol and the crude extract was kept in -20°C until further analyzation by HPLC or bioactivity testing.

7.6.2. Disc Diffusion Assay

To test antimicrobial activity of extracts, growth inhibition assays were performed. (for test organisms used in this project see Material 6.1.3)

- 1) First, labeled plates were prepared with about 4mm thick of the corresponding agar.
- 2) Then 150-300µl of test organism cell suspension was spread on the solid media, depending on the size of the plate.
- 3) In total 50µl of extract were pipetted on paper discs (2 times 20µl and 1-time 10µl, each time allowing them to dry for 10-20 minutes) before they were placed on the plates with a sterile tweezer.
- 4) 50µl of the solvent (Methanol) and 50µl of the extracted media were placed on the plates as negative controls. 10µl of 40µg/ml Kanamycin was used as a positive control in some cases.
- 5) The plates were placed with agar down and lid on the top into the incubator overnight at 28°C or 37°C.
- 6) After incubation, the discs were examined for the appearance of a surrounding zone of growth inhibition, indicating the presence of an antimicrobial substance within the extract.

7.6.3. High Performance Liquid Chromatography (HPLC)

All fermentation broth extracts were analyzed by HPLC, performed on a Shimadzu system. The column Acclaim® 120 C18, 4.6x150mm, 5µm (DIONEX, Thermo Fisher Scientific®) was used as stationary phase. Two solvents (solvent A = 0.1% aqueous solution of formic acid, solvent B = acetonitrile) were used as mobile phase with a flow rate of 0.5ml/min.

During gradient elution, solvent B was increased from 5% to 95% in 45 minutes.

After 95% of B was reached, the column was washed and re-equilibrated for 10 minutes with 5% of solvent B. The column oven temperature was set to 25°C. As detectors, a PDA- (photo diode array) detector, which scans from 190 – 800nm and an ELSD (Evaporative Light Scattering Detector) were used.

7.6.4. High Resolution Mass Spectrometry (HRMS)

Crude extracts of fermentation cultures were analyzed by HPLC combined with HRMS.

A maXis HD ESI-Qq-TOF mass spectrometer (microTOF-Q, Bruker Daltonics) was used to detect compounds from extracts in the corresponding mass spectra measured in the positive and the negative ion mode. Analysis was performed using an UltiMate 3000 RSLC-series system (DIONEX, Thermo Fisher Scientific®) coupled with a HCT 3D quadrupole ion trap mass spectrometer with an orthogonal ESI source (Bruker Daltonics). For ionization, the ESI ion source was applied with following parameters: 4500 V, nebulizer: 0.8 bar (N₂), dry gas flow: 7.0 l/min, dry temperature 200°C. As reference substances, which are used to evaluate the error of the measurement, dibutyl phthalate was used in the positive ion mode and stearic acid in the negative ion mode. The software Bruker Compass Data Analysis 4.2, which uses a SmartFormula algorithm, based on mass accuracy and isotopic pattern matching, was used to analyze mass spectra. The calculated masses and the chemical sum formulas were determined considering a mass accuracy with a maximum error of 10ppm.

The Dictionary of Natural Products, which can be found on the web under: <http://dnp.chemnetbase.com/>, were used to identify putative microbial compounds by comparing the obtained masses and sum formulas with this database.

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10. Appendix

10.1. Primers for Promoter Probe Vectors

Restriction sites are underlined. (*Sfi*I = GGCCCGGCGGGCC, *Hind*III = AAGCTT)

ADI 95-17 Cluster6 (1)

ADI 95-17 Cluster6 forward primer 1

5'-GATCGGCCCGGCGGGCCCGTCGCCTGAACCCGACGAA-3' (37)

ADI 95-17 Cluster6 reverse primer 1

5'-GATCAAAGCTTTCCGGTGCGGACGGACCG-3' (28)

→ PCR product: 177 bps (156 cut)

ADI 95-17 Cluster6 (2)

ADI 95-17 Cluster6 forward primer 2

5'-GATCGGCCCGGCGGGCCGAGCCGTCCGGGAGACCC-3' (35)

ADI 95-17 Cluster6 reverse primer 2

5'-GATCAAAGCTTGTCAGAGGCTGTTGGGTGGC-3' (30)

→ PCR product: 237 bps (216 cut)

ADI 95-17 Cluster29 (1)

ADI 95-17 Cluster29 forward primer1

5'-GATCGGCCCGGCGGGCCAACGAATTCCCTCTTCTG-3' (35)

ADI 95-17 Cluster29 reverse primer1

5'-GATCAAAGCTTCGAGTGTGGAATCAACAG-3' (28)

→ PCR product: 210 bps (189 cut)

ADI 95-17 Cluster29 (2)

ADI 95-17 Cluster29 forward primer2

5'-GATCGGCCCGGCGGGCCCGGTTCCGCATCGGCTG-3' (35)

ADI 95-17 Cluster29 reverse primer2

5'-GATCAAAGCTTTTATGGCCATGTTTGAAGGGCG-3' (32)

→ PCR product: 367 bps (346 cut)

GBA 94-01 Cluster6 (1)

GBA 94-01 Cluster6 forward primer 1

5'-GATCGGCCCGGCGGGCCGCGAGGTCGACCGTTT-3' (34)

GBA 94-01 Cluster6 reverse primer 1

5'-GATCAAGCTTGATCCGCGGACATTTCCCAG-3' (30)

→ PCR product: 184 bps (163 cut)

GBA 94-01 Cluster6 (2)

GBA 94-01 Cluster6 forward primer 2

5'-GATCGGCCCGGCGGGCCGGCACGAAGTACCCGCGG-3' (35)

GBA 94-01 Cluster6 reverse primer 2

5'-GATCAAGCTTGACCGATTGCCGCCTGCC-3' (28)

→ PCR product: 461 bps (440 cut)

GBA 94-01 Cluster16 (1)

GBA 94-01 Cluster16 forward primer 1

5'-GATCGGCCCGGCGGGCCGGACGTGAGTACGCGAGA-3' (35)

GBA 94-01 Cluster16 reverse primer 1

5'-GATCAAGCTTCGGTTCTCACGGCCCGGG-3' (28)

→ PCR product: 207 bps (186 cut)

GBA 94-01 Cluster16 (2)

GBA 94-01 Cluster16 forward primer 2

5'-GATCGGCCCGGCGGGCCACCCATCAGTCGGCGGAG-3' (35)

GBA 94-01 Cluster16 reverse primer 2

5'-GATCAAGCTTGAAAGGAATGTCCGTGTTCTGG-3' (32)

→ PCR product: 227 bps (206 cut)

GBA 94-01 Cluster24 (1)

GBA 94-01 Cluster24 forward primer 1

5'-GATCGGCCCGGCGGGCCACACGCGACTGTCGACCG-3' (35)

GBA 94-01 Cluster24 reverse primer 1

5'-GATCAAGCTTCAGCTGCCCCGAAAAGG-3' (28)

→ PCR product: 396 bps (375 cut)

GBA 94-01 Cluster24 (2)

GBA 94-01 Cluster24 forward primer 2

5'-GATCGGCCCGGCGGGCCCCGACCGTAGCGAACAC-3' (35/33)

GBA 94-01 Cluster24 reverse primer 2

5'-GATCAAGCTTGCACATCTCGATTTTTTAGATCG-3' (33/34)

GATCAAGCTTGATCGAAGAGGAAATAATACCAGC

→ PCR product: 451 bps (430 cut)

PVA 91-01 Cluster8 (1)

PVA 91-01 Cluster8 forward primer 1

5'-GATCGGCCCGGCGGGCCTGAGCCGTCGGGTGTC-3' (33)

PVA 91-01 Cluster8 reverse primer 1

5'-GATCAAGCTTGTTGTTGTCCACCGGTCC-3' (28)

→ PCR product: 269 bps (242cut)

PVA 91-01 Cluster8 (2)

PVA 91-01 Cluster8 forward primer 2

5'-GATCGGCCCGGCGGGCCCGCTCCGTC-3' (26)

PVA 91-01 Cluster8 reverse primer 2

5'-GATCAAGCTTCTCCCGTAGGCAGTGAGTGA-3' (30)

→ PCR product: 176 bps (149cut)

PVA 91-01 Cluster17

PVA 91-01 Cluster17 forward primer

5'-GATCGGCCCGGCGGGCCATGAGCCTG-3' (26)

PVA 91-01 Cluster17 reverse primer

5'-GATCAAGCTTAACAACTCCAGGTGATTCGTTGG-3' (33)

→ PCR product: 215 bps (188cut)

10.2. Primers for Knock-out Vectors

Restriction sites are underlined. (*AgeI* = ACCGGT, *EcoRI* = GAATTC)

ADI 95-17 Cluster6 knock-out

ADI 95-17 Cluster6 forward primer

5'-GATCACCGGTGAGCGGTCTGTCGCCATC-3' (28)

ADI 95-17 Cluster6 reverse primer

5'-GATCGAATTCTCCAGGACGAGCATCGCC-3' (28)

→ PCR product: 757 bps (743cut)

ADI 95-17 Cluster29 knock-out

ADI 95-17 Cluster29 forward primer

5'-GATCACCGGTGTACACCGGTGCAACCAT-3' (28)

ADI 95-17 Cluster29 reverse primer

5'-GATCGAATTCTGCACAGGTCTGGCGTGGAGGGGT-3' (32)

→ PCR product: 866 bps (852 cut)

GBA 94-01 Cluster6 knock-out

GBA 94-01 Cluster6 forward primer

5'-GATCACCGGTTGGTTCGAGGACTTCAGCCG-3' (30)

GBA 94-01 Cluster6 reverse primer

5'-GATCGAATTCTAACTCGGCGCAACCAGAA-3' (29)

→ PCR product: 585 bps (571 cut)

GBA 94-01 Cluster16 knock-out

GBA 94-01 Cluster16 forward primer

5'-GATCACCGGTTGGTCTCGTCGCCCCGGTTC-3' (28)

GBA 94-01 Cluster16 reverse primer

5'-GATCGAATTCTCCGTCAGGCAGTCCAGG-3' (28)

→ PCR product: 574 bps (560 cut)

GBA 94-01 Cluster24 knock-out

GBA 94-01 Cluster24 forward primer

5'-GATCACCGGTCACCATCGCGGGACTGCT-3' (28)

GBA 94-01 Cluster24 reverse primer

5'-GATCGAATTCTCGGATCGATTGGTCCTGCTCG-3' (31)

→ PCR product: 845 bps (831 cut)

PVA 91-01 Cluster 8 knock-out

PVA 91-01 Cluster8 forward primer

5'-GATCACCGGTGACGCACGTCAACGGCTG-3' (28)

PVA 91-01 Cluster8 reverse primer

5'-GGAGAATTTCGATGAAGGGCATGG-3' (23)

→ PCR product: 742 bps (732 cut)

PVA 91-01 Cluster 17 knock-out

PVA 91-01 Cluster17 forward primer

5'-GATCACCGGTGGCTGCGGAAGATGTGG-3' (27)

PVA 91-01 Cluster17 reverse primer

5'-GATCGAATTCCATCGTGCCGATGCCGTAC-3' (29)

→ PCR product: 1020 bps (1006 cut)