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„Activation of Secondary Metabolism in *Streptomyces* Bacteria by Extracts from Medicinal Plants“

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Corina Nermuth

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List of Abbreviations

°C	centigrade
µl	microliter
µg	microgramm
AM	Apramycin
AMP	Ampicillin
ARC	antibiotic remodeling compound
ASE	Accelerated Solvent Extraction
B. subtilis	*Bacillus subtilis*
BGC	biosynthetic gene cluster
C. albicans	*Candida albicans*
CAD	Charged Aerosol Detector
CML	Chloramphenicol
d	day
Da	dalton
DAD	Diode Array Detector
DER	drug extract ratio
dH_2O	distilled water
DMSO	Dimethylsulfoxide
DNP	Dictionary of Natural Products
E. coli	*Escherichia coli*
et al.	et alii
Et.Ac.	Ethylacetate
EtOH	Ethanol
ESI	Electro Spray Ionisation
F-Primer	forward primer

g	gramm
h	hour
HDAC	Histonedeacetylase
HPLC	High Performance Liquid Chromatography
HYG	Hygromycin
KAN	Kanamycin
kV	kilovolts
KO	knockout
L	liter
LC-MS	Liquid Chromatography – Mass Spectrometry
M	molar
MeOH	Methanol
min	minute
ml	mililiter
NAL	Nalidixic Acid
NP	natural product
NRPS	non-ribosomal peptide synthetase
NYS	Nystatin
ONC	overnight culture
OD_{600}	optical density, measured at a wavelength of 600 nm
PCR	Polymerase Chain Reaction
pDNA	plasmid DNA
PKS	Polyketide Synthase
psi	pound per square inch
rpm	rotation per minute
R_T	retention time

R-Primer reverse primer

S. cerevisiae *Saccharomyces cerevisiae*

sec second

SM secondary metabolite

SNOR *Streptomyces noursei*

SVEN *Streptomyces venezuelae*

tDNA total DNA

THIO Thiostrepton

T_M melting temperature

V volts

Abstract

Bioprospecting for natural products as remedies against human diseases has a long and successful history. Secondary metabolites (SMs) derived from natural sources, and especially antibiotics, dramatically increased life expectancies through effective treatments of infectious diseases. However, not long after their introduction to medical practice, first antibiotic resistances in pathogenic bacteria have been discovered. The search for new antibiotics is one of the most important tasks of science today. Frequent re-discovery of antibacterial agents, leading to a lack of novel antibiotic discovery, and the increasing rate of multi-drug-resistant microbes urge the discovery of new and improved antimicrobial drugs.

Actinomycetes, especially the genus *Streptomyces*, belong to the most important producers of SMs. Besides antibiotics, they are sources for many other natural medicinal products, like insecticides, herbicides, antitumor agents, immunosuppressant drugs and also cholesterol-lowering drugs.

With progress in genome sequencing, scientists discovered the hidden potential of streptomycetes biosynthetic gene clusters. Most of them remain silent under laboratory conditions but may lead to the discovery of novel natural compounds. Research focuses on a huge variety of methods for activation, induction and upregulation of these cryptic biosynthetic gene clusters and their corresponding biosynthesis pathways, through the manipulation of pleiotropic and pathway-specific regulators. Altering of chemical and physical conditions and genetic modification approaches have shown the huge potential of small changes in culturing.

In this project the effect of medicinal plant extracts on the production of SMs by the two well-researched engineered *Streptomyces* strains, *Streptomyces venezuelae* JZ2 and *Streptomyces noursei* NDA59 have been investigated

Comparative bio-assays and LC-MS analyses of the two bacterial cultures induced with various plant extracts versus control provided promising results in the change of the chromatographic profiles. This method may prove to be a useful and easy way for drug discovery from even well studied streptomycetes.

Zusammenfassung

Die Suche nach natürlichen Heilmitteln gegen humane Krankheiten hat eine lange und auch erfolgreiche Geschichte. Sekundärmetaboliten stammen aus verschiedensten biologischen Quellen und erst durch Antibiotika wurden effektive Behandlungen von Infektionskrankheiten möglich, wodurch ein positiver Lebenswandel erzielt werden konnte. Aber es dauerte nicht lange, bis erste Resistenzen von pathogenen Bakterienstämmen aufgedeckt wurden. Eines der wichtigsten Ziele der Forschung ist sicherlich die Suche nach und Entwicklung von neuen und effektiven Antibiotika. Das Auftreten resistenter Bakterien und häufiges Wiederentdecken bereits bekannter Substanzen zwingen die Wissenschaft, Lösungsansätze für die Entdeckung von neuen und vor allem verbesserten antimikrobiellen Arzneien zu finden.

Unter den Aktinomyceten erlangte allen voran die Gattung *Streptomyces* große Bedeutung bei der Produktion von Sekundärmetaboliten. Neben vielen bekannten Antibiotika sind diese auch Lieferanten vieler anderer Wirkstoffklassen, wie zum Beispiel Insektiziden, Herbiziden, Cholesterinsenkern, Immunsuppressiva und auch Krebstherapeutika.

Forscher entdeckten durch Fortschritte in bioinformatischen Analysen von Genomsequenzen das versteckte Potential von Biosynthese Genclustern der Streptomyceten. Viele Gencluster bleiben aber unter normalen Laborbedingungen „still gelegt“. Man nimmt an, dass diese Gencluster innerhalb des natürlichen Lebensraums der Bakterien bestimmte Funktionen aufweisen. Darum arbeiten Forscher an vielen Lösungsansätzen zur Anregung, Aktivierung und Hochregulierung dieser Cluster und den zugehörigen Biosynthesewegen, um die „still gelegten“ Maschinerien auch im Labor anzuregen. Vielleicht könnte dies die Lösung für die Entdeckung neuer Sekundärmetaboliten sein, denn schon kleine Veränderungen der Kulturbedingungen, wie die Manipulation chemischer und physikalischer Bedingungen, aber auch genetische Modifikationen, zeigen dabei großes Potential.

In der vorliegenden Arbeit wurde der Effekt von verschiedenen Pflanzenextrakten auf die Produktion von Sekundärmetaboliten der beiden gentechnisch veränderten Bakterienstämme Streptomyces venezuelae JZ2 und Streptomyces noursei NDA59 untersucht.

Mit der Hilfe von Wachstumsinhibitionstests und LC-MS Analysen wurden die mit Planzenextrakten getriggerten Bakterienkulturen im Vergleich zu ihren Kontrollen analysiert. Dies zeigte einige vielversprechende Ergebnisse auf und könnte somit eine

weitere einfache und kostengünstigere Methode für die Wirkstoffforschung mit gut untersuchten Streptomyceten liefern.

1 Introduction

1.1 Bioprospecting of Natural Products

Natural Products (NPs) are chemical compounds produced by living organisms that can be found in nature and are produced by plants, animals, fungi and bacteria. It´s known that there are many different classes of chemical compounds which vary in their structures and effects on biological systems. Usually, these chemical compounds show pharmacological or biological activities and therefore are used for pharmaceutical drug discovery and drug design (cf Katz & Baltz 2016; Campos et al. 2012). Historical tradition has provided much information that show the importance of natural products throughout our evolution. Hence it is well known that natural products have been used in human medicine for the treatment of diseases for thousands of years. Chewing on herbs to relieve pain, wrapping leaves around wounds and the use of honey for wound healing in ancient Egypt are only a few examples of ancient knowledge, which is still important for medicinal use and science today (cf Ji et al.; Simon et al. 2008). For decades, our knowledge and understanding of science enabled society to identify bioactive compounds at a molecular level, what should change with progress in chemical, molecular biological and pharmacological science (cf Zarins-Tutt et al., 2016). Today microbial natural products are an important source of already existing and also new drugs. Berdy (2005) points out three major ways to utilize microbial natural products. It is possible to directly apply natural products to medicine and other fields. Alternative methods are the use as starting material for chemical or microbiological modifications to get derivatives and the use as lead compounds for chemical synthesis of new analogues and templates in studies of rational drug design. That´s why many discovered metabolites, their second and continuing generations and semi-synthetic derivatives, are still in use today, what leads to a result of over 50% of all drugs on the market deriving from bioprospecting of natural sources (cf Katz & Baltz, 2016; Zotchev et al., 2012).

1.2 Antibiotic Era and its “Golden Age”

In 1928 Alexander Fleming, a Scottish scientist, opened up the antibiotic era by the discovery of Penicillin. A petri dish that contained *Staphylococcus bacteria* was contaminated with a fungus, later identified as *Penicillium notatum.* An inhibiting zone around a mold colony showed the antibacterial effect of the mold on the Gram-positive bacterium. The mold excreted a substance that apparently inhibited bacterial growth, hence A. Fleming accidentally discovered the antibiotic (cf Fleming, 1929). The medicinal use of Penicillin is due to Howard Florey and his assistants, who developed a process for the industrial production and brought it to market during the Second World War.

In the “Golden Age”, the early years of natural product discovery from microorganisms in the 1940s and early 1950s, many new antibiotics were found using convenient methods of fermentation and whole cell screening. In the 1940s, the Waksman group discovered Actinomycin, Streptomycin and Streptothricin by systematic screening of actinomycetes bacteria. Pharmaceutical companies then started to screen extracts of Actinomycetes and fungi for bioactive compounds against bacteria (cf Katz & Baltz, 2016). In the following years most of the important antibacterial antibiotics were discovered and isolated from *Streptomyces* species, for example Tetracyclines, Cephalosporins, Aminoglycosides, Macrolides (cf Bérdy, 2005).

1.3 Secondary Metabolites

Secondary metabolites (SMs) are low molecular weight chemical products from the secondary metabolism of plants, bacteria, fungi and marine organisms. Within bacteria especially the phylum of actinomycetes are the most important sources for secondary metabolites.

Reported by Solanki et al. (2008) and confirmed by Ruiz et al. (2010) and Vaishnav & Demain (2010) SMs cover a wide range of bioactive compounds. Secondary metabolism provides antibacterial, antifungal, antiparasitic, antitumor, cholesterol-lowering agents, immunsuppressants, tools for research and diagnostics. Besides this broad field of application in medicine SMs are also used in agriculture as pesticides, insecticides and herbicides (cf Bérdy, 2005).

Although discussions about the main functions of SMs for the producing organisms are still ongoing, it is known that they are, in contrast to primary metabolites, not required for growth (cf Bérdy, 2005; Solanki et al., 2008; Vaishnav & Demain, 2010). SMs provide survival benefits for their producer through many diverse functions. There are two major groups of SMs, those who have an intrinsic function within the producer strain and those which affect other organisms in the extrinsic surroundings. On the one hand, SMs are used as ecological deterrents, not only for defense against plants, insects or animals, but also as competitive weapons in nonselective environments, to win competition against other bacteria or fungi. SMs also act as agents of symbiosis and pathogenic agents between microbes and other organisms. On the other hand, studies have also shown that SMs serve as mineral scavengers and precursors of structural components (cf Vining, 1990; Demain & Fang, 2000). Detailed information of SM Biosynthesis is provided on the example of antibiotic biosynthesis in chapter 1.5.2.

1.4 Antibiotics

Antibiotics are natural products, which are synthesized by many species like bacteria, fungi, plants and animals. They are products from secondary metabolism and therefore have no function for the growth of the producing organism. But they can also derive from semi-synthetic production or chemical synthesis based on the structure of the natural products. It is well known that antibiotics can be classified by their effect on target structures in bacteria. Bacteria can be influenced negatively by the effect of antibiotics on the main drug targets cell-wall synthesis, DNA replication, transcription, protein synthesis and metabolic enzymes. Antibiotics have the characteristic to inhibit the growth or even kill living organisms at low concentrations, therefore it´s also important to differentiate between a bactericidal effect causing cell death, and a bacteriostatic effect inhibiting cell growth (cf Coates et al.,2002; Zotchev, 2008; Demain, 2009; Kohanski et al.,2010).

Figure 1 shows the main targets of antibiotics:

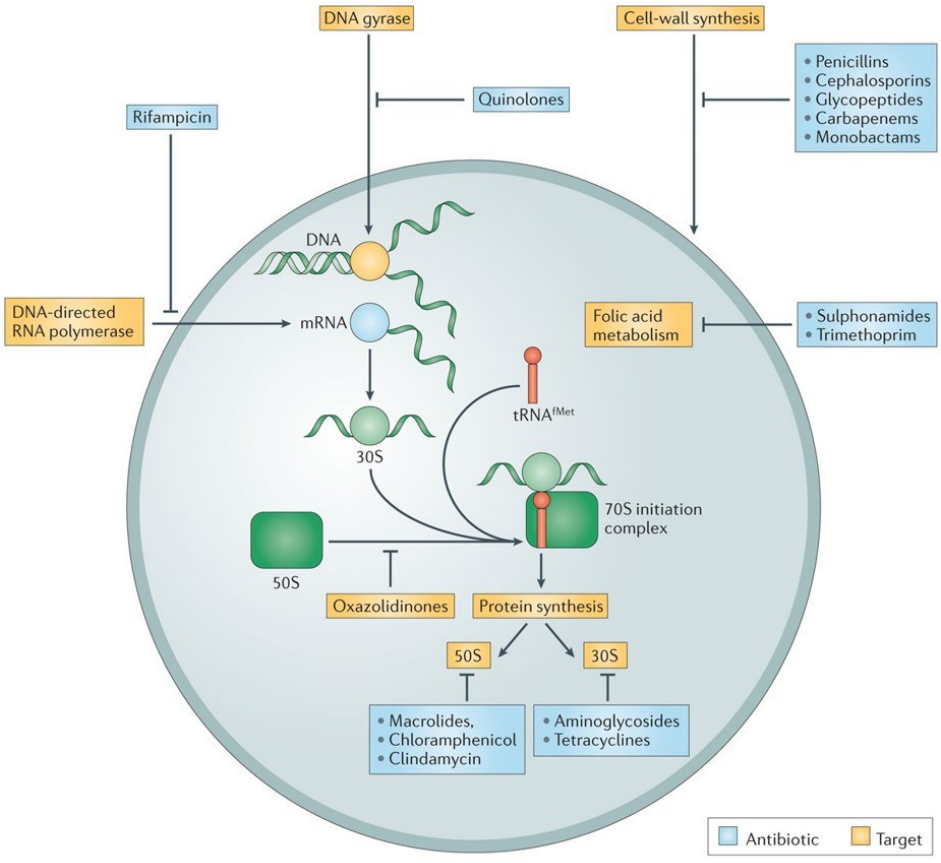


Figure 1: Main targets of antibiotics (Coates et al., 2002).

1.4.1 Antibiotic Resistance

Antibiotics have changed human life. In the first years of use they solved many problems of medicine and human health. Antibiotics made it possible to treat or prevent bacterial infections, that otherwise may have caused death. So, by changing the outcome of bacterial infections, antibiotics increased people´s life expectancies. Even today antibiotics help to decrease morbidity and mortality more than ever.

But all the benefits and improvements in medicine and health can be devastated by the development of antibiotic resistance by bacteria. It is obvious that, soon after the development of an antibiotic drug, first resistances may be detected. Resistance is due to overuse of antibiotics, incorrect prescribing, wrong treatment of diseases and indication, and due to a wide-spread use in agriculture and farming. It is also important to mention that resistance can be caused by spontaneous mutation of bacteria and the requirement of new genes. The latter can be achieved by horizontal gene transfer, which allows a transfer of (resistance) genes among different species of bacteria (cf Ventola, 2015). Antibiotics inhibit bacterial growth when interacting with their target. Therefore, it is necessary that antibiotics are available in a specific concentration and that they also recognise their target. Bacteria avoid growth inhibition or even being killed by antibiotics through different modes of resistance, which can be divided into active mechanisms like inactivation by enzymatic reactions, efflux pumps (antibiotics are pumped out of the cells), and passive mechanisms like a decreased uptake of the antibiotic, modifications of the antibiotic target (affects the binding efficiency of the antibiotic drug). Other methods are overproduction of the target or also a bypass of the metabolic pathway (cf Singh & Barett, 2006; Martinez & Baquero, 2014). An overview of the modes of resistance is shown in figure 2.

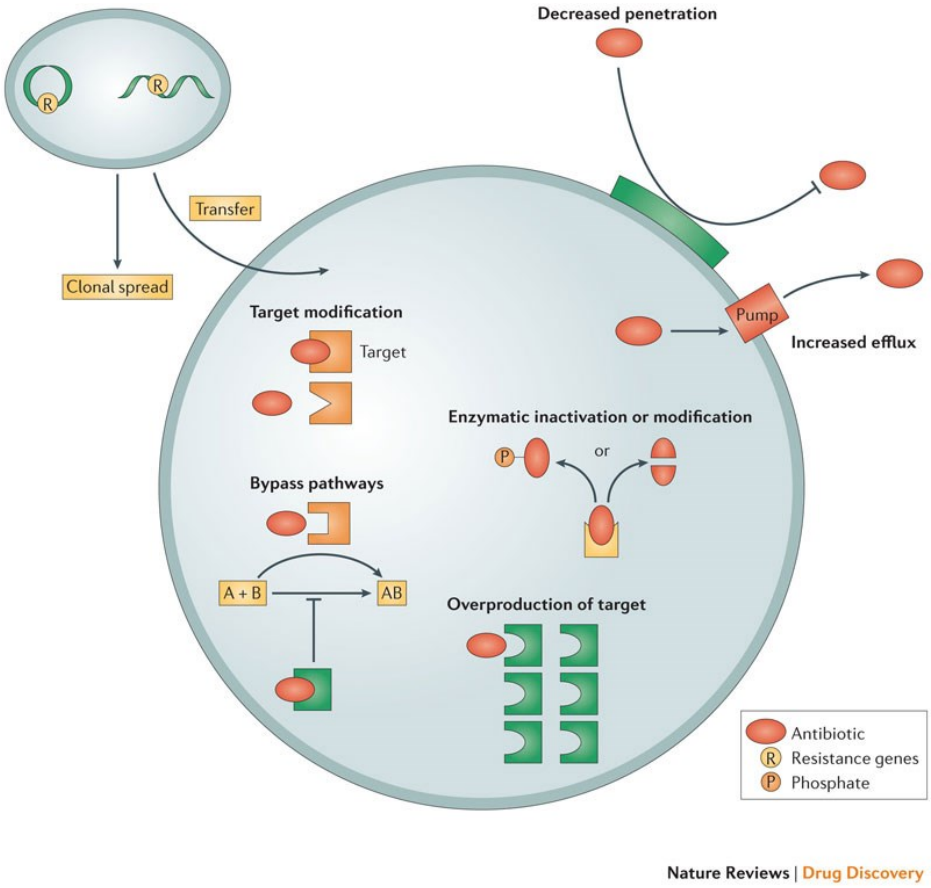


Figure 2: Modes of Resistance (Lewis, 2013).

1.4.2 The Urgent Need for New Antibiotics

Worldwide, society has to face the increasing problems of pathogenic bacteria with antibiotic resistance. Many multidrug resistant Gram-positive and Gram-negative bacteria are becoming resistant to nearly all antibiotics available, showing the urgent need for new antibiotics in this resistance crisis.

Due to various reasons, the discovery of new antibiotics has decreased in the last years. One major problem is the frequent re-discovery of already known compounds through traditional methods of bioactivity screening. Besides, long periods of clinical trials and admission procedures of new antibiotics, and the only use of new and effective antibiotics as “last line” drugs, have to be mentioned. New antibiotics are rarely prescribed, so that there are therapeutics left in case of emergency. For pharmaceutical industries antibiotic research is also an economic risk today. The investment on research and development of new antibiotics is not lucrative in comparison to many other pharmaceutical substances (cf Ventola, 2015 a).

Facing this global crisis national and international organizations are working together on management strategies to reduce the risk of antibiotic resistance. Adoption of antibiotic

stewardship programs, improvement of prescribing practice, optimization of therapeutic regimens and improvement of diagnosis and diagnostic tools, prevention of infections, improvement of tracking methodologies and international initiatives shall reduce the spread of antimicrobial resistance. But also efforts on the discovery of new antibiotics are of high priority (cf Ventola, 2015 b).

1.5 Actinomycetes as Producers of SMs

Actinomycetes were thought to be a transitional form between fungi and bacteria, hence the name derives from the Greek words for ray (aktis or aktin) and fungi (mukes). Actinomycetales is one of the largest orders within the bacteria domain. They are GC-rich, Gram-positive, filamentous bacteria and most of them are aerobic. Actinomycetes are inhabitants of aquatic and terrestrial ecosystems. They can also be found in the intestinal flora and living in symbiosis with plants.

As already mentioned, actinomycetes belong to the most important producers of SMs. They are sources for many natural products, like antibiotics, insecticides, herbicides, antifungals, antitumor agents, immunosuppressant drugs and others. Some examples of antibiotics in clinical use, that are produced by actinomycetes are aminoglycosides, tetracyclines, macrolides, beta-lactams, beta-lactamase inhibitors, chloramphenicol just to mention a few (cf Solanki et al.,2008; Barka et al.,2016).

1.5.1 *Streptomyces* Bacteria

Streptomyces is a genus of the order Actinomycetales. Their extraordinary life-cycle shows that these bacteria have a mycelial lifestyle and reproduce by sporulation.

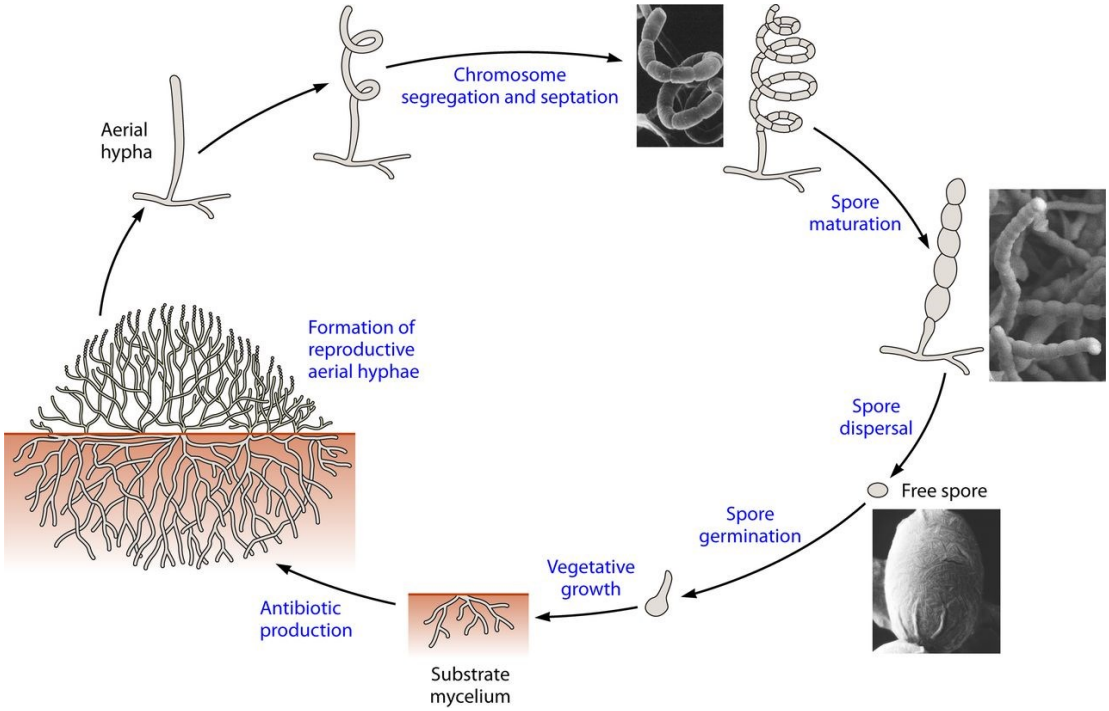


Figure 3: Schematic view of the life cycle of *Actinomycetes* (Barka et al.,2016).

When the conditions for a spore are favorable and advantageous, the spore is able to germinate. Through vegetative growth it forms hyphae that branch out in the substrate and result in a mycelial network of vegetative hyphae. When the nutrient level decreases, the vegetative mycelium differentiates and reproductive aerial hyphae are formed. The production of SMs is connected to the formation of aerial hyphae, which are characteristic for the appearance of the colonies. After doubling of the genome cross walls appear and aerial hyphae differentiate into spores. The spores outlast in a state of dormancy until favorable conditions are found and make it possible for the spore to germinate again (cf Barka et al., 2016).

Due to technical improvements in isolation and separation techniques, about a million natural compounds that derive from microbes, plants and animals, were described at the end of 2003. About 250 000 compounds show bioactive properties, of which nearly one-tenth (22 500) originate from microbes. Nearly 50 % of microbial SMs derive from the fermentation of Actinomycetes and within this phylum the genus of *Streptomyces* produces about 75 % of the known bioactive SMs (cf Berdy, 2005; Solecka, 2012).

Examples of antibiotics produced by *Streptomyces* strains are shown in Table 1.

Bacterial strain	Antibiotic	Chemical class	Target
S. albus	Salinomycin	Polyether	Membrane (ionophore)
S. aureofaciens	Tetracycline	Tetracycline	Ribosome
S. azureus	Thiostrepton	Thiopeptide	Ribosome
S. clavuligerus	Clavulanic acid	β-lactam	B-lactamase inhibitor
S. fradiae	Neomycin	Aminoglycoside	Ribosome
S. griseus	Streptomycin	Aminoglycoside	Ribosome
S. hygroscopicus	Hygromycin B	Substituted aminoglycoside	Ribosome
S. kanamyceticus	Kanamycin	Aminoglycoside	Ribosome
S. lavendulae	Streptothricin	N-glycoside	Ribosome
S. niveus	Novobiocin	Coumeringlycoside	DNA gyrase (β-subunit)
S. noursei	Nystatin	Polyene Macrolide	Membrane (pore former)
S. roseosporus	Daptomycin	Lipopeptide	Lipoteichoic acid?
S. venezuelae	Chloramphenicol	-	Ribosome
S. verticillus	Phleomycin	Glycopeptide	DNA strand breakage

Table 1: Examples of antibiotics produced by Streptomyces bacteria (cf Kieser et al., 2000).

1.5.2 Antibiotic Biosynthesis in *Streptomyces* Bacteria

As mentioned before, antibiotics are SMs and they represent a variety of chemical structures. The biosynthesis of some SMs have been studied in detail on the example of antibiotic biosynthesis.

In surface grown cultures of streptomycetes bacteria antibiotic production is linked to the development of aerial hyphae. In liquid cultures many *Streptomyces* strains undergo different developmental stages, which are known as a first exponential phase of rapid growth (RG1 phase), a transition phase (T phase), a second phase of rapid growth (RG2) and a final stationary phase (S phase). The T phase is a “metabolic shift” between primary and secondary metabolism, due to diauxic growth. This transient lag-phase has

been associated with the activation of antibiotic biosynthetic genes (cf Vohradsky et al.,2000).

This information reflects the fact that, at least under laboratory conditions, antibiotic production is growth-phase dependent. Apart from nutrient limitation at the end of the growth phase, antibiotic biosynthesis is influenced by many different environmental and physiological signals (cf Bibb, 2005; Zotchev, 2008). The biosynthetic pathway of SMs is linked to primary metabolism. Chemical compounds deriving from important pathways of the primary metabolism serve as precursors, that are assembled to antibiotic molecules by specific enzymes. Antibiotics are synthesized by living organisms, so it is obvious that enzymes with different catalytic activities are important for biosynthesis (cf Zotchev, 2008).

There are two major stages in antibiotic biosynthesis. In a first step the primary metabolism provides precursors, which are assembled into antibiotic scaffolds, with little or even no antibiotic activity. Precursors can already be activated, or their activation is reached through different chemical reactions, for example adenylation or phosphorylation. In a second step the scaffolds are modified to generate an active antibiotic by specific enzymes. Polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) are only two examples of major enzymes for scaffold biosynthesis and there are also different enzymes for scaffold modification like hydroxylases, methylases, acyltransferases, halogenases and others (cf Zotchev, 2008).

1.6 Genomic Era

The first genes connected to the antibiotic biosynthesis were discovered in the 1970s and 1980s. The starting point for understanding the genetic background of SM production was the identification of the first biosynthetic genes and also their specific manipulation. With the start of genome sequencing, dating back to the 1990s, scientists gained first access to complete genome sequences. Afterwards methods of genome sequencing developed very fast and therefore improved the search for novel natural products, hence there is plenty of genetic data available for research nowadays. Through genome sequencing it is possible to gain fast and automated access to almost whole genomes of numerous microorganisms in a very short period of time. Genomes containing numerous novel biosynthetic gene clusters (BGCs) open up new advantages for the discovery of novel natural products, but due to a huge amount of data available, it also implicates a significant effort in organization and handling of these data (cf Zotchev et al.,2012; Ziemert et al., 2016).

The research of Bentley et al. (2002) revealed the complete genome of *Streptomyces coelicolor*. As the best researched *Streptomyces* strain as of today, it became evident that novel BGCs, which often remain silent under laboratory conditions, could potentially lead to the discovery of new SMs (cf Zotchev et al.,2012).

1.6.1 Antibiotic Biosynthetic Gene Clusters

It is known that genes, which are involved in antibiotic biosynthesis, are arranged in clusters in some organisms. So-called BGCs have been found in bacteria and eukaryotes, like filamentous fungi and plants. BGCs are defined as a group of co-localized genes that are involved in the multistage biosynthesis of natural products. The BGCs consist of genes for regulation, biosynthesis, export and protection. While genes that encode enzymes for scaffold biosynthesis of SMs can often be found in the centre of the BGCs, genes responsible for scaffold modification join in the close surroundings, as well as regulatory genes which are important for the regulation of the expression of biosynthetic genes. The clusters also contain resistance genes, which protect the cell from its own antibiotic by encoding enzymes which inactivate the antibiotic, modify its targets, or work as efflux pumps to remove harmful substances (cf Zotchev, 2008; Osbourn, 2010).

A typical antibiotic biosynthesis gene cluster is shown in Figure 4.

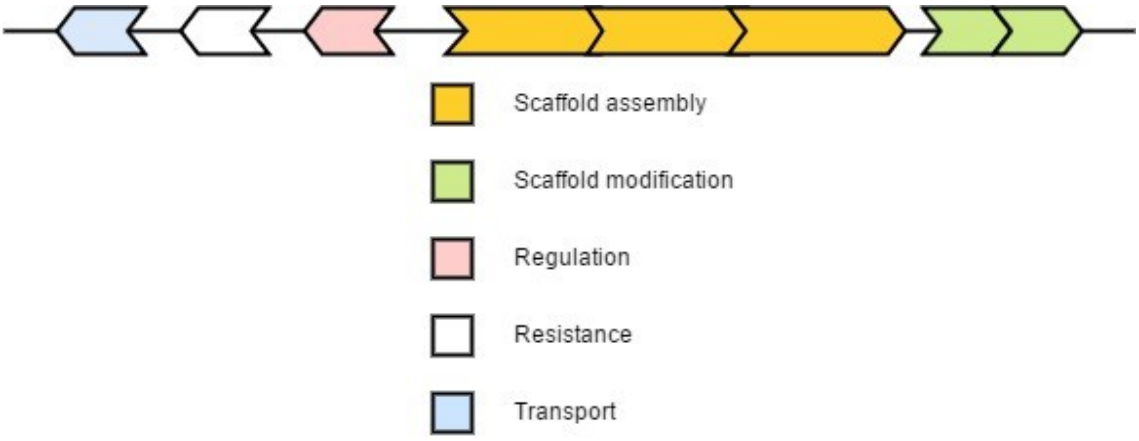


Figure 4: Schematic view of a typical antibiotic BGC (modified from Zotchev, 2008).

1.6.2 Regulation of Antibiotic Production

As mentioned before, the onset of antibiotic biosynthesis is dependent on many different environmental and physiological signals.

Culture conditions like the change of pH, change of temperature, aeration and media composition can broaden the spectrum of produced SMs. Nutrient limitation and the presence of other microorganisms are alternative approaches to obtain a different SM production (cf Zotchev,2008).

Based on research articles Pettit (2011) reports the use of small-molecules as elicitors of microbial SMs. Histone deacetylase inhibitors (HDAC inhibitors), enzyme inhibitors, solvents and heavy metals have all been reported to affect antibiotic production.

Bibb (1996) shows a variety of factors that may affect the onset of antibiotic production in *Streptomyces* bacteria. The factors include gamma-butyrolactone signalling molecules, imbalances in metabolism and stress response, shown in Figure 5.

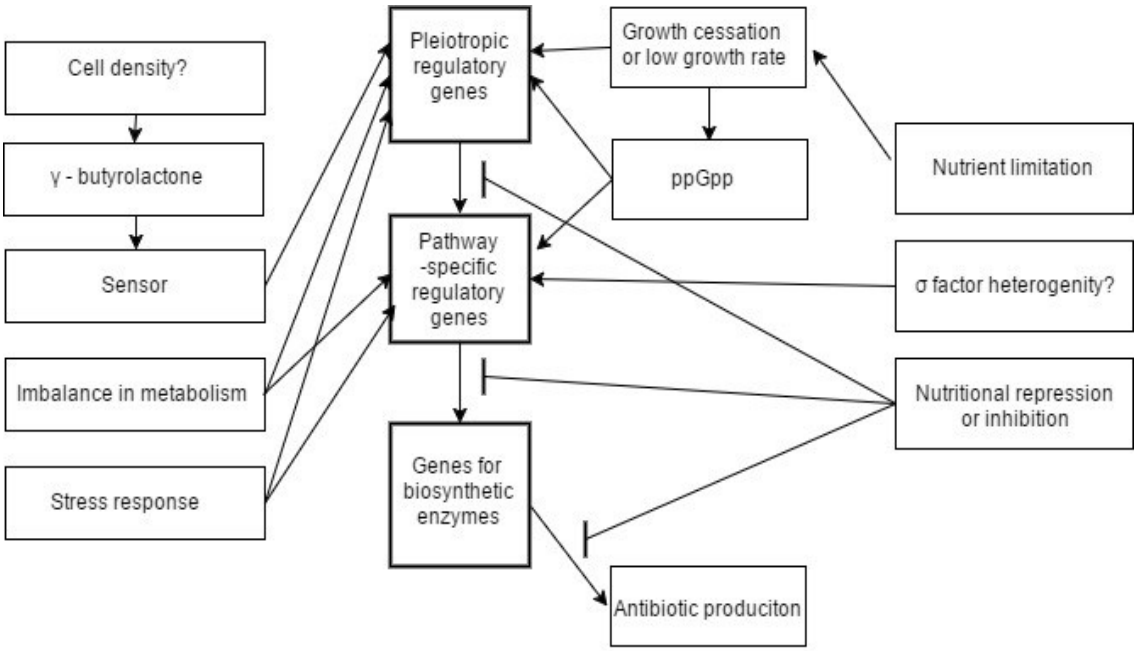


Figure 5: Factors potentially determining the onset of antibiotic production in Streptomyces (modified from Bibb, 1996).

There has been much research on these complex regulation systems. Thinking of the huge variety of environmental and physiological signals and also numerous streptomycetes bacteria, their gene clusters and many associated regulatory genes, influencing a huge variety of biosynthetic enzymes, it is obvious that the understanding of regulation of antibiotic production and biosynthetic pathways still remains a major topic of science. Two major regulation systems have been investigated in *Streptomyces*. The pleiotropic and the pathway-specific regulators control antibiotic production through the response to a large variety of “triggers” (Bibb, 1996).

Bibb (1996) also notes, that it is an important goal for the future to understand physiological and environmental conditions that not only trigger the expression of the genes, but also influence the activity of their products and the corresponding signal transduction pathways, and therefore are responsible for the activation of antibiotic biosynthesis.

1.6.2.1 Pleiotropic Regulators

Pleiotropic regulators, also referred to as global or higher-level regulators, are located outside the BGCs. Diverse changes in environment or nutrition level affect these regulators. On the one hand they can affect secondary metabolism indirectly by modulation of pathway-specific regulatory genes or directly affecting gene expression, and on the other hand they can also affect morphogenesis of *Actinomycetes*. Many of these regulators are two-component systems (TCS), consisting of a histidine-kinase, sensing environmental stimuli, and a cognate regulator, which is responsible for the cellular response through transcriptional regulation of genes. The PhoR-PhoP system, a major transduction system for phosphate control in *Streptomyces*, is one of the best known examples of TCSs. PhoR (phosphate regulon sensor protein) is a membrane associated protein kinase, that phosphorylates PhoP, a DNA binding response regulator, when phosphate is at a low level. Activated PhoP leads to an increased expression of pho regulon genes by binding to PHO boxes (consensus phosphate boxes in the promotor regions), and it also inactivates important metabolic pathways by interaction with other regulators. Therefore, changing phosphate concentrations can influence the control of gene expression and therefore also antibiotic production (cf Martín, 2004; Martín & Liras, 2010; Rodríguez et al.,2013).

1.6.2.2 Pathway-specific Regulators

Pathway-specific regulators are also known as cluster-situated regulators (CSRs). They are located within a dedicated BGC and therefore regulate the biosynthesis genes. They may respond to a pyramidal network of collaborating autoregulatory proteins and their related ligands. Often a γ-butyrolactone binds to a dedicated receptor. In response to external signals, these butyrolacton-receptor systems trigger regulatory genes. Well known examples of CSRs are proteins of the SARP- (*Streptomyces* antibiotic regulatory protein). As part of this family *Str*R is controlling the production of Streptomycin in *Streptomyces griseus*. *Cca*R of *Streptomyces clavuligerus*, is involved in the regulation of the biosynthesis of cephamycin and clavulanic acid and *Cda*R, *Red*D and *Act*II-ORF4

are regulators that control the production of actinorhodin, CDA and Red in *S. coelicolor* (cf Martín & Liras, 2010; van Wezel & McDowall, 2011).

1.7 Strategies for the Discovery of new Antibiotics

The discovery of the first antibiotic BGCs in the 1970s and 1980s and the establishment of *Streptomyces* genetics was the first progress in understanding the background of SM biosynthesis. The fast development of genome sequencing, and progress in analysing of the genomes, and also in bioinformatics revealed, that the biosynthetic potential of many strains has been underestimated. Sequencing studies of bacteria and fungi showed, that strains contain gene clusters with the potential to synthesize a plethora of SMs. But scientists soon recognized that these gene clusters, possibly useful in natural environments, remain silent under laboratory conditions and therefore only a small fraction of SMs is produced during fermentation (cf Pettit, 2011; Zotchev et al.,2012; Ochi & Hosaka, 2013).

1.7.1 Activation of Cryptic Gene Clusters

Silent or “cryptic” gene clusters may be linked to the biosynthesis of novel SMs. The conditions required to trigger their expression are yet unknown, so they are of great interest today, because specific manipulation of these silent gene clusters can reveal many novel bioactive compounds and probably lead to an increase of their chemical diversity. Under laboratory conditions the use of molecular or cultivation-based methods enable access to the silent metabolic pathways. There has been much research on many different concepts for the activation of the cryptic pathways (cf Osbourn 2010; Pettit, 2011; Ochi & Hosaka, 2013). Some of these concepts with focus on altering of chemical and physical conditions and genetic modifications will be discussed in the following chapters and serve as basis for this work.

1.7.2 OSMAC

It is known that different natural environments affect the SM biosynthesis. Special requirements like the pH of soil, food supply, oxygen supply or temperature lead to different development and evolution of microorganisms. These conditions result in changes in transcriptomes, proteomes and metabolomes, all of which affect survival of the organisms.

The OSMAC approach (One Strain Many Compounds) involves systematic alteration of cultivation parameters in order to increase SM production from one microbial source. Manipulation can occur at the transcriptional level, the translational level, the enzyme level and the metabolite level (see Figure 6).

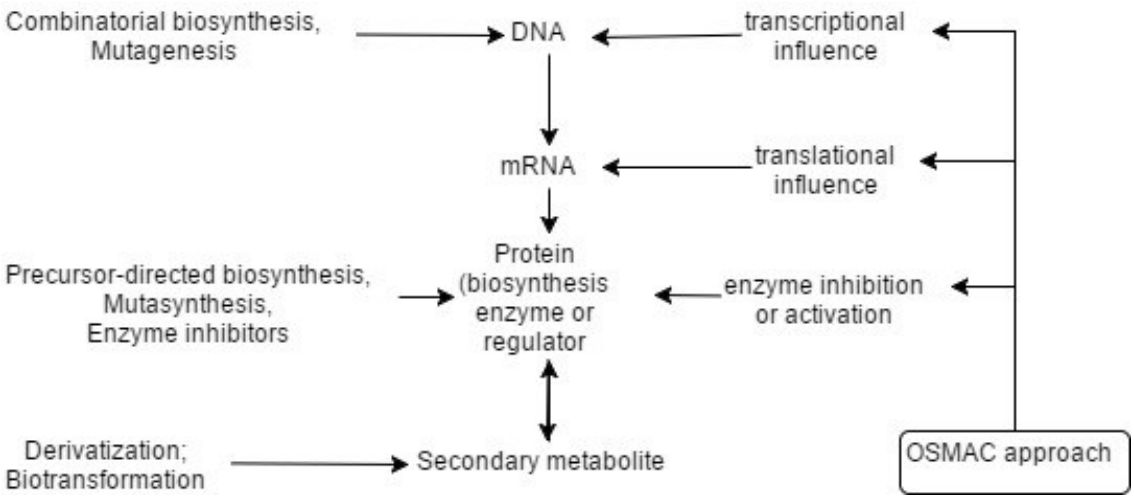


Figure 6: Different ways to influence SM biosynthesis. Manipulation can occur at the transcriptional, translational and enzyme level, with the help of the OSMAC approach (modified from Bode et al., 2002).

Based on the fact, that even small changes in the culture medium may affect SM biosynthesis and the metabolic profile of a single organism, different strategies were developed to increase SM production using this approach. These strategies include the variation of media composition, change of pH, hydrostatic pressure, temperature and aeration, addition of other microorganisms, addition of precursors and inhibitors of SM biosynthesis and also genetic approaches. The OSMAC approach with all of its methods focuses on the potential of a single microorganism to release the chemical diversity of its natural products. It is adapted for the activation of silent metabolic pathways (cf Bode et al., 2002; Abdelmohsen et al.,2015). Some of the modifications in culture conditions are discussed in the following chapters.

1.7.3 Co-Culturing

Co-cultivation is an OSMAC based way to elicit SM biosynthesis. In this approach a microorganism is grown together with one or more microbial strains. Many published co-cultivation experiments show the potential effects of this technique, yielding already known, but also many novel SMs. In their natural environment, many bacteria coexist besides each other. They live in communities in various habitats and communicate through different mechanisms. Co-existence allowing interspecies crosstalk, and also the fight for predominance over a different strain and competition for limited resources may affect the production of natural products. Co-cultivation is thought to mimic the

natural situation and therefore it could trigger production of novel SMs which are not produced in monocultures. It is also reported that this approach may increase the concentration of natural products, that could not be detected because of a too low titre (cf Marmann et al., 2014; Abdelmohsen et al., 2015; Zarins-Tutt et al., 2015).

Co-cultivated strains	**SMs reported**	**Source**
Streptomyces cinnabarinus PK209 and *Alteromonas* sp. KNS-16	Lobocompactol (increased production)	Cho & Kim, 2012
Streptomyces coelicolor and *Myxococcus xanthus*	Actinorhodin (increased production)	Pérez et al., 2011
Tsukamurella pulmonis and *Streptomyces endus*	Alchivemycin (new SM)	Onaka et al.,2011

Table 2: discovered SMs based on co-cultivation and corresponding source.

Mechanisms of cell-cell interaction are poorly understood and therefore are still being investigated. Abdelmohsen et al. (2015) reported different potential interactions between microorganisms, where secondary metabolism of the producer strain is directly triggered by the inducer-producer interaction. Besides physical cell to cell interactions (A), microorganisms can affect the secondary metabolism of the producer strain by small molecule mediated interactions (B). Alternative ways are the production of enzymes, which may activate precursors of the producer strain (C), and also horizontal gene transfer (D).

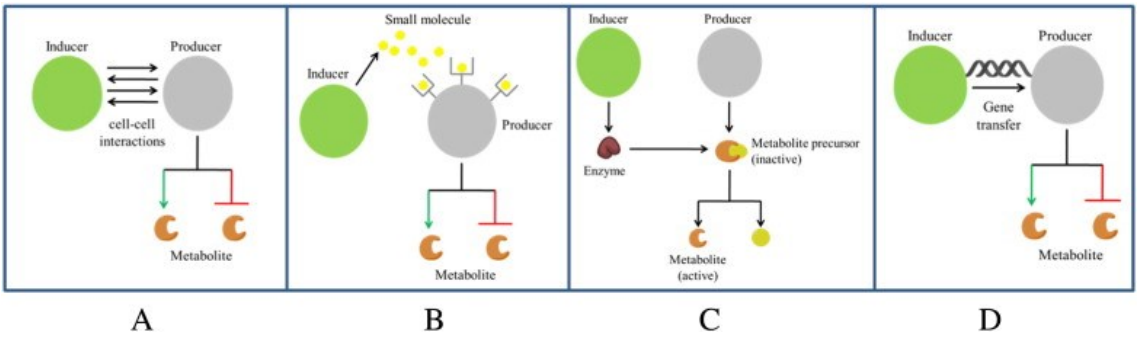


Figure 7: Consequences of co-cultivation (Abdelmohsen et al., 2015).

1.7.4 Antibiotic Production as Stress Response

As already mentioned in chapter 1.7.1 and 1.7.2 there are diverse methods for the manipulation of fermentation cultures that may affect SM biosynthesis and enable access to cryptic metabolic pathways. In their natural habitats Streptomyces are always exposed to environmental changes. Thus, it is obvious that stress can also be used to trigger SM biosynthesis under laboratory conditions, when actively applied to a culture. Physical methods like the use of different vessels, oxygen limitation versus high aeration, change of temperature (heat shock) and change of hydrostatic pressure, as well as chemical methods that are based on changes in the culture media can induce stress response (cf Pettit, 2011; Yoon & Nodwell, 2014). Vohradsky et al. (2000) revealed some insights into stress induced regulatory networks of *S. coelicolor* by two-dimensional gel analysis of gene expression, focusing on five main stress simulons, but the accurate mechanisms on how these diverse stress signals trigger antibiotic production are still poorly understood.

Table 3 and 4 show different physical and chemical experiments with Streptomyces bacteria resulting in a change of SM production due to stress response.

Physical methods		
Method	**Microorganism**	**Reference**
Oxygen limitation	*Saccharopolyspora erythraea*	Clark et al., 1995
Hydrostatic pressure	*S. parvulus*	Sattler et al., 1998
Heat shock	*S. hygroscopicus*	Liao et al., 2009

Table 3: shows examples of physical changes in cultivation of different Streptomyces strains. More information about the experiments can be found in the corresponding references.

Chemical methods		
Method	**Microorganism**	**Reference**
pH shock	*S. coelicolor*	Hayes et al., 1997
Addition of solvents	*S. venezuelae*	Chen et al., 2000
Salinity change	*S. coelicolor*	Bursy et al., 2008

Table 4: shows examples of chemical changes in culture media to trigger SM production of different Streptomyces strains. More information about the experiments can be found in the corresponding references.

1.7.5 HDAC Inhibitors and other Elicitors

Another concept for activation of cryptic BGCs and antibiotic production is the addition of chemicals or small-molecules to culture media.

HDAC inhibitors become more and more interesting for research. They repress HDACs (Histone deacetylases) and can cause up- or downregulation of genes. Histone deacetylases (HDACs) are a large and ubiquitous family of zinc-containing enzymes, which play an important role in the control of gene expression in eukaryotes. Through deacetylation of histone proteins, HDACs influence the accessibility of regulatory genetic elements. It is obvious that changes in accessing a gene may modify the transcription of this gene. As the name implies, early projects recognized the effect of HDAC inhibitors on chromatin of fungi. Due to these works, it was just a matter of time to apply HDAC inhibitors to *Actinomycetes* bacteria, which could have a nucleoid structure comparable to that of eukaryotes (cf Moore et al.,2012; Zarins-Tutt et al., 2016). Moore et al (2012) also reported, that most of sequenced streptomycetes genomes encode orthologues of HDACs. This fact reveals the huge potential of the application of HDAC inhibitors on bacterial SM biosynthesis, a cheap and easy way to potentially increase titres of already known SMs or activate cryptic BGCs.

Genome informed culturing is an approach that enables prediction and isolation of SMs. Structural and physico-chemical features of a novel metabolite can be used for the determination of enzymes involved in SM biosynthesis and are therefore used for the optimization of culture media. Thus, the addition of proper precursors, resulting in changes of metabolism, enhance the yields of known products as well as the production of novel compounds (cf Ochi & Hosaka, 2013; Zarins-Tutt et al., 2016). Craney et al. (2012) reported small-molecules affecting SM production by modulating fatty acid biosynthesis. Antibiotic remodeling compounds (ARCs) were demonstrated to induce actinorhodin production in *S. coelicolor* and to yield novel compounds produced by *Streptomyces peuceticus*. Feeding experiments of *Streptomyces griseoviridis* with carboxylic acids varying in their chemical structures, probably acting as signal molecules or co-factors, yielded thirty different compounds in addition to the strains´ main metabolites (cf Bode et al., 2002).

The use of subinhibitory concentrations of antibiotics is reported to have stimulatory as well as inhibitory effects on the production of (novel) SMs. The exact mechanism on how these low concentrations of antibiotics influence SM biosynthesis is still under discussion. Induced SM production is possible to be a consequence of activation or elevated transcription of regulators or probably also due to a natural stress response mechanism (cf Zarins-Tutt et al., 2016). Goadsporin for example induces secondary

metabolism and morphogenesis in various Streptomyces strains (cf Onaka et al., 2001) and the treatment of a marine Streptomyces strain with a low concentration of tetracyclin or bacitracin enabled the biosynthesis of streptophenazines (cf Mitova et al., 2008).

As already mentioned in chapter 1.7.3 many bacteria live in multispecies communities in their natural habitats. In response to environmental changes, especially to changes in cell-density, Gram negative and positive bacteria use quorum sensing to modulate the behavior of their whole cell community by the regulation of gene expression. Quorum sensing is based on the communication through “autoinducers”, naturally produced hormone-like small diffusible signaling molecules, that may activate their own synthesis at nanomolar concentrations in order to modulate diverse cell activities. The autoregulators are classified into five major groups including the γ-butyrolactones, diketopiperazines, furans, γ-butenolids and PI-factor (cf Matselyukh et al., 2015; Niu et al., 2016; Zarins-Tutt et al., 2016). The γ-butyrolactone A-factor was the first regulator discovered in *Streptomyces griseus* by Khaklov et al. in 1967. A-factor leads to induced streptomycin biosynthesis in *S. griseus* by activating a specific regulatory cascade (cf Ohnishi et al., 1999). Isolate from *Streptomyces natalensis*, the autoregulator PI-factor was reported to stimulate the production of pimaricin in the corresponding wild type strain (cf Recio et al., 2004). Four years later methylenomycin production of *S. coelicolor* was shown to be induced by methylenomycin furans that are products from the *mmfLHP* genes (cf Corre et al., 2008). Thus, synthetic and natural cognate analogues of the autoregulators could be a rich source for the identification of new chemical elicitors of SM production (cf Yoon & Nodwell, 2014).

The addition of solvents, heavy metals and rare earth elements have also been shown to affect secondary metabolism in *Streptomyces* and therefore provide even more possibilities to manipulate SM production (cf Pettit, 2011).

1.7.6 Genetic Modifications

1.7.6.1 Genome Based Mining for new SMs

Keeping in mind thousands of bacterial genome sequences and even more BGCs, which may be utilized to produce diverse SMs, it is obvious that there is a wealth of genetic data available today. For that reason, research focuses on data mining and the connection of BGCs and their cognate natural products.

The idea of genome mining is to predict and isolate natural products based on the genetic background information, as well as to activate silent BGCs via a genetic approach. Genomic information provides a basis to get a general idea of the genetic potential of

the microorganism for SM production. After the identification of putative BGCs in the genome sequence and their encoded enzymes, a prediction of a chemical class and sometimes even structure of cognate SMs becomes possible. Diverse software tools for genome mining are supposed to facilitate the discovery and characterization of genes which are involved in biosynthesis, the prediction of novel SMs and the identification of respective biosynthetic pathways (cf Weber, 2014; Ziemert & Alanjary, 2016).

1.7.6.2 Metabolic Engineering

“Metabolic engineering” derives from the words metabolism and engineering and is referred to as targeted alteration of diverse organisms´ metabolic pathways by design and implementation of genetic modifications. Strain development plays a central role in the improvement of biotechnological production of bioactive SMs. Metabolic engineering is needed for the improvement of cellular properties, extension of substrate and product range, improvements of yields, elimination of by-products and improvement of process-performance. To improve SM titers from bacterial sources, many approaches in metabolic engineering have been developed and have gathered considerable interest (cf Kumar & Prasad, 2011). A scheme of these diverse approaches that have been used for the improvement of SM production is shown in Figure 8.

Due to the fact, that regulators like activators and repressors often control SM production within a BGC, a main target of metabolic engineering is the manipulation of regulatory networks in order to improve SM titres. While manipulation of global regulators may increase the production of many SMs, manipulation of pathway specific regulators may increase the titer of a specific compound by activating transcription of genes to their biosynthesis (cf Chen et al., 2010).

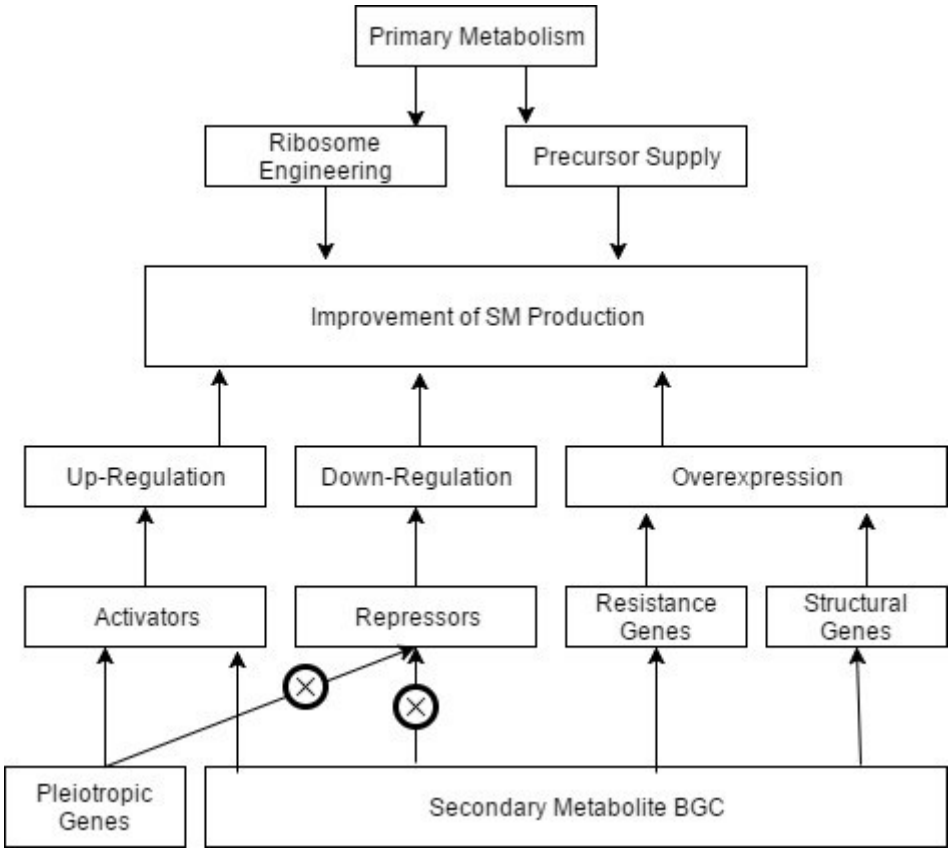


Figure 8: Different approaches for the improvement of SM production (modified from Olano et al. 2008).

1.7.6.3 Deletion of Repressors

Pathway specific regulators can act as activators or repressors. Repressors have a negative effect on the expression of gene cluster elements. When antibiotic production is regulated by a repressor, inactivation of this gene may improve the SM titre (cf Chen et al., 2010).

The following works illustrate the effects of the deletion of a repressor on SM production. Smanski et al. (2009) reported, that the production of platensimycin and platencin of *Streptomyces platensis* MA7327 is linked to the *ptmR1* gene, which encodes GntR-like transcriptional repressor. The deletion of this gene resulted in a 100-fold overproduction of platensimycin and platencin in the mutant strain, compared to the wild-type strain. But it is also reported, that deletion and inactivation of a gene can lead to the discovery of novel products deriving from secondary metabolism. Gottelt et al. (2010) reported production of the novel yellow pigment yCPK (coelimycin) in *Streptomyces coelicolor* A3(2) after deletion of the pathway-specific repressor gene *scbR2* within the *cpk* gene cluster.

1.7.6.4 Overexpression of Activators

As activators, pathway specific regulators may have a positive effect on the expression of gene cluster elements. In many cases activators are involved in SM pathways. Overexpression of these pathway-specific activators, by changing the promoter, may improve the production of SMs (cf Chen et al., 2010; Baltz 2011).

pSOK804 based integration vectors were introduced into *S. noursei* ATCC1144 (wild type) to determine a putative increase of nystatin production. While there was no effect on *nysRI* and *nysRIII*, *ermE**p based overexpression of the regulatory gene *nysRII* resulted in a 21 % increase of nystatin production (cf Sekurova et al., 2004). The addition of second copies from tylS and tylR were reported to increase the production of tylosin in *Streptomyces fradiae*, when overexpressed under transcriptional control of the ermE* promoter (cf Stratigopoulos et al.,2004). By overexpression of an activator within a PKS gene cluster of *Streptomyces ambofaciens* ATCC23877, Laureti et al. (2011) reported the production of stambomycins, novel macrolides with antitumor activity.

1.7.6.5 Construction of Genome-reduced Strains

Combination of the whole genome sequencing and progress in synthetic biology provided new possibilities to design novel biological systems with desirable properties. It has become evident, that a bacterial genome contains large blocks of non-essential genes. Hence, specific deletion of genes that are not essential for cellular metabolism can lead to new advantageous properties of the cell. Thus, genome minimization has become an important strategy for the construction of “super-hosts” for heterologous production of SMs. Minimizing of bacterial genomes can lead to increased genome stability, reduced production of unwanted byproducts, improved growth and streamlined metabolic pathways. Engineering of genome-reduced hosts can also improve the production of (novel) SMs (cf Weber et al., 2015; Gomez-Escribano and Bibb, 2011; Gao et al., 2010; Lee et al., 2009). Through comparative metabolite profiling, culture extracts of genome reduced strains have been shown to yield simplified HPLC chromatograms. A simple extracellular metabolite profile or low metabolite background can therefore make it easier to detect novel compounds or compounds produced at low levels (cf Gomez-Escribano and Bibb, 2011).

1.8 Background Information on the Study Material

1.8.1 Streptomyces noursei NDA59

S. noursei ATCC 11455 (wild type) is a producer of the antifungal polyene antibiotic nystatin (Nys). In this study the mutant NDA59 was used for experiments on triggering the antibiotic production. SNOR NDA59 is an in-frame deletion mutant based on the wild type, where a large part of the gene required for initiation of Nys biosynthesis has been deleted. Therefore, *S. noursei* NDA59 can no longer synthesize Nys, which is the major SM produced by *S. noursei* (cf Brautaset et al., 2003). The genome of *S. noursei* has been completely sequenced, revealing at least 43 BGCs (unpublished data).

1.8.2 Streptomyces venezuelae JZ2

S. venezuelae ATCC 10712 (wild type) is a producer of the antibiotics chloramphenicol (Cml) and jadomycin (Jad). SVEN JZ2 is a recombinant strain unable to biosynthesize Cml and Jad due to the deletions of particular biosynthetic and regulatory genes (cf Sekurova et al., 2016). The genome sequence of *S. venezuelae* is available (cf Pullan et al., 2011). It encodes, besides Jad and Cml clusters, 28 BGCs (unpublished data).

1.8.3 Plant extracts

According to the Pharmacopoeia Europaea (2017), extracts from medicinal plants (Plantarum medicinalium extracta) are defined as liquid, semisolid or solid preparations that are extracted from fresh or dried herbal drugs (plantae medicinales) with the help of an appropriate solvent.

According to the physical condition plant extracts are differentiated into the three major groups praeparationes fluidae ab extractionae, extracta spissa and extracta sicca.

Liquid extracts (praeparationes fluidae ab extractionae) are prepared by maceration, percolation (at room temperature) or solving of a viscous or dry extract with an appropriate solvent and dry extracts (extracta sicca) are defined as solid preparations, that are obtained by evaporation of the solvent used for the extract preparation.

1.8.3.1 Quality of Plant Extracts

The quality of the plants affects the quality of the herbal drug and thus also the quality of herbal preparations. Major aspects, that have an impact on the quality of the extract are the selection of the starting material, the origin of the starting material and the production of the herbal drug material.

Plant material can be obtained by wild harvesting or farming. Hence plants grow on different places, they can be collected by different gatherers. Determination mistakes of gatherers and the heterogeneity of the plant material due to different growth places and growth conditions are the main disadvantages of wild harvesting. In contrast, farming may provide high quality plant material with almost constant composition.

As already mentioned, the collected plant material can vary in its composition. The composition of compounds and SMs is dependent on the genetic variability of the plant, environmental factors like climate, soil conditions, fertilization, the growth state of the plant and also on the harvest time.

Post-treatments like washing or peeling, the type and terms of drying, grinding and also the storage of the drug material affect the SM composition of the drug material (cf Hänsel & Spiess, 2010; Veit, 2010).

1.8.3.2 Extraction Method

The chemical composition and therefore also bioactivity of an extract is influenced by many factors (see chapter 1.8.3.1) and especially dependent on the extraction method.

The extraction of herbal SMs is a solid sample extraction. Extraction enables dissolving of extractive components out of destroyed cells and out of intact cells by diffusion. For an effective extraction method, the Ph. Eur. requires a small and homogenous particle size of the sample.

There are different extraction methods like maceration and percolation, which are performed at room temperature and digestion and Soxhlet-extraction, which are performed at 40-50 °C. As compounds of herbal extracts are very sensitive, percolation as an exhaustive extraction technique is the method of choice to prepare plant extracts.

As a time saving option, the Accelerated Solvent Extraction (ASE) was the method of choice for this project. The ASE is a rapid and automated method, which allows an efficient extraction of organic compounds from solid and semisolid matrices. Common solvents, like MeOH in this project, may be used for extraction. The extraction is performed at elevated temperature and pressure to increase efficiency of the extraction.

Evaporation yields the concentrate that is later dried to the native dry extract. All adjustments of the extraction process can be found in methods 4.2 (cf Mottaleb & Sarker, 2012; Heilmann, 2010; Veit, 2010).

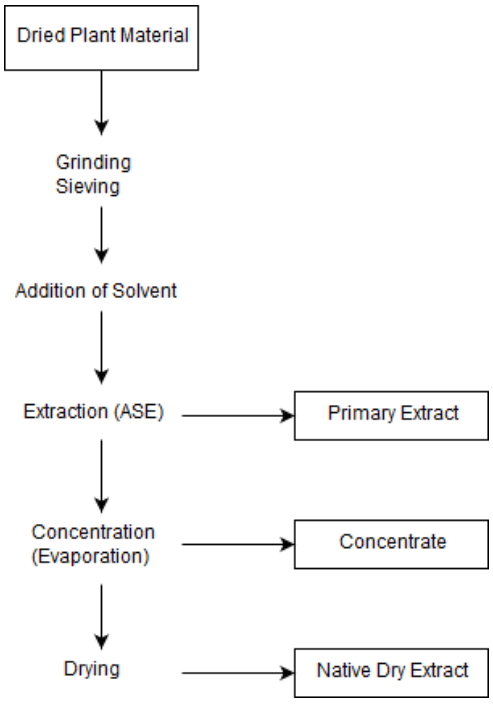


Figure 9: Simplified scheme of the preparation of a dry extract.

1.8.3.3 Selection of the Solvent

A solvent is meant to dissolve desired components of the herbal drug. As is well known, the selection of the solvent is dependent on solubility parameters and polarity of the desired target compound and the solvent itself. The content of extractive compounds and the drug extract ratio (DER) are dependent on the polarity of the solvent. To maximize the yield of a specific target compound, the solubility parameters of the compound and the solvent should be equal or at least as close as possible.

In addition, pharmaceutical and food regulations are also a limiting factor for the selection of the solvent, as well as the environmental burden, operational safety and operating efficiency.

Therefore the choice of the extractant is dependent on parameters like quantity and rate of extraction, extraction period, variety of extracted constituents and the handling of the extract.

Generally, mixtures of ethanol or methanol and water are used as solvents, since these mixtures enable the extraction of a broad range of bioactive compounds. As an intermediate stage, the primary extract can also be prepared with methanol or organic solvents like acetone, ether or hydrocarbons (cf Atanasov et al, 2015; Veit, 2010; Eloff 1997).

In this project Methanol, an ambiphilic solvent and therefore dissolving a broad spectrum of hydrophilic and also lipophilic components, is the extractant of choice, in order to achieve a variety of constituents of the plant material. The chance to affect bacterial secondary metabolism should increase with a larger variety of extracted compounds.

The preparation of the dry extracts and the corresponding stock solutions of this project are described in chapter 4.2, 4.2.1 and 4.2.2.

1.8.3.4 Effect of Herbal Extracts

Extracts from herbal drugs are complex mixtures of various compounds.

Veit (2010) differentiates analytical markers, active markers and active pharmaceutical ingredients. Analytical markers are non-ubiquitous sources and they are important for pharmaceutical quality assurance and analytical purposes. Active markers are compounds that affect activity of an extract for example by improving resorption, stability or solubility. Compounds that have a study-proved biological effect are called active pharmaceutical ingredients, for example alkaloids, anthranoids or cardiac glycosides.

Atanasov et al (2015) also refer to plant extracts as complex mixtures of various active, partially active and inactive compounds. Different approaches can be used to identify bioactive compounds and to characterize the extract, for example bioactivity-guided fractionation, metabolic profiling, direct phytochemical isolation and synergy-directed fractionation. Sometimes a single bioactive compound can be identified, but it is also possible that the effect of an extract results from interactions between various compounds.

1.8.3.5 Induction of Culture Extracts with Extracts from Medicinal Plants

As already mentioned in chapter 1.7 and its subchapters, there are many factors that influence SM production of a bacterial strain.

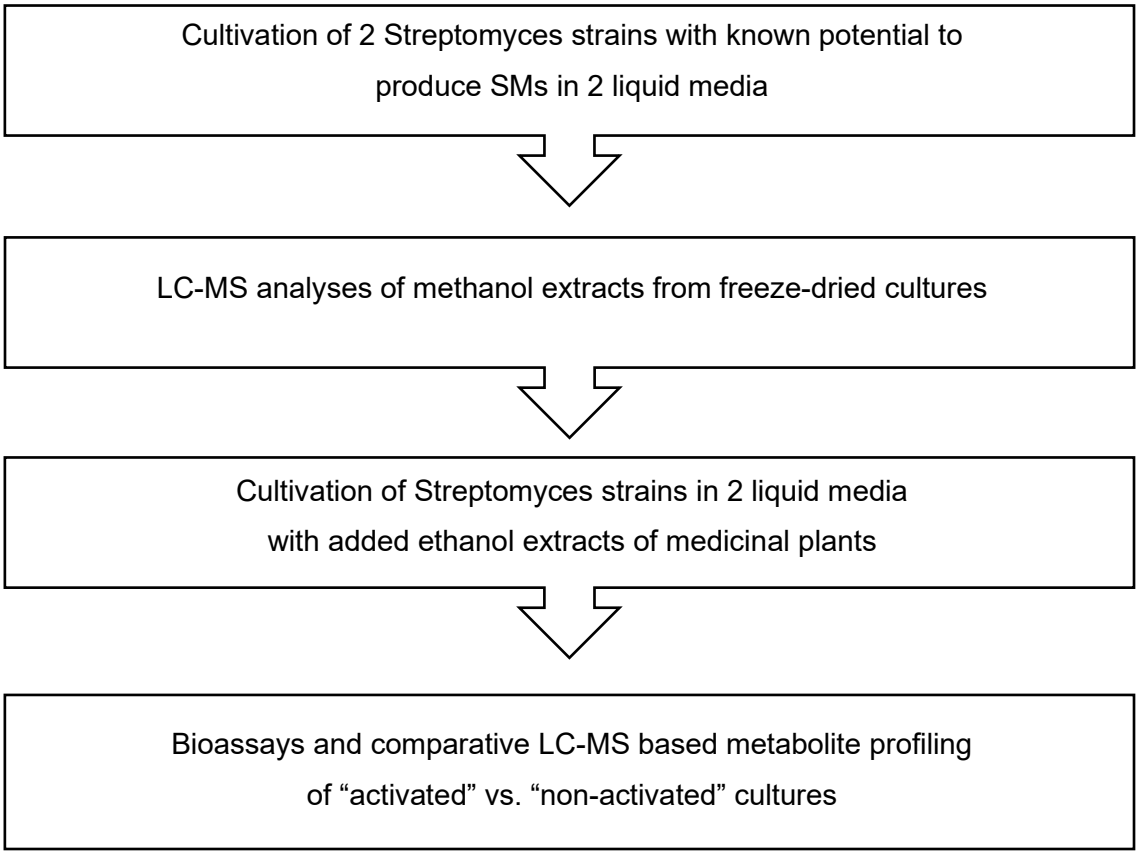
Plants are living species with a primary and secondary metabolism. Hence living plants produce natural products and their SMs are highly conserved molecules it is obvious that these herbal SMs serve specific biological functions within specific pathways of organisms (cf Atanasov et al, 2015; Hunter, 2008).

Reported by Moore et al (2012) and also referred to by Zarins-Tutt et al (2016), phytochemical compounds may affect SM production of microorganisms. The plant derived flavonoid Quercetin has a slight inducing effect on the production of actinorhodin (ACT) and undecylprodigiosin (RED) by *S. coelicolor*. Likewise, resveratrol, a phytoalexine, has been reported to affect actinorhodin production of this *Streptomyces* strain.

Based on these facts, it was decided to test the effect of herbal extracts derived from well characterized medicinal plants on fermentation cultures of two Streptomyces strains.

2 Aim of the Work

2.1 Activation of Secondary Metabolism in Streptomyces Bacteria by Extracts of Medicinal Plants



Society of today has to face increasing problems of multidrug resistant bacteria. Hence, the search for new (bioactive) secondary metabolites (SMs) has become a major goal of science in this postgenomic era, to contain and fight these problems.

Altering of chemical and physical conditions, as well as genetic modification approaches have shown that small changes in culturing can affect antibiotic biosynthesis. Hence there are many diverse ways to trigger SM production of microorganisms, specific manipulation of fermentation cultures, affecting the associated biosynthetic gene clusters (BGCs), may reveal many novel bioactive compounds. But the research on conditions required to trigger the expression of new or silent SMs is still in its infancy.

The goal of this diploma work was to trigger SM production of two engineered and well researched *Streptomyces* strains and to detect potential novel SMs or changes in the metabolic profile of the culture extracts. In this project *S. venezuelae* JZ2 and *S. noursei* NDA59, were meant to be cultivated in two different liquid media. As HDAC inhibitors and other chemical small-molecule elicitors have shown stimulatory and inhibitory effects

on the expression of biosynthetic genes, ten well researched medicinal plants should be processed to ethanol extracts and then added to the liquid cultures during fermentation. It should be promising that not only a single component of the extracts, but also the extracts as a whole could manipulate the SM biosynthesis. In *Streptomyces noursei NDA59* a gene knockout of the PKS (polyketide synthase) in cluster C14 should be performed, to exclude false positive results in the following detection methods. Comparative paper disc assays should detect possible antibiotic and antimycotic inhibition zones of the extracts and LC-MS analyses should point out the differences and changes in the substance profile of triggered culture extracts in comparison to a blind value without added herbal ethanol extract.

The extracts of fermentation cultures should also be provided to other research projects, using different bioassay methods to maybe detect other effects than the antibiotic or antimycotic effects described here.

3 Materials

3.1 Plant Material

Pharmaceutical-grade medicinal herbal drugs were purchased from the company Kottas (Kottas Pharma GmbH, Eitnergasse 8, A-1230 Wien). Powdered material of the following ten medicinal plants and the corresponding herbal drug was used for the preparation of plant extracts (see methods 4.2).

Extract name	**Parent plant**	**Herbal drug**
A	*Betula pendula*	Betulae folium
B	*Calendula officinalis*	Calendulae flos
C	*Chelidonium majus*	Chelidonii herba
D	*Cola nitida*	Colae semen
E	*Digitalis purpurea*	Digitalis purpureae folium
F	*Hypericum perforatum*	Hyperici herba
G	*Glycyrrhiza glabra*	Liquiritiae radix
H	*Rheum palmatum*	Rhei radix
I	*Rosmarinus officinalis*	Rosmarini folium
J	*Valeriana officinalis*	Valerianae radix

Table 5: shows the parent plant, the corresponding herbal drug used in this project and the nomenclature of the plant extracts during this project.

The letters from A-J were used for the nomenclature of herbal extracts, corresponding stock solutions and also for the marking of induced culture extracts that should be prepared later.

3.2 Bacterial Strains and Plasmids

3.2.1 Bacteria

Bacteria	Relevant Properties	Reference
E. coli DH5α	General cloning host	Lab inventory; Sekurova et al., 2016
E. coli ET12567	Strain used for conjugational DNA transfer with the helper plasmid pUZ8002 (KanR, CmR)	Lab inventory; Sekurova et al., 2016
B. subtilis	Test organism for bioassay	Lab inventory
C. albicans	Test organism for bioassay	Lab inventory
S. cerevisiae	Test organism for bioassay	Lab inventory
SNOR-CN	PKS deleted in Cluster C14	This project
SNOR NDA59	Wild type derivative with *nysA* in-frame deletion, nystatin non-producer	Lab inventory; Zotchev et al., 2003
SVEN JZ2	Cml gene cluster deletion in the JZ1 mutant; Cml and Jad non-producer	Lab inventory; Sekurova et al., 2016

Table 6: shows the bacterial strains used in this project and a short background information deriving from the corresponding reference.

3.2.2 Plasmids

Plasmid	Relevant Properties	Reference
pSOK201	Used to create the KO construct; Replication initioator protein gene, AmR, RP4 oriT, ColEI replication origin	Lab inventory; Sekurova et al. 2016
pSOK201 (3.1 kb fragm)	Used for KO of PKS in C14 of SNOR NDA59; replication initiator protein gene, AmR, RP4 oriT	This project
pSOK806	Used as a control for KO; ColEI replication origin, AmR, RP4 oriT, attP, int, ermEp*	Lab inventory

Table 7: shows plasmids used in this project and corresponding background information.

3.3 Primer

Primers were designed with the software tool Clone Manager 6 (see methods 4.8) and distributed by Eurofins Genomics, Ebersberg.

Forward Primer:

5´- GCAGGAATTCGCCTGGATGAAGAGTATTGG – 3´ (Oligoname = C14eryA-F)

Reverse Primer:

5´- CAGCCAAGCTTATGGTCGAAGACGACGGTAG – 3´ (Oligoname = C14eryA-R)

3.4 Media and Trace Element Solutions

3.4.1 Media for Cultivation of *Streptomyces*

ISP4

Soluble Starch .....10,0 g

Dipotassium Phosphate .....1,0 g

Magnesium Sulfate USP .....1,0 g

Sodium Chloride .....1,0 g

Ammonium Sulfate .....2,0 g

Calcium Carbonate .....2,0 g

Ferrous Sulfate .....1,0 g

Manganous Chloride.....1,0 g

Zinc Sulfate.....1,0 g

Agar.....20,0 g

dH_2O.....up to 1000,0 ml

pH 7.2 ± 0.2

ISP2

Yeast Extract .....4,0 g

Malt Extract.....10,0 g

Dextrose .....4,0 g

Agar.....20,0 g

dH_2O.....up to 1000,0 ml

pH 7.0 ± 0.2

SFM

Mannitol.....20,0 g

Soy flour .....20,0 g

Agar.....20 ,0g

dH_2O.....1000,0 ml

Mannitol was dissolved in 400 ml of dH_2O by short agitation. Soy flour was dissolved in 600 ml of water by constant agitation and at a temperature of 90-95°C for 30 min. After mixing the suspensions the agar was added. The medium was supplemented with $CaCl_2$ up to a concentration of 60 mM for conjugation experiments.

TSB

CASON Bouillon .....30,0 g

dH_2O.....up to 1000,0 ml

M5319

Glycerol .....30,0 g

Caseine Peptone .....2,0 g

Dipotassium hydrogenphosphate.....1,0 g

Sodium chloride.....1,0 g

Magnesium sulfate * 7 H_2O.....0,5 g

Trace element Solution for M5319 .....5,0 ml

dH_2O.....up to 1000,0 ml

pH 7.3 – 7.5

PM4-1

Gluose ..... 15 g

Soy Meal..... 15 g

Corn Steep Solids ..... 5 g

Calcium carbonate ..... 2 g

TMS1 (trace elements solution) ..... 6 ml

dH_2O ..... up to 1000 ml

A glucose solution (150 g/l dH_2O) was prepared and autoclaved. The corn steep solids were dissolved in 890 ml of dH_2O by agitation with a magnetic stirrer. Later soy flour and $CaCO_3$ were added. After 5 min of agitation the solution was autoclaved. After sterilisation, 100 ml of the autoclaved glucose solution and 6 ml of TMS1 solution were added. When in use, the medium was stirred while taking it out of the bottle.

3.4.2 Media for Cultivation of *E. coli* and *B. subtilis*

L Agar

Agar ..... 10 g

Difco bacto tryptone ..... 10 g

Sodium chloride ..... 5 g

Glucose ..... 1 g

dH_2O ..... up to 1000 ml

L Broth

Difco bacto tryptone ..... 10 g

Difco yeast extract ..... 5 g

Sodium chloride ..... 5 g

Glucose ..... 1 g

dH_2O ..... up to 1000 ml

2XYT Medium

Difco bacto tryptone 16g

Difco bacto yeast extract..... 10g

Sodium chloride 5g

dH_2O ..... up to 1000ml

3.4.3 Media for Cultivation of *C. albicans*

YPD Agar

Bacteriological Peptone .....20 g

Yeast extract.....10 g

Glucose .....20 g

Agar.....15 g

dH_2O.....up to 1000 ml

YPD Broth

Bacteriological Peptone .....20 g

Yeast extract.....10 g

Glucose * H_2O .....20 g

dH_2O.....up to 1000 ml

3.4.4 Trace Element Solutions

Trace element solutions were used as supplements for diverse media. Ingredients were dissolved in water by heating and stirring. The solutions were sterilized at 121 °C for 15 minutes and added to the media in the sterile bench. The solutions were stored at room temperature.

TMS1 (trace element solution)

$FeSO_4$ * 7 H_2O.....5000 mg

$CuSO_4$ * 5 H_2O.....390 mg

$ZnSO_4$ * 7 H_2O.....440 mg

$MnSO_4$ * H_2O.....150 mg

Na_2MoO_4 * 2 H_2O.....10 mg

$CoCl_2$ * 6 H_2O .....20 mg

1 M HCl.....50 ml

dH_2O.....up to 1000 ml

Trace Element Solution for M5319 media

$CaCl_2$ * 2 H_2O .....3,0000 g

Fe-III-citrate * H_2O .....1,0730 g

$MnSO_4$ * H_2O .....0,2230 g

$ZnCl_2$ (or $ZnSO_4$).....0,1000 g

$CuSO_4$ * 5H_2O.....0,0250 g

NaB_4O_7 * 10 H_2O .....0,3790 g

$CoCl_2$ * 6 H2O.....0,0040 g

$NaMoO_4$ * 2 H2O .....0,0117 g

dH_2O.....up to 1000 ml

3.5 Stock Solutions

Glycerol 20 %(V/V) Stock

Glycerol .....200 ml

dH_2O.....800 ml

1 M $CaCl_2$ Solution

$CaCl_2$ .....222 g

dH_2O.....up to 1000 ml

3.6 Buffers

Lysis Buffer for the isolation of total DNA from Streptomyces

200 mM Tris HCl (pH 8.0).....1 ml

100 mM EDTA (pH 8.0) ..... 1 ml

1.2 % Triton X-100.....120 µl

Lysozyme .....200 mg

dH_2O.....up to 10 ml

Aliquotes of 500 µl of the lysis buffer were stored at -20 °C.

TBE Buffer (10x)

Tris base.....108 g

Boric acid.....55 g

0.5M EDTA (pH 8.0) ..... 51 ml

dH_2O.....up to 1000 ml

TSS Buffer

PEG 8000.....5,0 g

1 M Magnesium chloride.....1,5 ml

DMSO.....2,5 ml

dH_2O.....up to 50,0 ml

The buffer was filter-sterilised and stored at +4°C.

3.7 Antibiotics

Powdered and pharmaceutical-grade powdered antibiotics were obtained by the company Sigma Aldrich Handels GmbH (Marchettigasse 7/2, 1060 Wien).

Ampicillin (AMP)

Apramycin (AM)

Chloramphenicol (CML)

Hygromycin (HYG)

Kanamycin (KAN)

Nalidixic Acid (NAL)

Thiostrepton (THIO)

3.8 Kits

Distributor	Title	Application
Quiagen GmbH, Hilden	Dneasy Tissue Kit	Isolation of total DNA
Promega GmbH, High-Tech-Park, Mannheim	Wizard PLUS SV Minipreps DNA Purification System	pDNA purification
Sigma-Aldrich Handels GmbH, Wien	GC-rich PCR Kit	PCR
New England Biolabs GmbH, Frankfurt am Main	Taq PCR Kit	PCR
New England Biolabs GmbH, Frankfurt am Main	Q5-High-Fidelity 5X Master Mix	PCR
Anopoli Biomedical Systems, Eichgraben	DNA Clean & Concentrator TM-5-Kit	DNA Purification
Anopoli Biomedical Systems, Eichgraben	Zymoclean Gel DNA Recovery Kit	Gel Recovery

Table 8: shows the application method of different Kits; includes information of the distributors.

3.9 Gels

Standard Agarose Gel 0,8 %

Agarose .....3,2 g

TBE buffer (1x) .....400,0 ml

GelRed (10,000x) .....20,0 µl

Agarose was dissolved in TBE buffer and microwaved to get a clear solution. The gel was stored at 60 °C in the incubator, to stay liquid for immediate use. Before pouring the gel the dye GelRed was added.

4 Methods

4.1 Preparation of Antibiotic Stock Solutions

Powdered antibiotics, purchased from the company Sigma Aldrich (see materials 3.7), were used to prepare antibiotic stock solutions by dissolving in the corresponding solvent. Stock solutions had to be prepared under the sterile bench. When water was used as a solvent the solutions had to be sterile filtered using a 0,22 µm filter. Preparations with DMSO were performed with gloves in the chemical hood. Stock solutions were stored at -20 °C because of heat and light sensitivity. While using the stock solutions they were kept on ice or in cold boxes.

Antibiotic	**Concentration**	**Solvent**
AM	100 mg/ml	Sterile dH_2O
KAN	40 mg/ml	Sterile dH_2O
CML	25 mg/ml	96 % EtOH
THIO	30 mg/ml	DMSO
HYG	100 mg/ml	Sterile dH_2O
NAL	30 mg/ml	0.1 M NaOH
AMP	100 mg/ml	Sterile dH_2O

Table 9: shows the concentration of antibiotic stock solutions and the corresponding solvent.

4.2 Preparation of Plant Extracts

Ten different medicinal plants were chosen because of well research and information about their active components especially according to their application (see materials 3.1). Dried and powdered plant material of these medicinal plants was used for the preparation of plant extracts, which were expected to induce secondary metabolism of fermentation cultures of two Streptomyces strains in two different media. It was unknown if a single component of the extracts or the whole composition would cause an effect on the fermentation cultures and it was also possible that the plant extracts would cause the opposite effect.

The extracts were prepared as follows: In a first step liquid methanol extracts of the powdered herbal drugs (A-J) were prepared by accelerated solvent extraction (ASE) (see methods 4.2.1), yielding the primary extracts of the plant material. Out of these MeOH extracts the corresponding dry extracts were obtained by evaporation. The dry extracts

were used to prepare EtOH stock solutions (A-J) of the plant extracts in a final concentration of 20 mg/ml.

4.2.1 Accelerated Solvent Extraction

As already mentioned in chapter 1.8.3.2 the ASE is a fast and automated method allowing an efficient extraction of organic compounds from solid and semisolid matrices with common solvents at elevated temperature and pressure (cf Mottaleb & Sarker, 2012).

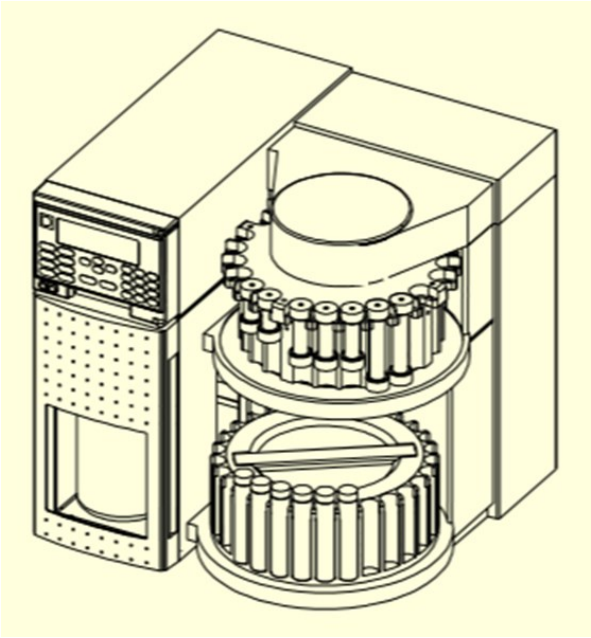


Figure 11: ASE 200 accelerated solvent extractor © 1999 Dionex Corporation.

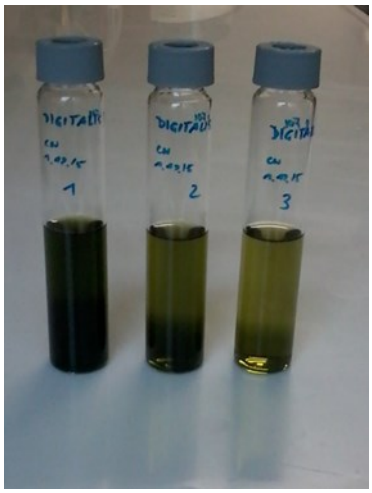


Figure 10: Collection tubes. Three 20 ml vials were used for the collection of one plant extract.

The ASE was performed with the ASE 200 accelerated solvent extractor from the company DIONEX. Depending on the weight and consistency of the powdered plant material, an initial weight of about 3,00 g, 4,00 g or 5,00 g of the powdered herbal drug was mixed up with siliceous earth. The mixture was applied to 40 ml steel-cells and extracted with 100 % MeOH into three 20 ml collection vials at a pressure of 1500 Psi and a temperature of 40 °C.

Data of the method adjustment are listed in table 10.

Preheat	40 °C, 5 min
Heat	40 °C, 5 min
Static	5 min
Flush	60 % vol
Purge	60 sec
Cycles	1
Solvent	100 % MeOH
Pressure	1500 Psi

Table 10: adjustment of the ASE method.

4.2.2 Stock Solutions of Plant Extracts

EtOH stock solutions of plant extracts (A-J) were meant to have a final concentration of 20 mg/ml. An initial weight of 1,00 g of herbal dry extract was therefore put into a 5 ml volumetric flask and filled up with 70 % EtOH, that should dissolve the material of the dry extracts. If needed, the ultrasonic bath should dissolve remaining solid material. The obtained stock solutions were filter sterilized and stored at -20 °C.

4.3 Preparation of Media

Media were prepared by dissolving their corresponding ingredients in 1000 ml dH_2O by heating and stirring. In some cases, the pH had to be adjusted. After getting a clear solution, media got sterilized at 121 °C for 15 min. For immediate use solid media was poured into petri dishes. For storage liquid and solid media were kept in sterile conical flasks at +4 °C. To reuse solid media, it was melted in the microwave. Preparation methods of special media can be found at the corresponding recipes in chapter 4.4.

4.4 Culture Terms and Conditions

4.4.1 Cultivation of *Streptomyces*

Liquid cell cultures of *Streptomyces* bacteria were performed in conical flasks with 15 ml TSB in the shaking incubator at 28 °C and 250 rpm overnight. Media, when appropriate, were supplemented with antibiotics using the following concentrations:

Antibiotic	**Concentration**
AMP	100 µg/ml medium
AM	50 µg/ml medium
CML	25 µg/ml medium
HYG	100 µg/ml medium
KAN	20 µg/ml medium
NAL	30 µg/ml medium

Table 11: concentration of antibiotics added to the cultivation media of Streptomyces strains.

For the cultivation of *Streptomyces* bacteria on solid media 100-200 µl of spore suspension were spread on petri dishes, containing 25 ml of medium. The media were supplemented with the amount of antibiotics listed in table 11. Petri dishes were incubated at 28 °C for 3-7 days, depending on the rate of growth of the strains.

For sporulation of *Streptomyces*, SVEN JZ2 was grown on ISP4 while SNOR NDA59 and the SNOR-CN mutant were grown on ISP2 medium.

4.5 Storage of microorganisms

4.5.1 Spore Suspensions of *Streptomyces*

Frozen *Streptomyces* cultures were melted. 100 µl of the cultures were plated out on the corresponding agar plates, followed by an incubation at 28 °C for 3 to 7 days, until sporulation level was high enough. After sporulation, spores were washed out with 4,5 ml of Glycerol 20 %(V/V) by scratching the surface carefully with the pipette. The suspension was filtered through sterile cotton wool to remove agar and mycelia. The filtered suspensions were transferred in tubes and stored at -80 °C.

4.5.2 Glycerol Stocks

20 % Glycerol Stocks were prepared for *E. coli* DH5a, *E. coli* ET12567, *B. subtilis*, *C. albicans* and *S. cerevisiae*. Aliquotes of these glycerol stocks were used to inoculate overnight culture (ONCs) for bioassays.

In a first step ONCs of the mentioned microorganisms were prepared. 20 ml of LB or YPD media and 50 µl of bacteria or yeast were added to conical flasks. *B. subtilis* and *E. coli* were incubated at 37 °C, while *S. cerevisiae* and *C. albicans* were incubated at 30 °C and 250 rpm in the shaking incubator. In a second step the ONCs of the bacteria and molds were spinned down in a centrifuge for 5 min and 4000 rpm to get a pellet. The supernatant was removed and the pellet was resuspended in 20 ml of 20 % Glycerol. 500 µl of this cell suspension were transferred into cryotubes and filled up to 1 ml with another 500 µl of 20 % Glycerol. After resuspending for homogenization, the 1 ml cell suspension stocks were stored at -80 °C.

4.6 Isolation of Plasmid and total DNA

4.6.1 Isolation of total DNA from *Streptomyces*

The Isolation of total DNA from *S. noursei* was performed with the Dneasy Tissue Kit from the company Quiagen. It should be used as template DNA for the production of vector inserts.

In a first step an ONC from well sporulated colonies of SNOR NDA59 was prepared in 2 ml TSB media and incubated on the shaker at 28 °C and 250 rpm overnight. To prepare a pellet, 1,5 ml of the ONC were mixed with 1 ml 50 µM sterile EDTA solution (pH 8) and spinned down at 4500 rpm for 3 min in the centrifuge. The supernatant had to be discarded completely. To wash the pellet, it was resuspended in 1 ml 50 M EDTA. After another centrifuging step at 10000 rpm EDTA was discarded. For cell lysis the pellet was resuspended in 180 µl Lysis Buffer. The suspension was incubated at 37 °C for 15 min and mixed by tipping the tube every 5 min. For DNA isolation 25 µl Proteinase K and 200 µl of Buffer AL were added and mixed by pipetting up and down gently. The mix was incubated at 55 °C for 30 min. 200 µl 96 % EtOH were added and the mixture was applied onto a column placed in a collection tube and centrifuged at 10000 rpm for 2 min. EtOH should help to bind nucleic acid to the column. The column then was washed twice, by application of 500 µl buffer AW1 and a spinning step of 10000 rpm for 1 min and a following application of 500 µl AW2 spinned down at 10000 rpm for 3 min. In the last step the column was transferred to a new Eppendorf tube and 125 µl preheated buffer AE (50 °C) were applied on the column. After incubation at room temperature for

15 min the sample was centrifuged for 1 min at 10000 rpm. The column was discarded and 2,5 µl of the DNA sample had to be checked by gel electrophoresis afterwards. Purified solutions containing DNA were stored at -20 °C.

4.6.2 Isolation of pDNA from *E. coli*

The Isolation of pDNA from *E. coli* was performed with the Wizard Plus SV Minipreps DNA Purification System.

1,5 ml of ONC from *E. coli* were centrifuged at 10000 rpm for 5 min. After discarding of the supernatant, the pellet was resuspended in 250 µl Cell Resuspension Solution. 250 µl of Cell Lysis Solution were added and mixed by inverting the tube. Then 10 µl of Alkaline Protease Solution were added and after mixing the sample was incubated for 5 min at room temperature and the solution clarified. 350 µl Neutralization Solution were added and mixed that proteins precipitate. Then the suspension was centrifuged at 10000 rpm for 5 min. The clear supernatant was applied onto a spin column placed in a collection tube. The column was centrifuged at 10000 rpm for 1 min at room temperature and the flowthrough was discarded. The column was washed with 750 µl of Wash Solution, centrifuged for 1 min at 10000 rpm. After discarding the flowthrough the washing step was repeated with 250 µl of Wash Solution and centrifuged for 2 min at 10000 rpm. The dry column was transferred to a sterile Eppendorf tube and 75 µl of warmed up Nuclease-Free Water were applied. After incubating for 10 min (to increase the yield) the purified pDNA solution was spun down for 1 min and stored at -20 °C. Gel electrophoresis was used to check the quality of purified pDNA.

4.7 PCR

For PCR reaction the Kit Q5-High-Fidelity 2X MASTERMIX from New England Biolabs was used. The composition of the PCR reaction was prepared as shown in table 12. The reaction was carried out using the PCR programme shown in table 13.

Component	**Volume µl**
dH2O	9 µl
Q5 master mix	12,5 µl
F-Primer	1,25 µl
R-Primer	1,25 µl
t-DNA	1 µl
Total volume	25 µl

Table 12: shows the composition of PCR reaction.

PCR step	**Temperature °C**	**Time sec**	**Cycles**
Initial denaturation	98 °C	30 sec	25
Denaturation	98 °C	10 sec	
Annealing	60 °C	30 sec	
Extension	72 °C	120 sec	
Final Extension	72 °C	120 sec	
Hold	4 °C	-	

Table 13: shows the PCR method.

4.8 Primer Design

Specific primers were designed to enable synthesis and amplification of the knockout (KO) insert CN.

The Primer Design was performed with the help of the software Clone Manager 6. A DNA file of *S. noursei* NDA59 was used in order to select the gene of interest. The KO vector was planned to cover around 70 % of the genes sequence. PCR products for cloning were planned to have compatible ends. Cutting of vector and insert with the same enzymes would enable a connection of the molecules via ligation later. Therefore, cutting sites of specific restriction endonucleases were attached to the 5´ end of each primer sequence. To enable the correct orientation of the insert, it was planned to have two different cutting sites (*EcoRI* and *HindIII*). GCAG (primer 1) and CAGC (primer 2) were

added to the 5´ end of the primer sequences as additional shoulders to facilitate the binding of restriction enzymes. Primer 1 and primer 2 were designed to achieve a melting temperature (T_M) between 60 and 75 °C. The adjustment of the two primers should ensure a maximum difference in melting temperature (T_M) of 5 °C. The sequences of the designed primers can be found in chapter 3.3.

4.8.1 Preparation of Primer Stock and Working Solutions

The primer stock solutions were prepared as prescribed in the manufacturers manual, also indicated at the delivered tubes. The instructions included the amount of water that had to be added to reach a primer stock solution in a concentration of 100 pmol/µl. The content of the tubes was spinned down for 10 sec. and the corresponding amount of dH_2O was added to the tubes (Forward Primer: 274 µl dH_2O; Reversed Primer: 225 µl dH_2O). For the working solutions 5 µl of the stock solution were diluted with 45 µl of dH_2O.

4.9 Product Purification

4.9.1 DNA Purification

Purification from desired DNA products was performed with the Genomic DNA Clean & Concentrator TM Kit from Zymo Research.

Five volumes of DNA Binding Buffer were added to each volume of DNA sample within an Eppendorf tube and mixed by vortexing. The resulting mixture was transferred to a column placed in a collection tube. The column was centrifuged at 10000 rpm for 30 sec and the flowthrough was discarded. 2 washing steps followed by addition of 200 µl of DNA Wash Buffer, followed by centrifuging at 10000 rpm for 30 sec. Then 10 µl DNA Elution Buffer were directly applied to the column and incubated at room temperature for 1 min. The column was then transferred to a sterile Eppendorf tube and centrifuged for 30 sec to elute the DNA. Purified DNA was stored at -20 °C.

4.9.2 DNA Recovery from Agarose Gels

After separation from PCR derived DNA products by gel electrophoresis, bands from the desired samples were cut out from the gel. To improve visibility of the products, this step was performed under UV light. The purification and recovery of the samples from the gel were performed with the Zymoclean TM Gel DNA Recovery Kit.

DNA fragments of interest were excised from the gel under UV light using a scalpel. The pieces containing the DNA sample were put to sterile weighed Eppendorf tubes. The samples were weighed and 3 volumes of ADB were added to each volume of weighed gel. To dissolve the gel, the mixture was incubated at 50 °C (37 °C- 55 °C) for 5 min. The melted solution was transferred to a column placed in an Eppendorf tube and centrifuged at 10000 rpm for 30 sec. When the flowthrough was discarded two washing steps were performed by addition of 200 µl of DNA Wash Buffer and centrifuging at 10000 rpm for 30 sec. The two flowthroughs were discarded. Then 8 µl of DNA Elution Buffer were directly applied to the column and incubated for 1 min to increase the yield. The DNA was eluted into a sterile Eppendorf tube by centrifuging for 30 sec at 10000 rpm and also stored at -20 °C.

4.10 Restriction Digest

Restriction enzymes cut DNA at or near specific recognition sites. In this work these enzymes were used for cloning, to create compatible ends of insert and vector, and also analytics of DNA products. The composition of the mixture was dependent on the total volume of the reaction setup. The mixture included insert or vector, reaction buffer, restriction enzymes and water.

4.10.1 Restriction Digest for Cloning

For cloning, insert and vector were cut at 37 °C for 2 h. Gel electrophoresis was used after restriction digest to analyse the fragments by size and concentration.

Ingredients	**Insert in µl (CN 2045 bps)**	**Vector in µl (pSOK201 3.1 kb fragment)**
DNA	10 µl	5 µl
Cut Smart Buffer (10x)	2 µl	2 µl
EcoRI	0,5 µl	0,5 µl
HindIII	0,5 µl	0,5 µl
dH_2O	7 µl	12 µl
total volume	20 µl	20 µl

Table 14: shows the reaction setup for cloning.

4.10.2 Restriction digest - Analysis of Isolates from transformed Cloning Hosts

For analysis of isolates from transformed cloning hosts, pDNA was cut at 37 °C for 30 min at a total volume of 20 µl. Plasmid isolates from putative clones were checked for their correct composition before transformation to E. coli ET12567. Isolated pDNA from transformed DH5α cells was cut in fragments with the same enzymes to enable a conclusion from 2 predicted pieces of 3.1 kb and 2045 bps to the correct construct.

Ingredients	**Volume µl**
pDNA	2 µl
Cut Smart Buffer (10x)	2 µl
EcoRI	0,5 µl
HindIII	0,5 µl
dH_2O	15 µl
total volume	20 µl

Table 15: reaction setup for assessment of pDNA from cloning hosts.

4.11 Ligation of DNA

Ligation was used to create the vector pSOK201-CN for the construction of the knockout mutant SNOR-CN. Gel electrophoresis was used to analyse and estimate the concentrations of prepared vector and insert. Equal volumes of vector and insert were necessary for an efficient ligation. The enzyme T4 ligase was used for ligation in order to create the right knockout construct. The reaction setup (Table15) was mixed gently, spinned down for 2 sec. and placed on ice overnight to get a graduation of temperature.

Ingredients	**Volume µl**
pSOK201 3.1 kb vector	1,0 µl
CN-insert (2045 bp)	9,0 µl
10x T4 DNA Ligase Buffer	2,0 µl
T4 DNA Ligase	0,5 µl
dH_2O	7,5 µl
Total volume	20,0 µl

Table 16: reaction setup for ligation.

4.12 Gel Electrophoresis

Gel electrophoresis separates biological macromolecules by size. While the charge of molecules, for example a negative charge of DNA fragments, is used to make them move through the gel, the distance of the movement is affected by the size of the fragments, with smaller fragments moving faster than bigger ones. In this project this technique was used to check PCR products, quality and quantity of total DNA, fragments of restriction digest and also to compare concentrations of insert and vector for ligation.

A comb was placed in a casting tray to form pockets and the gel (0,8 % Agarose see materials 3.9) was poured into it, cooled down for 20 min and then covered with TBE (1x) buffer. DNA samples were staggered with 1 µl loading dye and filled up to 10 µl with dH2O (= DNA sample mix). 10 µl of DNA sample mix and 1 µl of DNA ladder were applied to separate wells with a micro pipette. Quick-Load 1 kb Ladder was used as a reference to determine the size of separated DNA molecules. Adjustments for the gel run were 100 V at a runtime for 45 – 60 min. DNA bands were visualized with the imaging device Gel Doc. To view DNA under UV light GelRed was applied to the gel (see materials 3.9). The determination of DNA size was enabled by the use of Quick-Load 1 kb or 100 bp Ladder, showing marker bands as a reference.

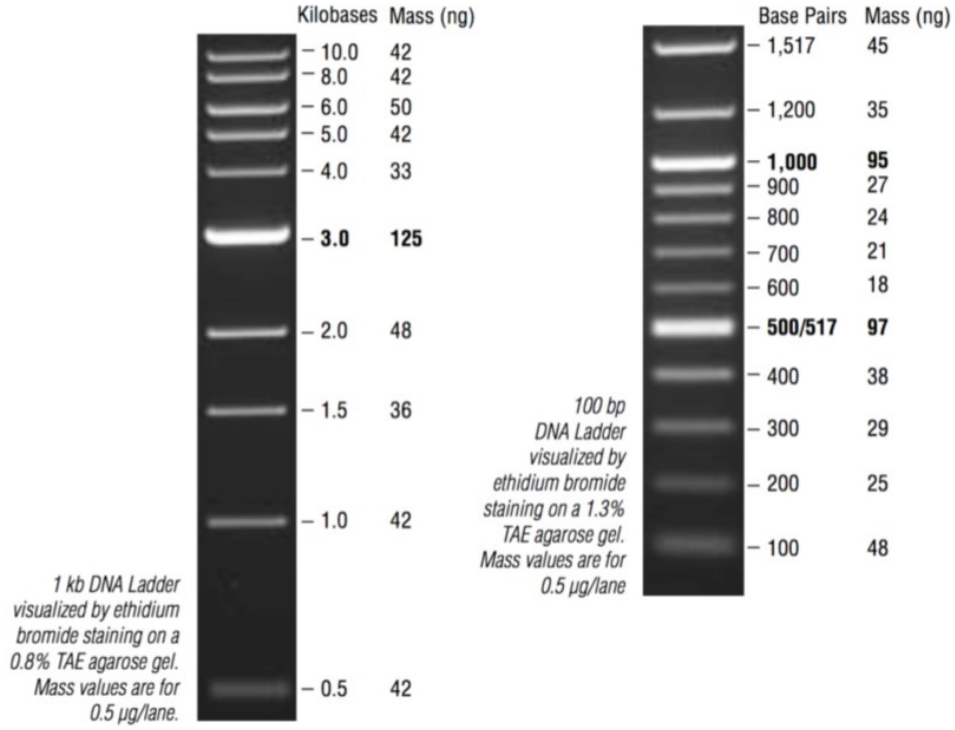


Figure 12: Quick Load® 1 kb and 100 bps DNA Reference Ladders. DNA size was determined by comparison to the marker bands of the reference ladders.

4.13 Transformation of DNA to E. coli and Cloning

4.13.1 Preparation of Chemically Competent Cells

Competent cells of *E. coli* DH5α and *E. coli* 12567 were prepared to achieve efficient transformation.

An ONC culture of *E. coli* was prepared in LB media. After homogenization 50 µl of *the E. coli* ONC were transferred to 50 ml of fresh LB medium. The culture was incubated at 37 °C and grown until an OD_{600} of about 0.2 – 0.5 was reached. (The OD_{600} therefore was controlled every half hour). When the cell density was reached, cells were kept on ice. All following working steps were performed on ice and all used material had to be cooled down. The cell suspension was put into a falcon tube and centrifuged at 3000 rpm for 10 min at 4 °C. After pelleting the supernatant was discarded and the pellet was carefully resuspended in 5 ml cooled TSS buffer (volume of TSS should be 10 % of culture volume). The resulting homogenous suspension was used to prepare aliquots of 100 µl in Eppendorf tubes. They were stored at -80 °C.

4.13.2 Transformation of *E. coli* by Heat Shock

Transformation by heat shock was the method used to introduce the constructed plasmid pSOK201-CN into competent cells of *E. coli*. *E. coli* DH5α was used for cloning and *E. coli ET*12567 was used for conjugation into *Streptomyces* later.

First, frozen competent cells were melted on ice. Then 1 µl of pDNA solution was mixed with 100 µl of competent cells and the mixture was incubated on ice for 15 min. The cells were heat shocked at 42 °C for 45 sec and put back on ice for another 5 min. 500 µl LB medium were added to the samples and mixed gently. Then the samples were put in the shaking incubator at 37 °C and 250 rpm for 40 min. For the selection of transformants, an aliquot of 100 µl of this mixture was plated out on a petri dish containing 25 ml of LA media supplemented with AM (100 µg/ml medium). The rest was spread on a second dish with the same amount of supplemented media. The plates were incubated at 37 °C overnight. For storage and immediate use glycerol stock solutions were prepared (see methods 4.5.2). Isolates from the transformed cloning hosts were analysed with the help of restriction digest (see methods 4.10.2).

4.14 Conjugative DNA Transfer from E. coli to Streptomyces

The methylation deficient *E. coli* ET12567 enables the introduction of foreign DNA into *Streptomyces* as a donor for intergeneric conjugation. The latter is reached when the correct vector contains oriT of *Streptomyces*. In addition, ET12567 cells harbour the helper plasmid pUZ8002, which is a RK2 derivative and contains the tra gene, a function that enables intergeneric transfer of any plasmid containing oriT from *E. coli* ET12567 *to Streptomyces* (cf Du et al.,2012).

After transformation, *E. coli* ET12567 harboured the correct plasmid pSOK201-CN, which should be transferred to *S. noursei* NDA59, in order to achieve a gene knockout, to reduce SM background of *S. noursei* NDA59.

As a first step over night cultures of E. coli ET12567, containing the correct plasmid, were prepared on LA plates. The LA medium was substituted with the three antibiotics AM (15 µg/ml), KAN (20 µg/ml) and CML (25 µg/ml), in order to select only ET12567 cells, containing the right plasmid pSOK201-CN, now carrying an additional AM-resistance. The plates were incubated overnight at 37 °C.

On the next day ET12567 cells of the clone that grew the best were used to prepare cell suspensions in 500 µl 2 X YT medium. 50 µl of a fresh SNOR NDA59 spore suspension were added to 350 µl 2 X YT medium and heat shocked 5 min. After cooling down at room temperature 400 µl SNOR NDA59 spores were mixed with 100 µl of ET12567 cell suspension in an Eppendorf tube. The mixture was centrifuged for 1 min at 4000 rpm. 250 µl of supernatant were discarded and the pellet was resuspended in the remaining 250 µl of supernatant. The suspension then was spread over a SFM agar plate without additional antibiotics, which was then incubated at 28 °C for 16 -18 h.

On the next day the SFM plate was treated with an aqueous antibiotic solution. NAL was added to suppress the growth of *E. coli* and AM to select SNOR-CN (SNOR NDA59 containing the correct plasmid). Prepared of dH2O, 30 µg/ml NAL and 15 µg/ml AM, 1 ml solution was gently spread to the SFM plate with the spatula, avoiding to scratch the spores. The treated plate was dried out for 20 min and later incubated at 28 °C for 5 days.

After 5 days the first transconjugants could be seen on the SFM plate. For a first selection step a transconjugant was picked with a sterile toothpick and transferred to a SFM plate with additional antibiotics by stamping. 30 µg/ml NAL should enable contamination and 15 µg/ml AM again assured the selection of SNOR-CN. The selection plates were incubated until spores appeared.

In a second selection step the transconjugants were plated on an ISP2 plate supplemented with 15 µg/ml AM in order to get good growth and sporulation conditions for SNOR-CN. The plates were again incubated at 28 °C for another 3 days. After the plates were well sporulated, spores were harvested in order to prepare spore suspensions (see methods 4.5.1).

4.15 Gene Knockout

The gene knockout is a result of homologous recombination between the target gene located in the host genome and an internal part of this gene fragment on the plasmid. Via single homologous crossover the plasmid integrates into the chromosome and disrupts the target gene (cf Kieser et al., 2000). Figure 12 demonstrates the mechanism of the gene knockout via homologous recombination.

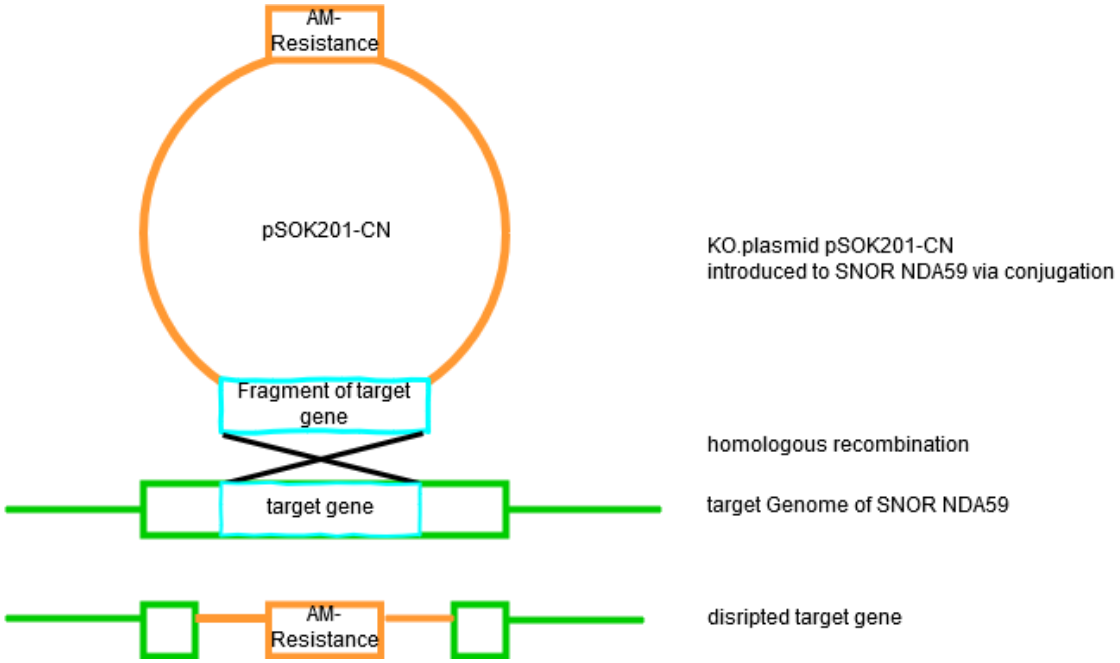


Figure 13: Gene knockout via homologous recombination. The KO vector pSOK201-CN carrying an AM resistance gene disrupts the target gene of SNOR NDA59 and is integrated into the genome of SNOR NDA59. Homologous recombination results in a KO mutant resistant to AM.

The 3.1 kb knockout fragment of pSOK201, carrying an internal part of the target gene, was introduced to Streptomyces via conjugation (see methods 4.14). This fragment of pSOK201 harbours an AM resistance gene. Therefore, homologous recombination between the vector-insert and the target gene results in a knockout mutant, resistant to AM, what enables an antibiotic selection of these mutants. Vector maps of pSOK201, the KO-insert CN and the KO-product pSOK201-CN can be found in the appendix.

The constructs and primers for the gene knockout were designed with Clone Manager 6, replicated with PCR, selected via gel electrophoresis and introduced to *Streptomyces* by conjugation (see 4.7, 4.8, 4.12, 4.14).

4.16 Fermentation

For SM production *Streptomyces* strains were grown in two different liquid media, M5319 and PM4-1 (see materials 3.4.1).

Two ONCs were prepared by applying 100 µl of spores from the *Streptomyces* strain to 15 ml of TSB media within a conical flask and put to the shaking incubator at 28 °C, 250 rpm overnight. Twelve shaking flasks were prepared for fermentation. Ten were signed with media name, *Streptomyces* strain and the letters A-J from the corresponding herbal extract. Two flasks were used for blind values named X and Y. For fermentation 12 shaking flasks were filled with 50 ml of media (M5319 or PM4-1). Then 2,5 ml of starting culture were added to each flask (named with media, Streptomyces strain and added extract A-J). Each of the twelve shaking flasks was prefilled with 50 ml of medium (M5319 or PM4-1). Then 2,5 ml of the starting culture (SNOR-CN or SVEN JZ2) were added. And in a last step 2 µl/ml plant extract (A-J) or 2 µl/ml of 70 % EtOH (X, Y) were applied to the appropriate flasks. The fermentation cultures were put into the shaking incubator for 72 h at a temperature of 250 °C and 250 rpm. The fermentation cultures were applied to sterile falcon tubes and stored at -20 °C until used for extraction.

4.17 Extraction of Secondary Metabolites

Via extraction, SMs from the fermentation cultures should be dissolved and also concentrated. 10 ml of melted and homogenous fermentation culture were transferred into a falcon tube and centrifuged at 5000 rpm for 5 min for a separation into pellet and supernatant. To dissolve SMs, the pellet was extracted with 3 ml MeOH one time by vortexing for 5 min. After centrifugation at 5000 rpm for 5 min the upper MeOH phase was divided into two parts (A and B) that were applied into 5 ml pear shaped flasks. Part A was evaporated to a dry extract and resuspended in 500 µl DMSO, while the volume of part B was only reduced to an amount of approximately 500 µl by evaporation. The supernatant was extracted in 3 steps. The extraction of the supernatant with 1 ml EtAc. by vortexing for 1 min, and a following centrifugation step yielded the sediment D1 and the upper phase U1. The sediment D1 was extracted and centrifuged in an equal step and yielded U2. In a final step the extraction and following centrifugation of the sediment D2 yielded the supernatant U3. The obtained upper phases U1, U2 and U3 were

collected in a 5 ml pear shaped flask, evaporated to a dry extract and resuspended in 500 µl MeOH. The samples were stored in Eppendorf tubes at -20 °C.

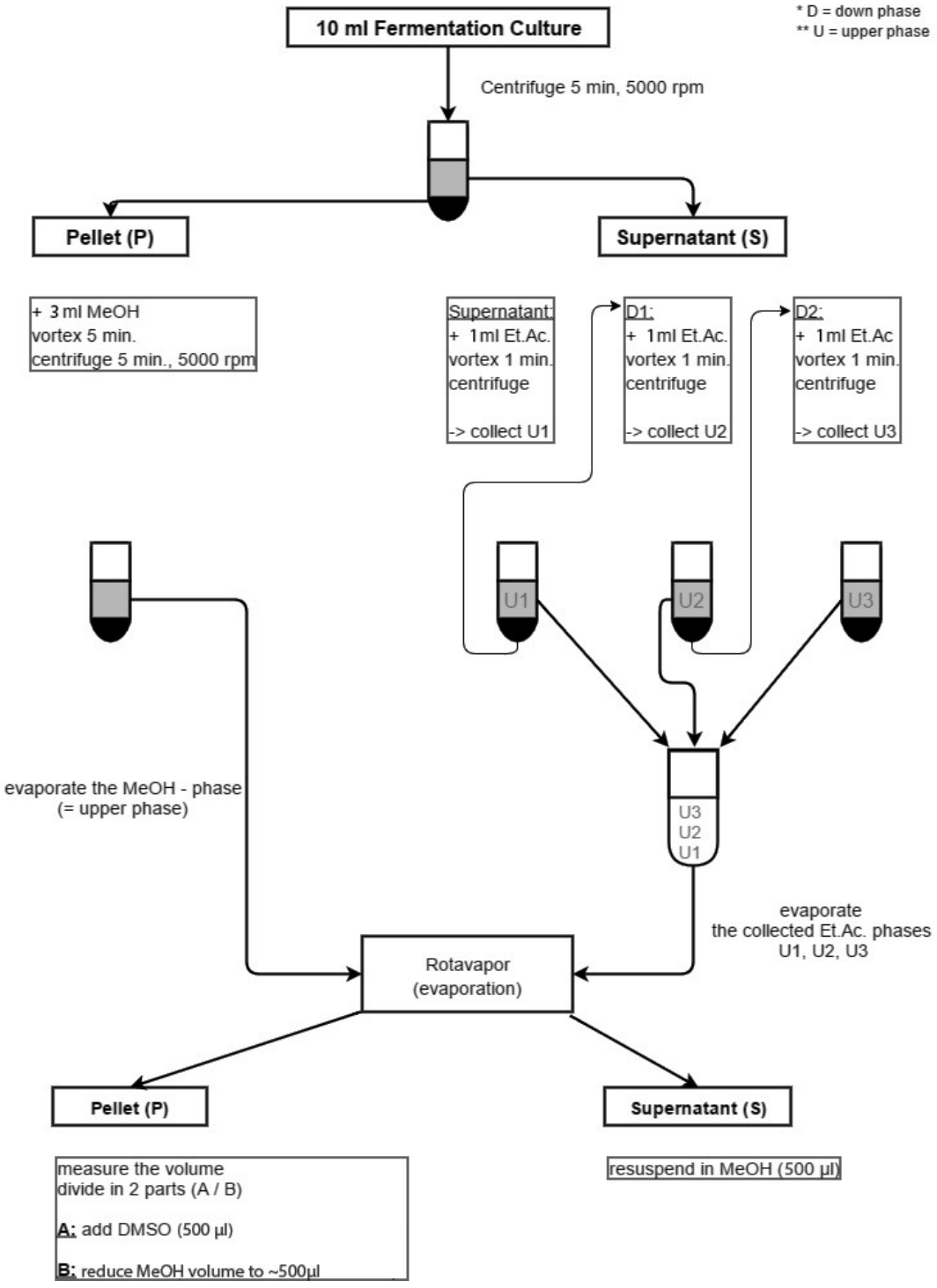


Figure 14: Scheme of the extraction method (detailed information is provided in chapter 4.17).

4.18 Analysis of the Culture Extracts

4.18.1 Growth inhibition bioassay

Growth inhibition bioassays should detect antimicrobial activity of extracts from induced fermentation cultures against the test organisms *E. coli, B. subtilis* and *C. albicans*. *E. coli* should represent Gram-negative bacteria, *B. subtilis* should represent Gram-positive bacteria and *C. albicans* was used to represent fungi.

For each bioassay 12 plates with 30 ml LA and 6 plates with 30 ml of YPD medium were prepared. *E. coli* and *B. subtilis* were plated and dried out on LA plates and *C. albicans* was plated and dried on YPD plates. 50 µl of extract from the pellet (AP-JP) or the supernatant (AS-JS) were applied to a paper disc. As controls on the one hand 50 µl of 1:250 diluted MeOH plant extracts (A-J) were added to a paper disc. And on the other hand, 50 µl extract from the pellet or the supernatant of two blind cultures with added EtOH extract (XS and XP) were applied to other paper discs. Discs had to be dried for 45 min and transferred to the prepared plates. While assays of *B. subtilis* and *E. coli* were incubated at 37 °C overnight, plates containing *C. albicans* were placed at 28 °C overnight. On the next day, the paper discs were examined for inhibition zones in comparison to their controls, to detect antimicrobial compounds of the extracts.

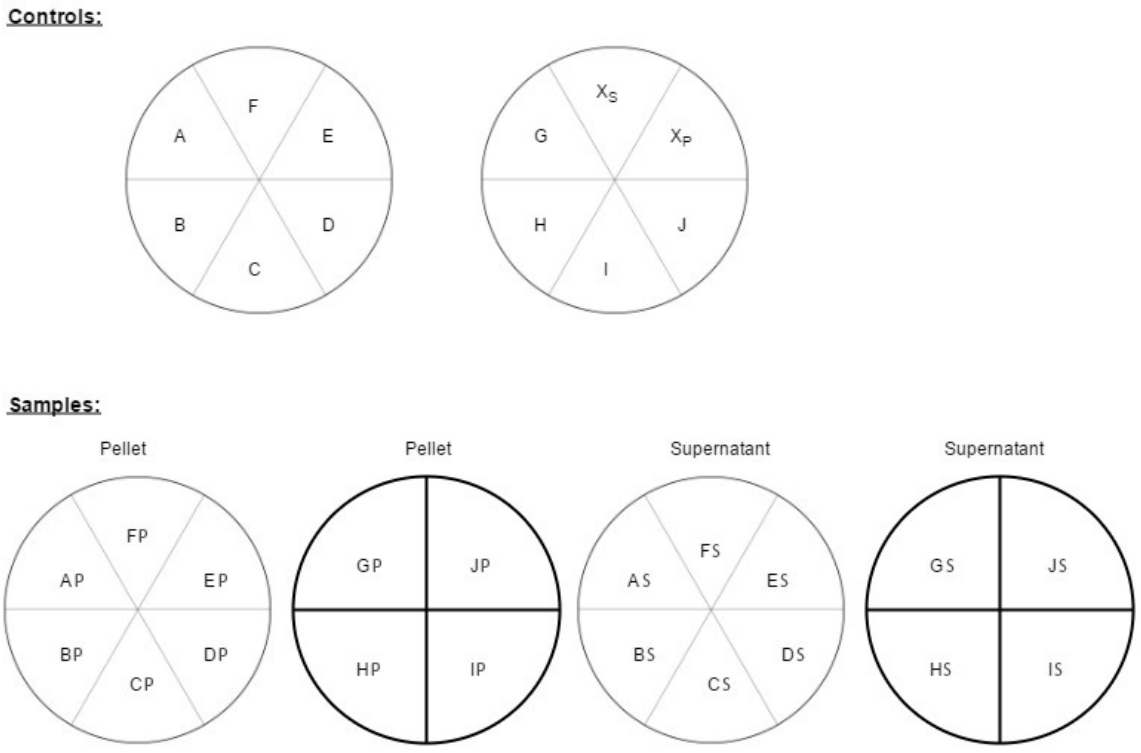


Figure 15: Growth inhibition bioassay. 6 plates were prepared for each test strain (E. coli, B. subtilis, C. albicans*). 2 plates for the application of controls (A-J 1:500 diluted herbal MeOH extract, and extracts of pellet and supernatant from blind cultures XP and XS), 2 plates for the application of extracts deriving from the pellet (AP-JP) and 2 plates for extracts deriving from the supernatant (AS-JS). The samples were placed as shown in figure.*

4.18.2 HPLC-MS Methods

To separate the constituents of the culture extracts and to determine the molecular masses of the components, LC-MS analyses were performed with the Utimate 3000 RSLC-Series System (Dionex). It was linked to a 3D-ion trap mass spectrometer with an ESI-source (HCT; Bruker Daltonics).

4.18.2.1 HPLC

The high performance liquid chromatography (HPLC) can be used for the separation of mixture constituents and also for the identification and quantification of substances. The separating capacity is dependent on the distribution of components between a solid stationary phase and a liquid mobile phase.

The obtained induced culture extracts of pellet and supernatant (see chapter 5.4) were measured with the help of HPLC Ultimate 3000 RSLC-Series System (Germering, Germany). The separation of components was performed via reversed-phase chromatography on an Acclaim 120 C18, 2,1 x 150 mm, 3 µm HPLC column (Dionex) at 25 °C. The flowrate was set to 0,5 ml/min. Two mobile phases A (0,1 % aqueous formic acid) and B (acetonitrile) were used for a gradient elution, in order to achieve an optimal separation of the sample. Within 45 min during gradient elution phase B was increased linearly from 5 % to 95 %. Substances were detected with the help of a charged aerosol detector (CAD; Corona Ultra RS by Thermo Fisher Scientific). In addition, a diode array detector (DAD) detected substances at a wavelength ranging from 190 to 400 nm.

4.18.2.2 MS

Mass spectrometry (MS) is an analytical method for the determination of masses deriving from gaseous ions. MS can be used for identification and quantification of known substances and also for structural analysis of unknown substances. MS is a sensitive method to gain information of molecular weight and fragments of an analyte. It consists of an ion source, mass analysis and a detector. Dependent on the ion source, the sample can be introduced solid, liquid or gaseous. Gaseous ions are then separated via *m/z* ratio and detected.

In this work a 3D quadrupole ion trap mass spectrometer, connected to an orthogonal ESI-source (HCT; Bruker Daltonics) was used as the second component of LC-MS for the identification of masses, deriving from interesting HPLC peaks at a specific retention time. The liquid analyzed samples deriving from HPLC analysis (see 4.18.2.1) were ionized with ESI (Electrosprayionisation). ESI ion source parameters were chosen as follows: capillary voltage +3,5/-3,7 kV, nebulizer 26 psi (N2), dry gas flow 9 l/min (N2)

and dry temperature 340 °C. Positive and negative ion mode mass spectra were obtained in data dependent acquisition mode.

5 Results

5.1 DER of Plant Extracts

The project started with the preparation of dry extracts from ten different medicinal plants (see methods 4.2). With the help of ASE, liquid methanol extracts were obtained as primary extract and later processed to dry extracts by evaporation. The corresponding drug extract ratio (DER) of the powdered plant material are provided in the table below. The dry extracts were used to prepare ethanol stock solutions of the plant extracts later (see methods 4.2.2).

Powdered Plant Material	**DEV**
Betulae folium plv.	6 : 1
Calendulae flos plv.	3 : 1
Chelidonii herba plv.	13 : 1
Colae semen plv.	12 : 1
Digitalis purpureae folium plv.	3 : 1
Hyperici herba plv.	4 : 1
Liquiritiae radix plv.	5 : 1
Rhei radix plv.	3 : 1
Rosmarini folium plv.	5 : 1
Valerianae radix plv.	3 : 1

Table 17: used powdered herbal drugs and the determined DEV.

5.2 Bioassay of SNOR NDA59 Fermentation Extract

The antibiotic effect of the recombinant SNOR NDA59 was examined in a growth inhibition bioassay in order to exclude a false positive outcome within the following experiments.

The results of this bioassay indicated, that both the culture extract from the pellet and the supernatant showed inhibition zones against *B. subtilis*, deriving from macrolide production of the polyketide synthase (PKS) in the cluster C14. No effects of the culture extracts against *C. albicans* and *E. coli*. could be detected. Due to these bioassay results, it was decided to perform a gene knockout to prohibit the production of macrolides, known to be produced by the strain, that are active against *B. subtilis* in order to avoid false positive results of putative bioactive SMs later. Geneclusters, which are responsible for the production of these macrolides, have been identified earlier (Zotchev et al., 2000), allowing the design of a gene inactivation (knock-out) strategy.

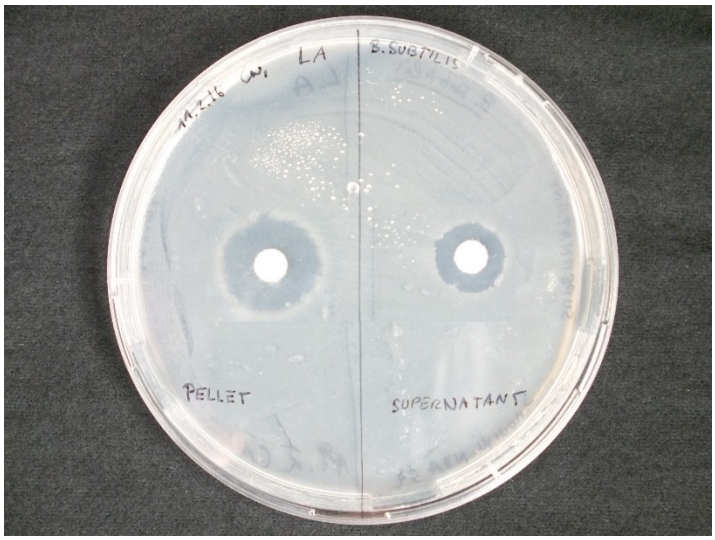


Figure 16: Growth inhibition Bioassay of SNOR NDA59 shows inhibition zones of pellet and supernatant extracts against B. subtilis.

5.3 Construction of the SNOR-CN Knockout Mutant

5.3.1 Isolation and analysis of *Streptomyces* total DNA

For the production of the KO insert, genomic DNA from SNOR NDA59 was used as a template for PCR. The isolation of total DNA was performed with the Dneasy Tissue Kit from Quiagen (see methods 4.6.1). Isolated DNA was then analyzed via gel electrophoresis for integrity (see methods 4.12).

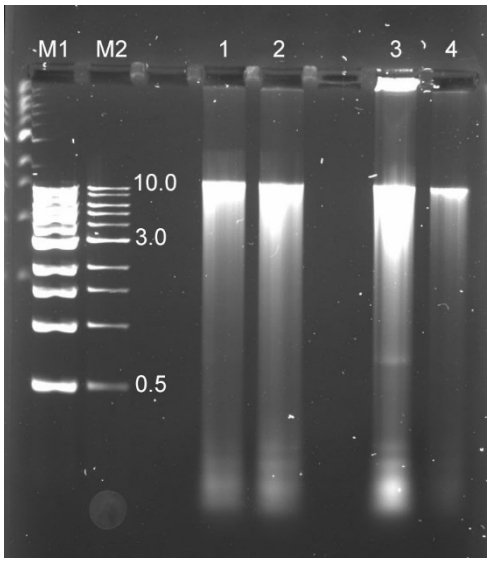


Figure 17: Gel electrophoresis from 18.2.2016: Analysation of tDNA isolated from SNOR NDA59. M1: Quick Load 1 kb DNA Ladder (1 µl); M2: 1 kb DNA Ladder (0,5 l) DNA Ladder was marked at 0.5, 3.0 and 10.0 kb; 1, 2, 3, 4: tDNA isolated from four SNOR NDA59 colonies.

5.3.2 Production and Analysis of the KO Insert

For synthesis and amplification of the KO insert via PCR, specific PCR primers were designed with the help of Clone Manager 6 (see materials 3.3 and methods 4.8). The expected KO-Insert is shown in figure 18.

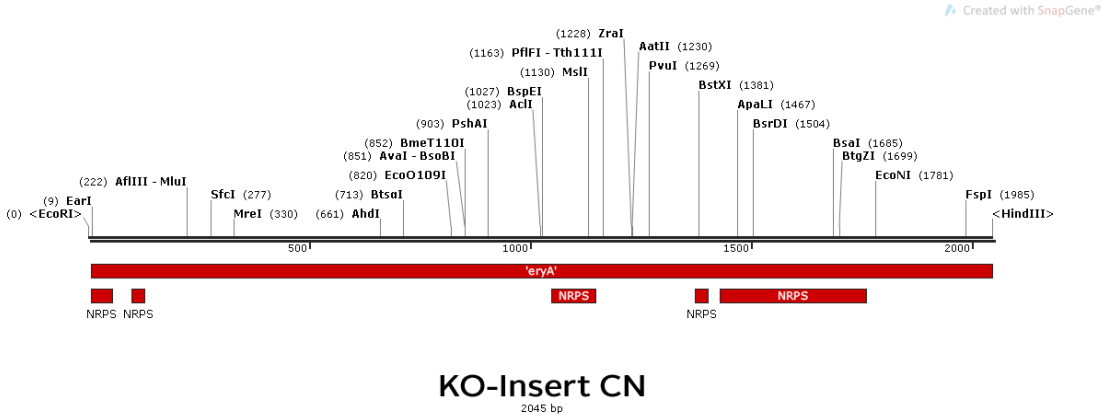


Figure 18: KO-insert CN. Designed with Clone Manager 6. Used for the production of the KO vector for SNOR NDA59.

PCR was carried out with the Q5 – High – Fidelity 2X MASTER MIX (see materials 3.8 and methods 4.7). The size of the KO-insert was checked by gel electrophoresis (see methods 4.12). Figure 19 shows that the obtained PCR products had the expected size of 2045 bps.

The correct KO inserts were cut out from the gel and purified with the Zymoclean Gel DNA Recovery Kit (see materials 3.8). Figure 20 shows the purified Insert CN after DNA recovery.

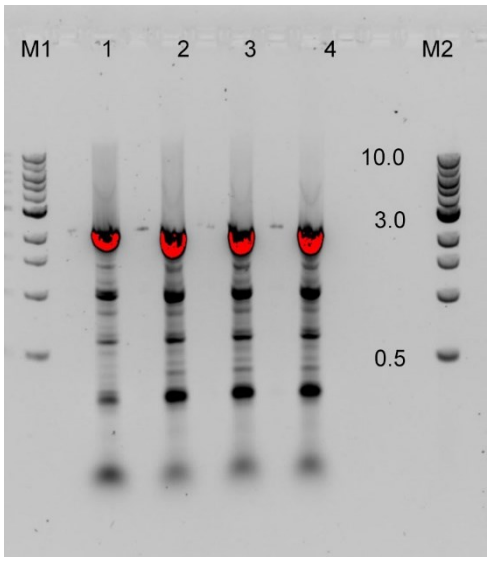


Figure 19: Gel electrophoresis 24.2.2016. Analysis of the PCR products from Q5 – High – Fidelity 2X MASTER MIX; The determined size of the KO insert (2045 bps) was confirmed via gel electrophoresis.

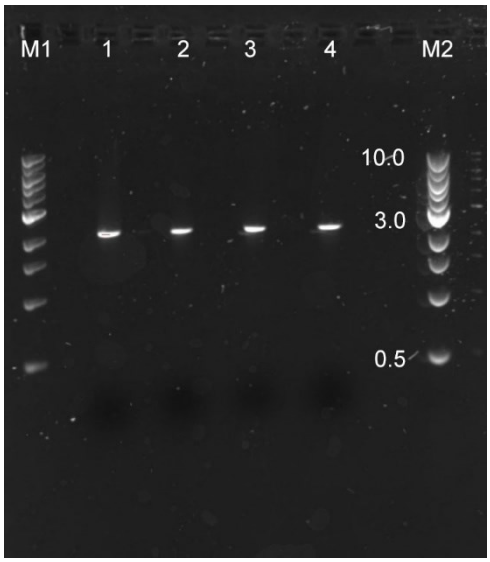


Figure 20: Gel electrophoresis from 24.2.2016. KO inserts (CN) after DNA recovery and purification with the Zymoclean Gel DNA Recovery Kit.

5.3.3 Isolation and Assessment of pSOK201

For the construction of knockout-constructs a 3.1 kb fragment of the plasmid pSOK201 was designed with Clone manager 6. The expected 3.1 kb fragment is shown in figure 21. The fragments were cut out with BamHI/HindIII and EcoRI/HindIII. For the isolation of the 3.1 kb fragments, the products were separated by preparative gel electrophoresis (cf Füreder,2017).

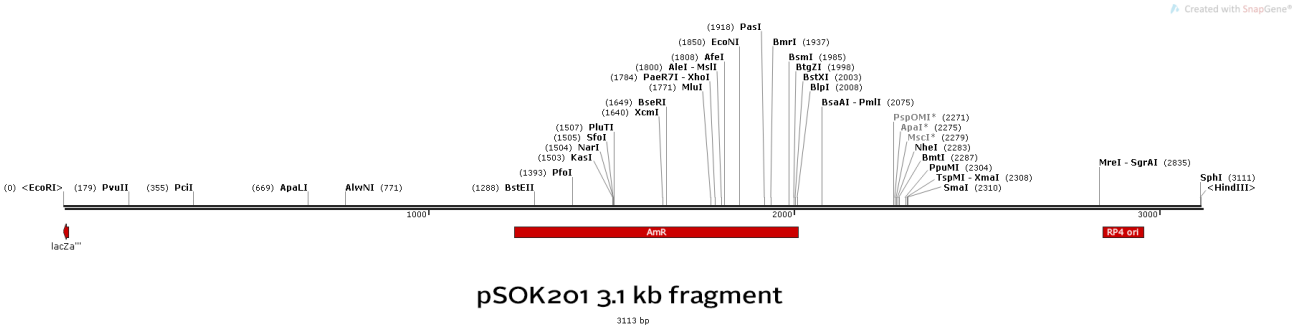


Figure 21: 3.1 kb fragment of pSOK201. Used for KO constructs; replication initiator protein gene, AmR , RP4 oriT; pSOK201 was therefore cut with EcoRI and HindIII.

3.1 kb fragments of pSOK201 were provided by M. Füreder (2017). In his work pSOK201 was isolated from transformants of *E. coli* DH5α. After isolation, the integrity of the pDNA was checked on a gel.

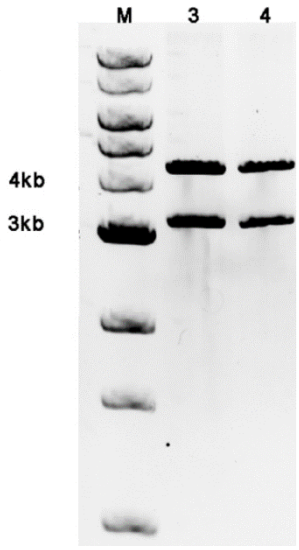


Figure 22: Preparation of pSOK201 3.1kb fragments for isolation (cf Füreder,2017).

5.3.4 Ligation of pSOK201 and CN

Restriction digest of the 3.1 kb fragment of pSOK201 and the KO-insert CN with the same enzymes (EcoRI and HindIII) guaranteed compatible ends for ligation. The ligation of the 3.1 kb fragment of pSOK201 (see chapter 5.3.3) with the KO-insert CN (see chapter 5.3.2) yielded the plasmid pSOK201-CN for the construction of the knockout mutant SNOR-CN. The composition of the mixture and settings of the method can be found in methods 4.11. The plasmid map of KO-vector pSOK201-CN is shown in figure 23.

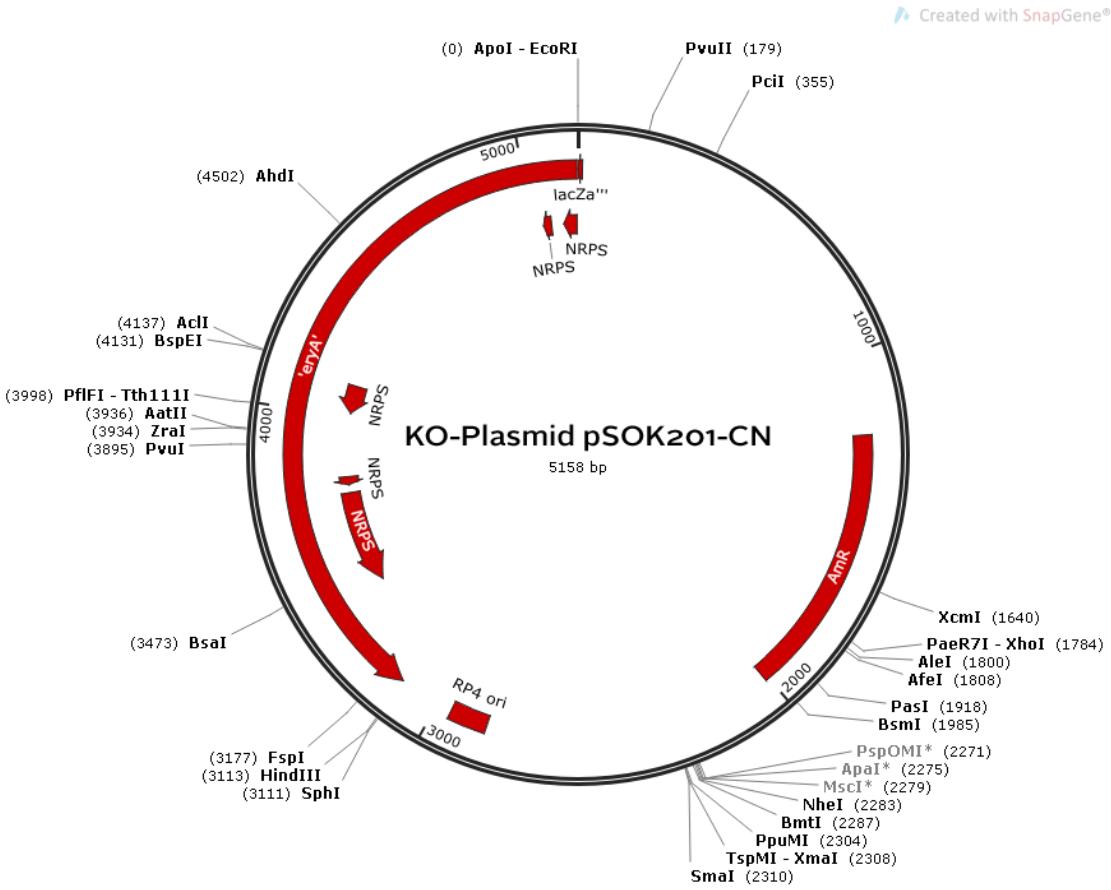


Figure 23: plasmid map of the final KO-vector pSOK201-CN.

5.3.5 Transformation, Isolation and Assessment of pSOK201-CN

The knockout construct was transformed into cloning hosts for amplification. Transformation by heat shock was the method used to introduce the obtained plasmid pSOK201-CN into competent cells of *E. coli*. *E. coli* DH5α was used for cloning and *E. coli* ET12567 was used for conjugation into *Streptomyces*.

For the assessment of pSOK201-CN, the isolated plasmid DNA from DH5α was isolated from *E. coli* DH5α cells with the Wizard Plus SV Minipreps DNA Purification System (see methods 4.6.2). Followed by a restriction digest, isolated pDNA was cut by *HindIII* and

EcoRI. The composition and reaction setup of the restriction digest for the assessment of the correct KO-construct can be found in chapter 4.10.2

The correct knockout construct was expected to produce fragments of 3113 bps (3.1 kb fragment of pSOK201) and 2045 bps (insert CN). Therefore, the size of the fragments was checked via gel electrophoresis (see methods 4.12). Figure 24 shows the results of the gel electrophoresis, depicting that the fragments had the correct size.

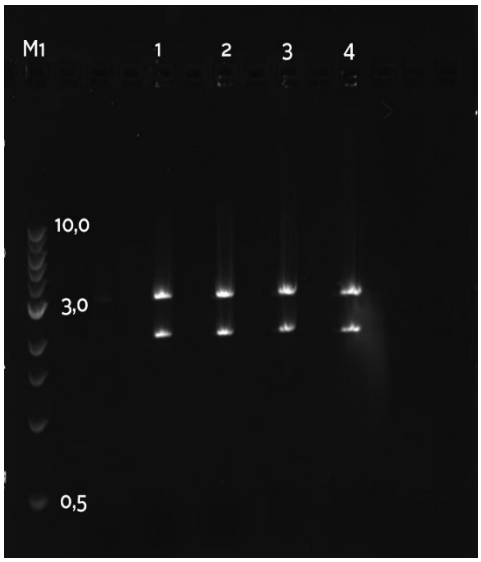


Figure 24: Gel electrophoresis from 25.2.2016: Assessment of the correct KO-construct pSOK201-CN. M1: Quick Load 1 kb DNA Ladder (1 µl); M2: 1 kb DNA Ladder (0,5 l) DNA Ladder was marked at 0.5, 3.0 and 10.0 kb; 1, 2, 3, 4 show the3.1 kb and the 2045 bps fragments of the isolated pSOK201-CN from 4 different KO-mutants (SNOR-CN).

5.3.6 Transformation of *E. coli* ET12567 and Conjugation of pSOK201-CN into *Streptomyces*

The correct KO-construct pSOK201-CN was verified by restriction digest and then transformed into *E. coli* ET12567 (see methods 4.13.2). Carrying the helper plasmid pUZ8002, this strain was used for the conjugational DNA transfer to SNOR NDA59, yielding the recombinant strain SNOR-CN (see methods 4.14). As a control pSOK806 was also transformed into ET cells and transferred to SNOR NDA59 via conjugation.

5.4 Preparation of Culture Extracts

Culture extracts were prepared by fermentation (see methods 4.16) and following extraction (see methods 4.17). Fermentation of SVEN JZ2 and SNOR-CN was performed in two different media, M5319 and PM4-1, and the addition of ethanol plant extracts (A-J), which should affect SM production. For reproducibility 70 % ethanol (2 µl/ml) was added to the blind culture X. The extraction of the corresponding pellets (AP-JP) and supernatants (AS-JS) yielded 88 culture extracts. These culture extracts were analysed by growth inhibition bioassay and HPLC-MS later (see 4.18.1and 4.18.2).

Medium M5319		**Medium PM4-1**	
Extracts Pellet	Extracts Supernatant	Extracts Pellet	Extracts Supernatant
SVEN AP M5319	SVEN AS M5319	SVEN AP PM41	SVEN AS PM41
SVEN BP M5319	SVEN BS M5319	SVEN BP PM41	SVEN BS PM41
SVEN CP M5319	SVEN CS M5319	SVEN CP PM41	SVEN CS PM41
SVEN DP M5319	SVEN DS M5319	SVEN DP PM41	SVEN DS PM41
SVEN EP M5319	SVEN ES M5319	SVEN EP PM41	SVEN ES PM41
SVEN FP M5319	SVEN FS M5319	SVEN FP PM41	SVEN FS PM41
SVEN GP M5319	SVEN GS M5319	SVEN GP PM41	SVEN GS PM41
SVEN HP M5319	SVEN HS M5319	SVEN HP PM41	SVEN HS PM41
SVEN IP M5319	SVEN IS M5319	SVEN IP PM41	SVEN IS PM41
SVEN JP M5319	SVEN JS M5319	SVEN JP PM41	SVEN JS PM41
SVEN XP M5319	SVEN XS M5319	SVEN XP PM41	SVEN XS PM41

Table 18: Induced culture extracts from Streptomyces venezuelae JZ2. M5319 and PM4-1 are the used culture media for fermentation. The letters A-J mark the plant extract added to the fermentation cultures. X stands for the non-induced culture with addition of 2 µl/ml 70 % ethanol. P signs culture extracts deriving from the pellets and S from the supernatants.

Medium M5319		Medium PM4-1	
Extracts Pellet	Extracts Supernatant	Extracts Pellet	Extracts Supernatant
SNOR AP M5319	SNOR AS M5319	SNOR AP PM41	SNOR AS PM41
SNOR BP M5319	SNOR BS M5319	SNOR BP PM41	SNOR BS PM41
SNOR CP M5319	SNOR CS M5319	SNOR CP PM41	SNOR CS PM41
SNOR DP M5319	SNOR DS M5319	SNOR DP PM41	SNOR DS PM41
SNOR EP M5319	SNOR ES M5319	SNOR EP PM41	SNOR ES PM41
SNOR FP M5319	SNOR FS M5319	SNOR FP PM41	SNOR FS PM41
SNOR GP M5319	SNOR GS M5319	SNOR GP PM41	SNOR GS PM41
SNOR HP M5319	SNOR HS M5319	SNOR HP PM41	SNOR HS PM41
SNOR IP M5319	SNOR IS M5319	SNOR IP PM41	SNOR IS PM41
SNOR JP M5319	SNOR JS M5319	SNOR JP PM41	SNOR JS PM41
SNOR XP M5319	SNOR XS M5319	SNOR XP PM41	SNOR XS PM41

Table 19: Induced culture extracts from the Streptomyces noursei *NDA59 mutant SNOR-CN. M5319 and PM4-1 are the culture media used for fermentation. A-J mark the plant extract added to the fermentation cultures. X stands for the non-induced culture with addition of 2 µl/ml 70 % ethanol. P signs culture extracts deriving from the pellets and S from the supernatants.*

5.5 Bioassay Based Analysis of Culture Extracts

The obtained induced culture extracts from SVEN JZ2 and SNOR-CN were analyzed by growth inhibition bioassays (see methods 4.18.1). They were expected to yield new bioactive substances or an increase of production level of SMs. Bioactive compounds should be detected by comparison of inhibition zones of culture extracts and the corresponding controls.

All extracts deriving from cultures of SVEN JZ2 (M5319 and PM4-1) showed no effect on any of the test strains compared to the corresponding non induced cultures. The blind culture extracts of the mutant SNOR-CN XP/XS PM4-1 still showed small inhibition zones against *B. subtilis*. Triggered culture extracts (AP-JP and AS-JS) from the PM4-1 media showed different sizes of the inhibition zones against *B. subtilis* in comparison to the blind values. Smaller zones were thought to derive from inhibition and bigger zones probably derived from induction of secondary metabolism. No inhibition zones of the triggered extracts were detected on *E. coli* and *C. albicans* plates, neither from the

extract derived from the pellet nor from the supernatant. The same effects were detected in case of the triggered M5319 culture extracts of SNOR-CN compared to the corresponding blind values.

5.5.1 Bioassay Based Analysis of Extracts from SNOR-CN PM4-1

As already mentioned, the induced culture extracts (A-J PM4-1) showed different sizes of inhibition zones against *B. subtilis* in comparison to the corresponding blind values XP and XS (PM4-1). To demonstrate the changes, the diameters of the inhibition zones of the culture extracts were measured and compared to the diameters of the control zones. For the determination of the correct diameter, the diameter of the paper disc was subtracted.

Induced culture extract of SNOR-CN (PM4-1)	Diameter of inhibition zones for extracts prepared from the pellets	Diameter of inhibition zones for extracts prepared from the supernatants
A	0,90 - 0,6 = 0,30 cm	-
B	0,90 - 0,6 = 0,30 cm	-
C	0,80 - 0,6 = 0,20 cm	-
D	0,75 - 0,6 = 0,15 cm	-
E	0,85 - 0,6 = 0,25 cm	-
F	0,80 - 0,6 = 0,20 cm	0,65 - 0,6 = 0,05 cm
G	1,00 - 0,6 = 0,40 cm	0,70 - 0,6 = 0,10 cm
H	1,00 - 0,6 = 0,40 cm	-
I	1,00 - 0,6 = 0,40 cm	0,65 - 0,6 = 0,05 cm
J	1,00 - 0,6 = 0,40 cm	0,70 - 0,6 = 0,10 cm

Table 20: Assessment of growth inhibition bioassay from induced SNOR-CN (PM4-1) culture extracts against B. subtilis. Diameter of paper disc = 0,60 cm, Diameters of non-induced cultures XS = 0,25 cm; XP = 0,40 cm.

5.5.2 Bioassay Based Analysis of Extracts from SNOR-CN M5319

In the culture extracts prepared with M5319 medium the same effect, as mentioned in chapter 5.5.1., was detected. The diameter of the inhibition zones from extracts A-J (M5319) were measured in comparison to the corresponding blind values XS and XP (M5319).

Induced culture extract of SNOR-CN (M5319)	Diameter of the pellets	Diameter of the supernatants
A	1,75 - 0,60 = 1,15 cm	1,70 - 0,60 = 1,10 cm
B	1,50 - 0,60 = 0,90 cm	1,50 - 0,60 = 0,90 cm
C	1,70 - 0,60 = 1,10 cm	1,00 - 0,60 = 0,40 cm
D	1,60 - 0,60 = 1,00 cm	1,10 - 0,60 = 0,50 cm
E	1,60 - 0,60 = 1,00 cm	1,10 - 0,60 = 0,50 cm
F	1,45 - 0,60 = 0,85 cm	1,40 - 0,60 = 0,80 cm
G	1,35 - 0,60 = 0,75 c	1,70 - 0,60 = 1,10 cm
H	1,40 - 0,60 = 0,80 cm	1,70 - 0,60 = 1,10 cm
I	1,30 - 0,60 = 0,70 cm	1,45 - 0,60 = 0,85 cm
J	1,40 - 0,60 = 0,80 cm	1,60 - 0,60 = 1,00 cm

Table 21: Assessment of growth inhibition bioassay from induced SNOR-CN (M5319) culture extracts against B. subtilis. Diameter of paper disc = 0,60 cm; Diameters of non-induced cultures XS = 0,90 cm; XP = 1,20 cm.

5.6 Results and Comparison of LC-MS Based Analysis of Culture Extracts

5.6.1 HPLC-CAD Analysis

Fermentation cultures (see methods 4.16) of engineered SVEN JZ2 and SNOR-CN in two different media (M5319 and PM4-1) were induced with ethanol extracts from 10 medicinal plants A-J (see materials 3.1). Extraction of induced fermentation cultures (see 4.17) yielded extracts from the corresponding pellets (P) and supernatants (S). These culture extracts were expected to yield new bioactive compounds and were therefore analysed with HPLC-CAD (see methods 4.18.2.1). To exclude substance peaks originating from the growth medium, HPLC chromatograms of the culture extracts (black) were compared with corresponding blind values XP and XS (blue), which were prepared with addition of 70 % ethanol in a concentration of 2 µl/ml. The chromatogram was also overlayed with the corresponding methanol plant extract (A-J) in the same concentration to exclude peaks, deriving from the extract.

The chromatograms showed changes in the metabolic profiles of induced culture extracts compared to the corresponding blind values. The most significant HPLC chromatograms and the change in metabolic profiles of induced culture extracts from SVEN M5319 and SNOR-CN can be found in the appendix.

The following newly emerged peaks of elected chromatograms were planned to be analyzed with MS (see table 22).

Induced extract (A-J)	**R_t Peak 1**	**R_t Peak 2**	**R_t Peak 3**	**R_t Peak 4**	**R_t Peak 5**	**R_t Peak 6**
SVEN M5319 BP	22.8					
SVEN M5319 DP	19.7	32.1	34.5			
SVEN M5319 EP	23.8	32.1	34.5			
SVEN M5319 HP	21.6	30.3				
SVEN M5319 IP	34.5	36.3	37.3	38.3		
SVEN PM4-1 BP	22.75	24.7				
SVEN PM4-1 EP	22.75	23.6				
SVEN PM4-1 HP	26.35	36	38.6	39.7		
SVEN PM4-1 IP	37.3	38.3				
SVEN PM4-1 GS	19.5					
SVEN PM4-1 JS	22.4					
SNOR M5319 BP	22.7	24.7				
SNOR M5319 AS	18.7	19.7	20.2	20.4	22.7	24
SNOR M5319 BS	22.4					
SNOR M5319 GS	20.4					
SNOR M5319 IS	16.3	20.4				
SNOR PM4-1 AS	29.8					

Table 22: shows the retention time (R_T) of promising peaks deriving from selected HPLC-CAD chromatograms.

5.6.2 MS Analysis

Comparative analysis of HPLC-CAD chromatograms of the induced extracts, corresponding controls and the diluted methanol plant extracts yielded a lot of new substantial peaks (see chapter 5.6.1, table 22). The masses of the corresponding peaks were determined with mass spectrometry. Promising masses were planned to be analyzed with high-resolution mass spectrometry (MS). Due to lack of time further analysis with high resolution MS and a following comparison of accurate masses to the Dictionary of Natural Products (DNP) could not be pursued.

The following tables show only ascertainable masses of the corresponding peaks at a specific retention time, because in many cases the masses of the peaks could not be detected because of high and distracting background signals probably deriving from culture media.

SVEN M5319

E	**P#**	**R_T (min)**	**M+/M-**	**Mass (Da)**	**Additional information**
DP		32.1	M+: M-: 571.2	572.2	Potassium adduct
EP		23.7	M+:1049.5 M-: ?	1050.5	1096 formate adduct 1073 sodium adduct Double charged
		32.1	M+: ? M-:571.2	572.2	
IP		34.5	M+: 1062.9 M-: 1060.9	2123	Double charged peak at 36.3 probably isomer

Table 23: Mass spectrometry of the culture extracts of SVEN JZ2 in M5319 media. The table assigns the induced extract (E) deriving from the pellet (EP and IP). It includes the peak number (P#) deriving from the corresponding HPLC-CAD chromatograms (see figures 26, 27 and 29) and the corresponding retention time (R_T), masses of its ions (M+ and M-), the determined mass and additional information that helped to find the exact mass of the compounds.

SVEN PM41

E	P#	R_T (min)	M+/M-	Mass (Da)	Additional information
BP		22.75	M+: 941 M-: 939.5	940.5	Sodium and potassium adduct
		24.7	M+: 795 M-: 793.4	794.0	Sodium and potassium adduct
EP		22.75	M+: 941. M-: 939.5	940.5	
		23.6	M+: ? M-: 1049.4	1050.0	Sodium and formate adducts
HP		36	M+:1062 M-: 1060	1061.0	

Table 24: Mass spectrometry of the culture extracts of SVEN JZ2 in PM41 media. The table assigns the induced extract (E) deriving from the pellet (BP, EP, HP). It includes the peak number (P#) deriving from the corresponding HPLC-CAD chromatograms (see figures 30, 31 and 32) and the corresponding retention time (R_T), masses of its ions (M+ and M-), the determined mass and additional information that helped to find the exact mass of the compounds.

SNOR M5319

E	P#	R_T (min)	M+/M-	Mass (Da)	Additional information
AS		18.5	M+: 257.0 M-: 255.0	256.0	
		19.7	M+: ? M-: 1243.0	1244.0	
IS		16.3	M+:509.0 M-: 507.2	508.0	
BP		24.7	M+: ? M-:793.4	794.4	Probably elimination of a sugar

Table 25: Mass spectrometry of the culture extracts of SNOR-CN in M5319 media. The table assigns the induced extract (E) deriving from the pellet (BP) or the supernatant (AS, IS). It includes the peak number (P#) deriving from the corresponding HPLC-CAD chromatograms (see figures 36, 37 and 40) and the corresponding retention time (R_T), masses of its ions (M+ and M-), the determined mass and additional information that helped to find the exact mass of the compounds.

6 Discussion and Outlook

Worldwide, society has to face increasing problems with pathogenic and multidrug resistant bacteria. Facing the global crisis and working on management strategies, to reduce the risk of antibiotic resistance, efforts on the discovery of new antibiotics are of high priority (cf Ventola, 2015 b). Actinomycetes are still an important source for the discovery of SMs. Genome sequencing of *Streptomyces* revealed the hidden potential of cryptic secondary metabolite BGCs. Sequencing of numerous genomes therefore could lead to the discovery of an even bigger number of novel SMs. Due to the fact, that cryptic BGCs remain silent under laboratory conditions, it is obvious that research focuses on activating the expression of cryptic or poorly expressed pathways (cf Baltz 2011; Baltz, 2016). The OSMAC approach showed that manipulation of biosynthesis can be performed at different levels. Manipulation can occur at transcriptional, translational, enzyme and metabolite level. Within this approach, diverse strategies have been developed to manipulate antibiotic production. Specific manipulation of culture media has shown promising results in triggering antibiotic production or in the increase of antibiotic titres (cf Abdelmohsen et al.,2015; Bode et al., 2002).

In the current study, SM production of the two engineered *Streptomyces* strains SVEN JZ2 and a new constructed mutant of SNOR NDA59 was triggered with extracts from ten well researched medicinal plants during fermentation. Although no new antimicrobial activities were revealed in this project, it has definitely shown extracts from medicinal plants as a cheap and promising method to trigger SM production. Comparative HPLC-CAD analysis of the induced culture extracts disclosed promising changes in the corresponding metabolic profiles. The comparison of HPLC-CAD chromatograms of induced extracts, corresponding controls and the diluted methanol plant extracts have shown a lot of new substantial peaks (see chapter 5.6.1, table 22.). The main focus of attention was put on newly emerged peaks of the induced culture extracts, that were not directly associated with the corresponding non-induced culture extracts and the pure 1:500 diluted methanol plant extract. Having a closer look at the HPLC-CAD chromatograms of the induced culture extracts, SVEN JZ2 turned out to be a highly sensitive strain to the triggering effects of the methanol plant extracts of B (*Calendula officinalis*), D (*Cola nitida*), E (*Digitalis pururea*); H (*Rheum palmatum*) and I (*Rosmarinus officinalis*). In addition, the methanol plant extracts B and I also had a triggering effect on the fermentation cultures of SNOR-CN. MS analysis of the newly emerged peaks of the HPLC-CAD chromatograms revealed the corresponding masses of the compounds (see chapter 5.6.2). Further questions, challenges and goals for future projects that could probably help to improve other experiments will be discussed in detail.

The challenge of genetic modification of SNOR NDA59:

Due to macrolide production, the bioassay of SNOR NDA59 culture extract showed activities against *B. subtilis*, prompting to perform a gene knockout of a PKS in Cluster C14, which specified production of antimicrobial macrolide. The knockout mutant SNOR-CN was engineered to achieve a lower SM background in HPLC-MS analysis, but demonstrated comparable results in growth inhibition bioassays. The culture extracts of the generated knockout mutant SNOR-CN still showed a rest of antibiotic activity against *B. subtilis*. Probably other antibacterial compounds contributed to the activity, and different or additional KO strategies could change the metabolite profile to easier detect novel antimicrobial SMs in later tests.

Optimization of culture media and detection of SMs:

As already mentioned in chapter 1.7.1, the metabolic profile of fermentation cultures is dependent on the media composition. It is well known that microorganisms produce various secondary metabolites, but often in poor quantity. In some MS spectra of the induced culture extracts too much background resulting from media components precluded determination of masses from smaller peaks deriving from the HPLC-CAD chromatograms. Upscaling of fermentation cultures and variations in fermentation media could probably help to avoid these problems. In this study, the medium M5319 turned out to have a lower background in HPLC analysis than PM4-1. Further optimization of fermentation medium can probably yield even better results.

If new potential SMs were traceable, bioactivity-guided fractionation and separation could also help, to easier detect and extract potential SMs, that are responsible for the effect of the culture extract.

Quality of plant extracts and the impact on SM production:

It has to be mentioned that changes in the quality of the plant extracts probably also affect their triggering effect. Reported by M. Veit (2010) and also mentioned by Hänsel R. and Spiess E. (2010), there are many aspects that affect the quality of the living plant and thus also of the herbal drug, mentioned in chapter 1.8.3.1. In this work, pharmaceutical-grade herbal drugs were used and processed to dry extracts. Since only the DEV and no quantitative analysis of the plant extracts was determined it is difficult to define the components. The Pharmacopoea Europaea 9.0 (2017a) guarantees the quality of extracts through the correct identification of the plant and the instructions of

the manufacturing process of the herbal drugs, the following procession to the extracts and in process controls of identity and purity of the preparations. The use of ready-made pharmaceutically approved herbal extracts could prevent this problem and would be helpful and time saving.

Due to the triggering effect of the methanol plant extracts B (*Calendula officinalis*) and I (*Rosmarinus officinalis*) on both *Streptomyces* cultures, it is of great interest for future projects which biosynthetic pathways are triggered by these methanol plant extracts or their components.

In the European Pharmacopoea 9.0 (2017b) *Calendulae flos* is defined as whole or cut, fully flowered, dried single flowers, detached from the inflorescence ground of the cultivated varieties of *Calendula officinalis L*. It contains at minimum 0.4% of flavonoids, calculated as hyperoside (C21H20O12, Mr 464.4) in reference to the dried herbal drug.

There has been much research on the constituents of the plant. According to Sticher (2010) and the referred literature the plant contains Triterpene alcohols and Triterpene saponins, Flavonoids, Carotenoids, water soluble polysaccharides, essential oil and other Substances like Coumarines, Phenolic acids, Sterols and Sterol glycosides, Ionon- and Sesquiterpene glycosides.

The European Pharmacopoea 9.0 (2017c) defines *Rosmarini folium* as whole leaves of *Rosmarinus officinalis L*. containing at least 12 $ml \cdot kg^{-1}$ volatile oil and 3 percent hydroxycinnamic derivatives, calculated as rosmarinic acid ($C_{18}H_{16}O_8$; M_r 360,3) in reference to the anhydrous herbal drug.

According to the European Medicines Agency (2010) the leaves of *Rosmarini folium* contain essential oil and compounds belonging to the chemical groups of Monoterpenes, Diterpenes, Triterpenes, Flavonoids, Cinnamic derivatives and Phenolic acids.

Yet, it is not known if the triggering effect is due to a specific (bioactive) molecule within the plant extracts or due to the extracts as a whole. Does the effect originate from pleiotropic or pathway-specific regulation? Does the manipulation occur at the transcriptional, the translational, the enzyme level or the metabolite level? Studies on the potential points of action could provide more information about the triggering effect of these plant extracts. And as mentioned above, the composition of the extract is an important aspect of its quality. Reported by Atanasov et al (2015) plant extracts are complex mixtures that contain a multitude of constituents. Sometimes a single active component is responsible for an effect on the target, but it is also possible that a high activity is a result of other partially active or even inactive components that support the effect of the active component on its target. Comparative analysis of plant extract-

induced versus lead (bioactive) substance-induced culture extracts could give more information about the triggering agent.

Bioassay methods and detection of putative novel SMs:

Growth inhibition bioassays of the culture extracts of SVEN JZ2 and SNOR-CN didn´t indicate putative bioactive compounds against *E. coli, B. subtilis* and *C. albicans* but it has to be mentioned that in the scope of this project the amount and diversity of bioassays was very limited. Diverse bioassay methods could reveal a variety of bioactive compounds with more than antimicrobial activities.

Identification of SMs:

The identification of the induced SMs detected in this project is still outstanding. To be certain about exact masses and for the prediction of putative novel bioactive compounds, it would be necessary to determine accurate masses of the substances with high-resolution mass spectrometry and compare them to masses listed in the Dictionary of Natural Products (DNP). This should later be followed by purification and structure elucidation using NMR.

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8 Appendix

8.1 HPLC – Spectra

Comparative analysis of extract SVEN M5319 BP

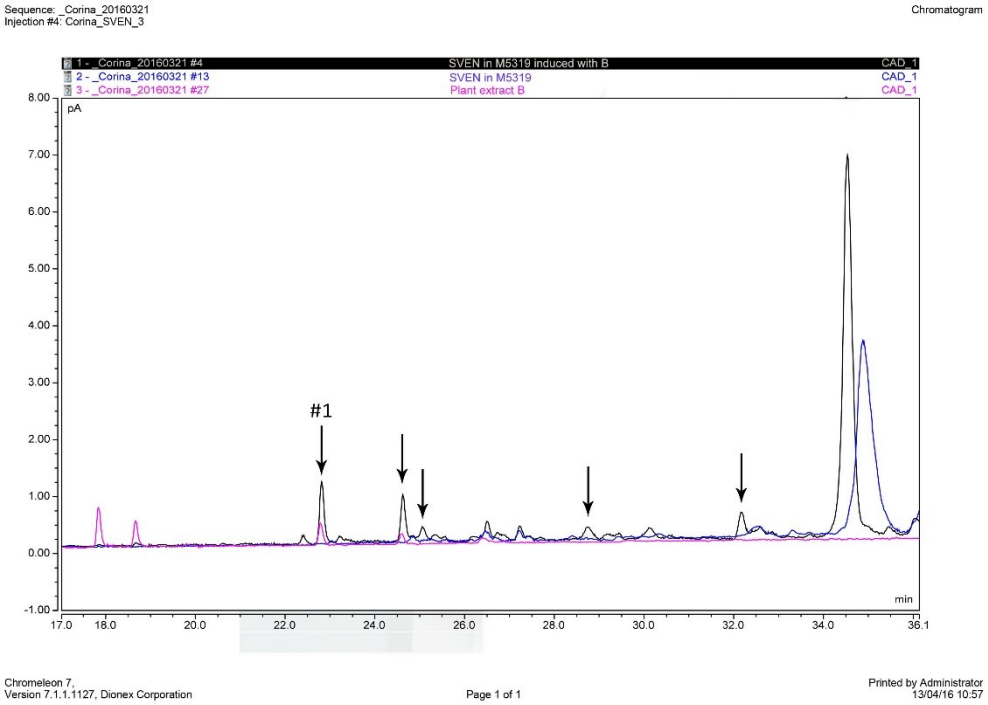

Figure 25: Comparative HPLC-CAD analysis of SVEN M5319 BP (black), the corresponding non-induced culture extract SVEN M5319 XP (blue) and the diluted MeOH plant extract B (pink) yielded newly emerged peaks. Peak #1 (R_T 22.8) was determined with MS.

Comparative analysis of extract SVEN M5319 DP

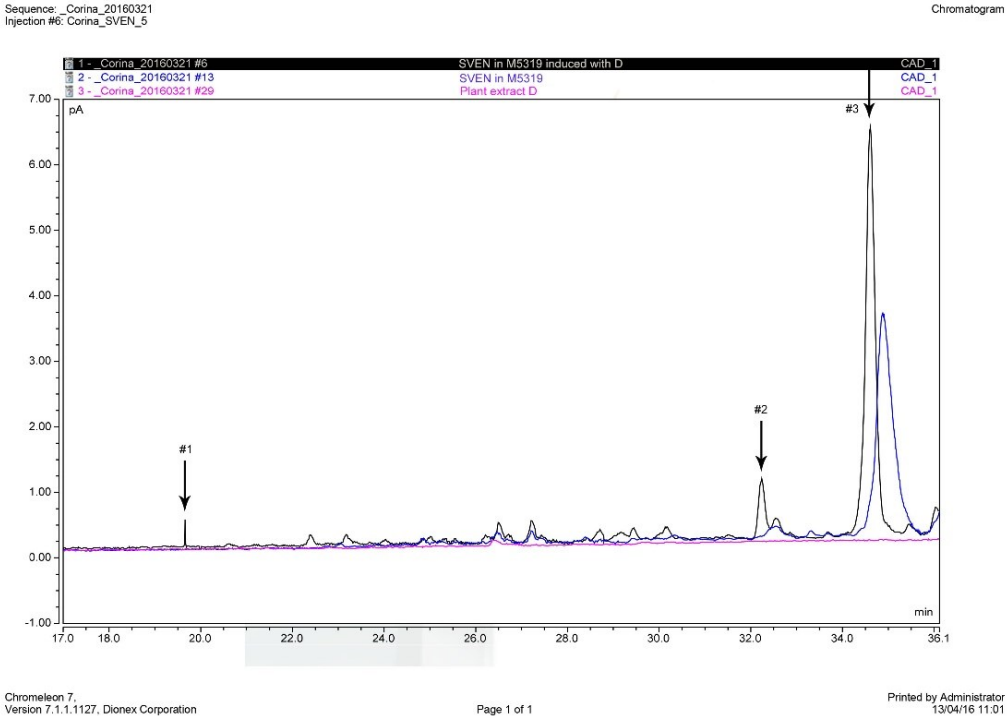

Figure 26: Comparative HPLC-CAD analysis of SVEN M5319 DP (black), the corresponding non-induced culture extract SVEN M5319 XP (blue) and the diluted MeOH plant extract D yielded the newly emerged peaks #1 (R_T 19.7), #2 (R_T 32.1) and #3 (R_T 34.5) that were determined with MS.

Comparative analysis of extract SVEN M5319 EP

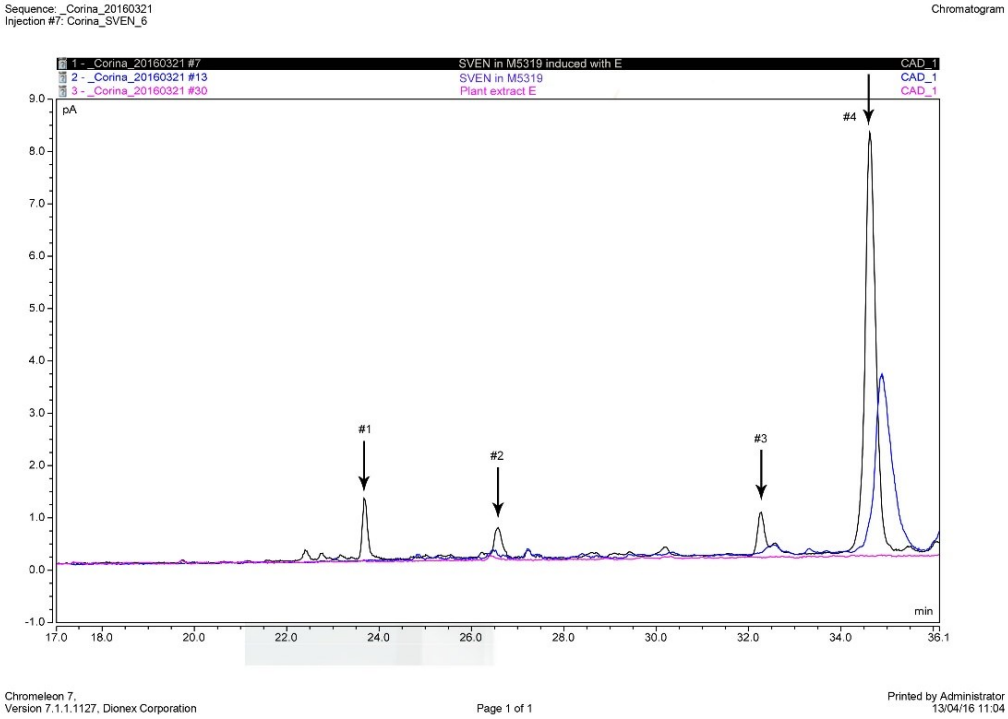

Figure 27: Comparative HPLC-CAD analysis of SVEN M5319 EP (black), the corresponding non-induced culture extract SVEN M5319 XP (blue) and the diluted MeOH plant extract E (pink), yielded the newly emerged peaks #1 (R_T 23.8), #2 (R_T 32.1) and #3 (R_T 34.5), that were determined with MS.

Comparative analysis of extract SVEN M5319 HP

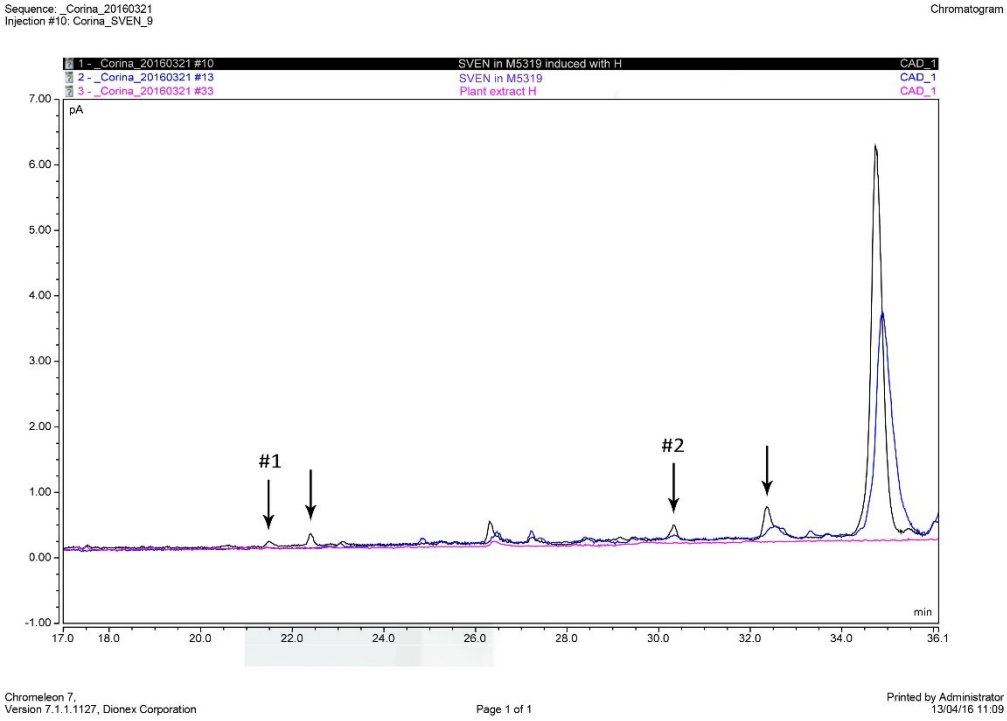

Figure 28: Comparative HPLC-CAD analyses of the extract SVEN M5319 HP (black), the corresponding non-induced culture extract SVEN M5319 XP (blue) and the diluted MeOH plant extract H yielded newly emerged peaks. The masses of peak #1 (R_T 21.5) and #2 (R_T 30.2) were determined with MS.

Comparative analysis of extract SVEN M5319 IP

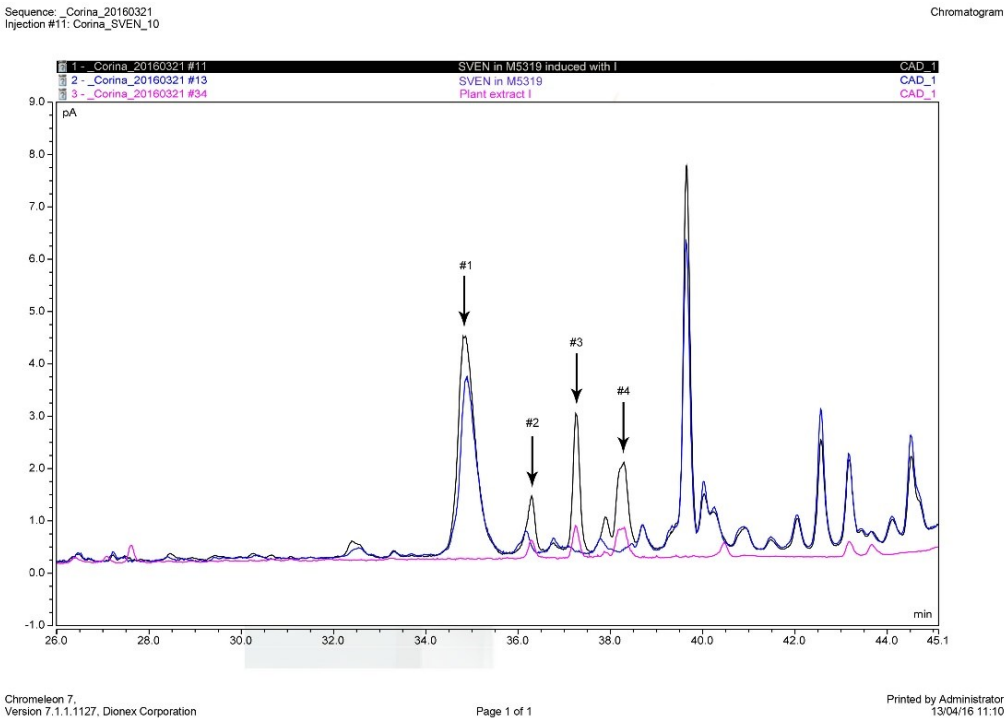

Figure 29: Comparative HPLC-CAD analysis of SVEN M5319 IP (black), the corresponding non-induced culture extract SVEN M5319 XP (blue) and the diluted MeOH plant extract I (pink). The masses of the newly emerged peaks #1 R_T t 34.5), #2 (R_T 36.3), #3 (R_T 37.3) and #4 (R_T 38.3) were determined with MS.

Comparative analysis of extract SVEN PM4-1 BP

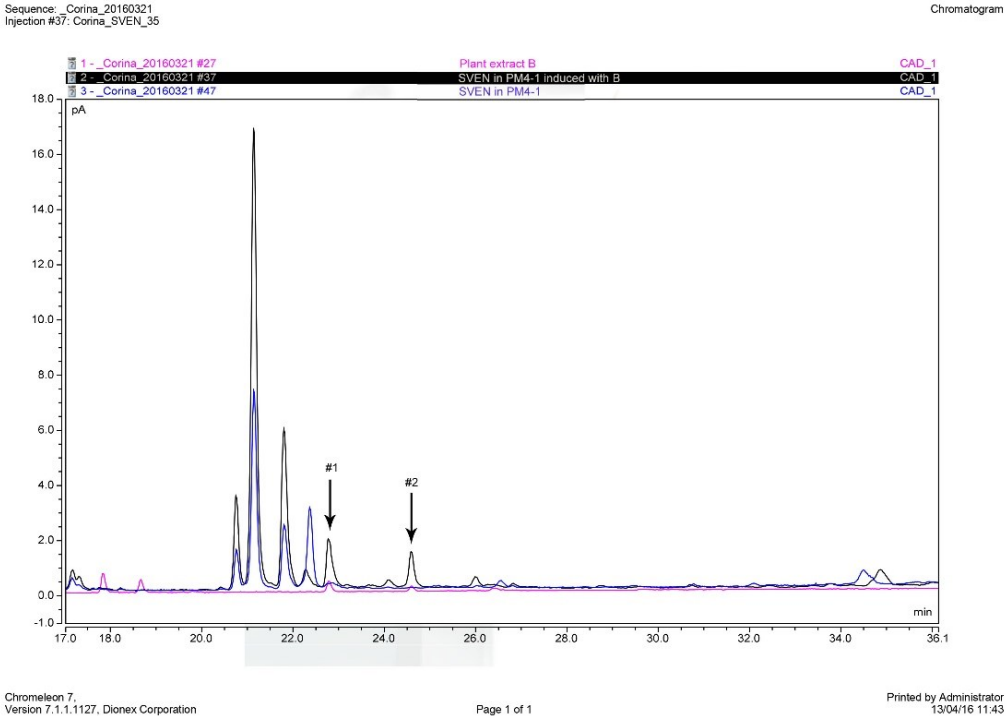

Figure 30: Comparative HPLC-CAD analysis of SVEN PM4-1 BP (black), the corresponding non-induced culture extract SVEN PM4-1 XP (blue) and the diluted MeOH plant extract B (pink). The masses of the newly emerged peaks #1 (R_T 22.75) and #2 (R_T 24.7) were determined with MS.

Comparative analysis of extract SVEN PM4-1 EP

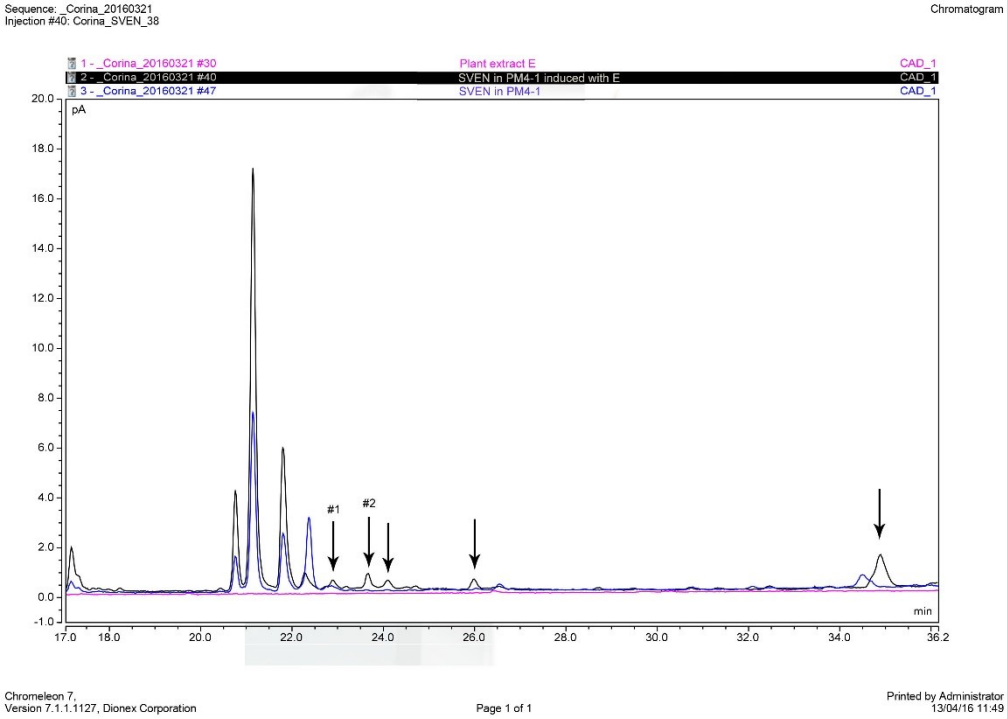

Figure 31: Comparative HPLC-CAD analysis of SVEN PM4-1 EP (black), the corresponding non-induced culture extract SVEN PM4-1 XP (blue) and the diluted MeOH plant extract E (pink). The masses of the newly emerged peaks #1 (R_T 22.75), #2 (R_T 23.6) were determined with MS.

Comparative analysis of extract SVEN PM4-1 HP

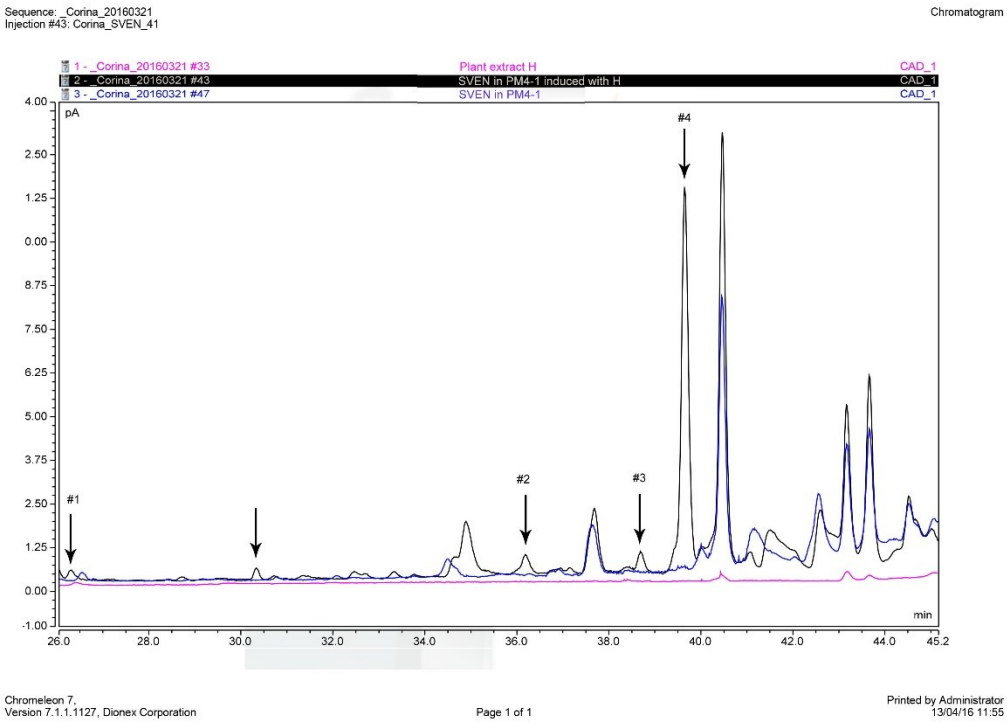

Figure 32: Comparative HPLC-CAD analysis of SVEN PM4-1 HP (black), the corresponding non-induced culture extract SVEN PM4-1 XP (blue) and the diluted MeOH plant extract H, yielded the newly emerged peaks #1 (R_T 26.4), #2 (R_T 36.0), #3 (R_T 38.6), #4 (R_T 39.7) that were determined with MS.

Comparative analysis of extract SVEN PM4-1 IP

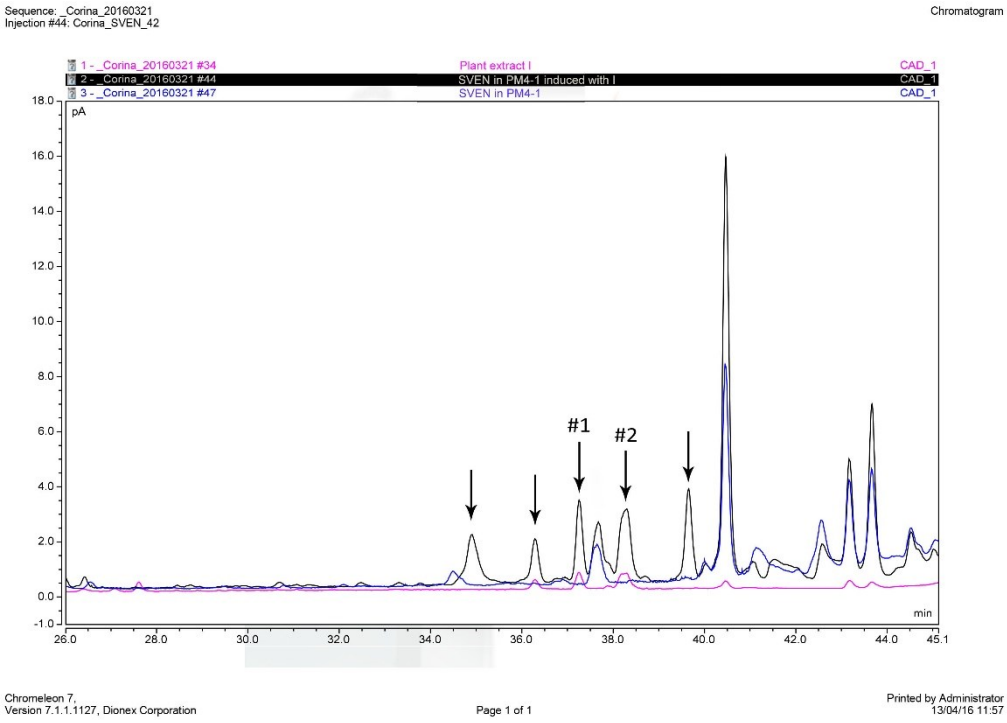

Figure 33: Comparative HPLC-CAD analysis of SVEN PM4-1 IP (black), the corresponding non-induced culture extract SVEN PM4-1 XP (blue) and the diluted MeOH plant extract I (pink), yielded newly emerged peaks. The masses of peak #1 (R_T 37.3) and #2 (R_T 38.3) should be determined with MS.

Comparative analysis of extract SVEN PM4-1 GS

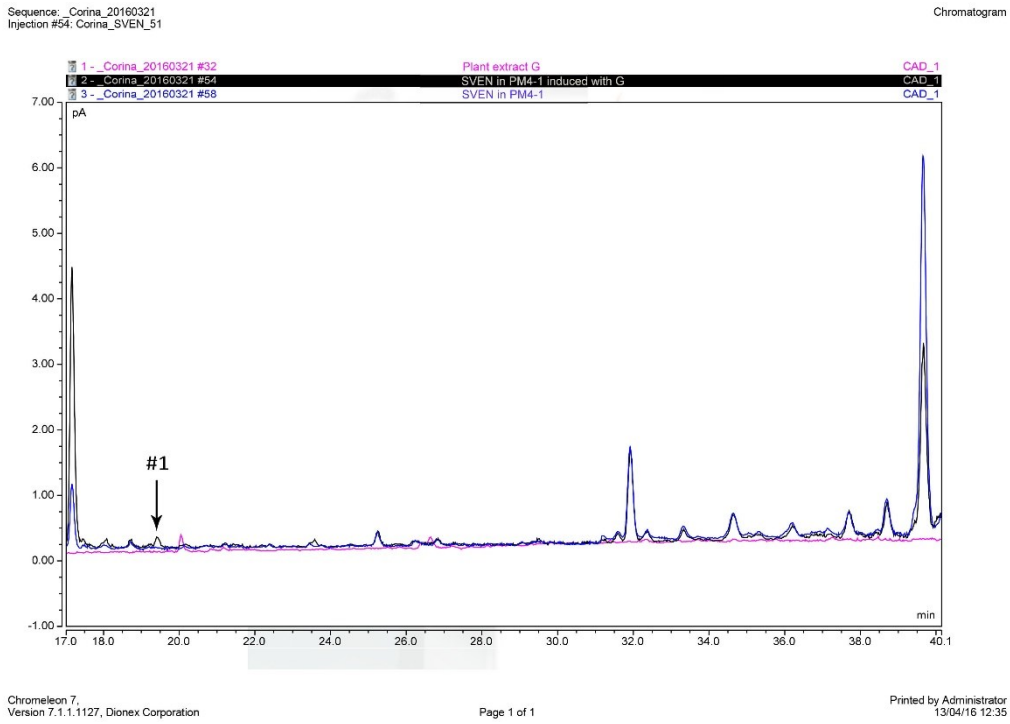

Figure 34 Comparative HPLC-CAD analysis of SVEN PM4-1 GS (black) the corresponding non-induced culture extract SVEN PM4-1 XS (blue) and the diluted MeOH plant extract G (pink), yielded the newly emerged peak #1 (R_T 19.5) that was determined with MS.

Comparative analysis of extract SVEN PM4-1 JS

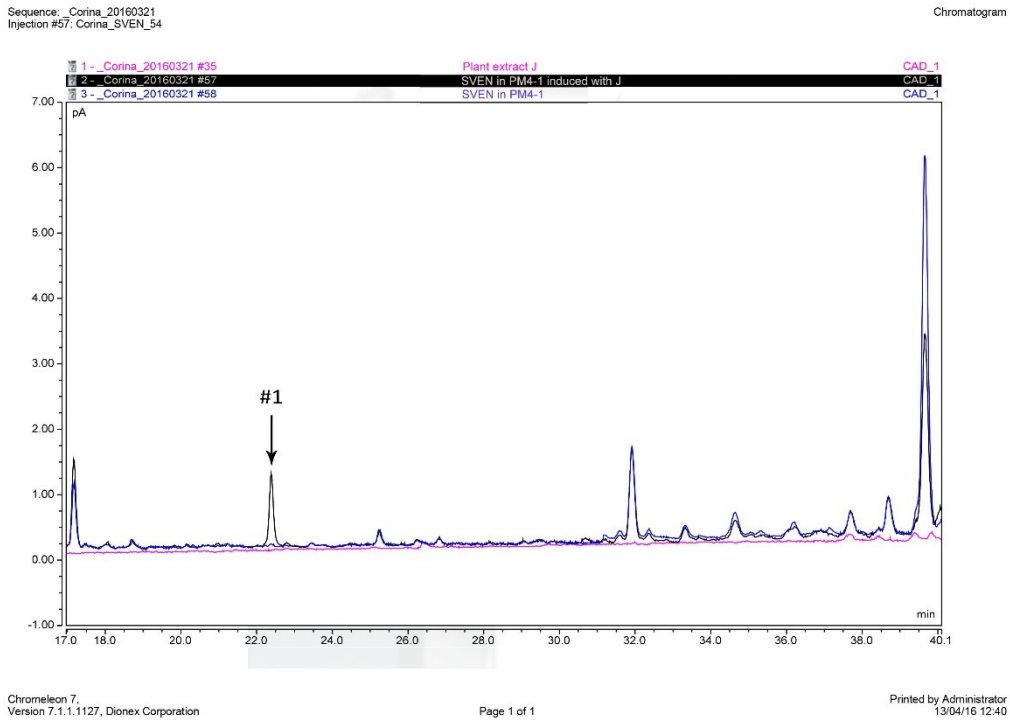

Figure 35: Comparative HPLC-CAD analysis of SVEN PM4-1 JS (black), the corresponding non-induced culture extract SVEN PM4-1 XS (blue) and the diluted MeOH plant extract J (pink) yielded the newly emerged peak #1 (R_T 22.4), that was determined with MS.

Comparative analysis of extract SNOR M5319 BP

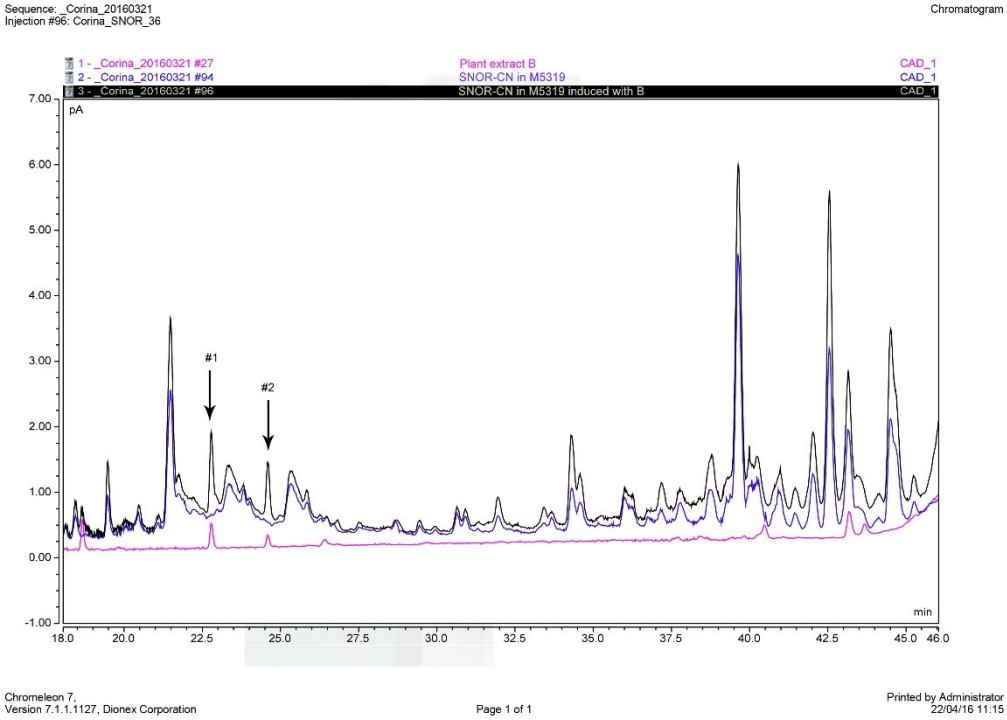

Figure 36: Comparative HPLC-CAD analysis of SNOR M5319 BP (black), the corresponding non-induced culture extract SNOR M5319 XP (blue) and the diluted MeOH plant extract B (pink), yielded the newly emerged peaks #1 (R_T 24.7) and #2 (R_T 22.7), that were determined with MS.

Comparative analysis of extract SNOR M5319 AS

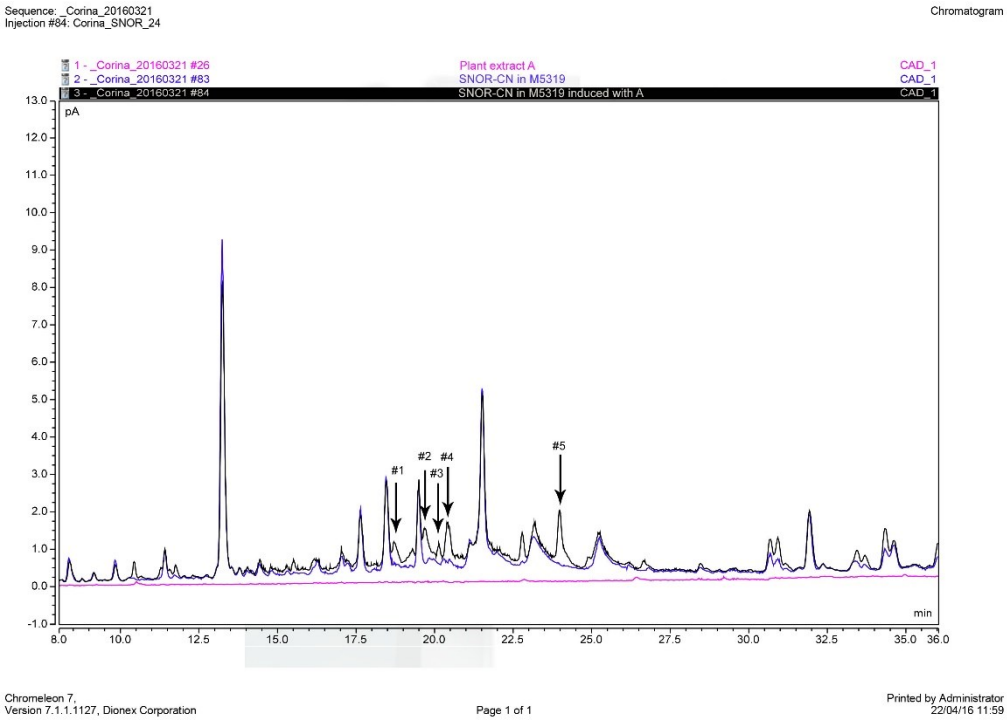

Figure 37: Comparative HPLC-CAD analysis of of SNOR M5319 AS (black), the corresponding non-induced culture extract I SNOR M5319 XS (blue) and the diluted MeOH plant extract A (pink), yielded the newly emerged peaks #1 (R_T 18.7), #2 (R_T 19.7), #3 (R_T 20.2), #4 (R_T 20.4), #5 (R_T 24.0), that were determined with MS.

Comparative analysis of extract SNOR M5319 BS

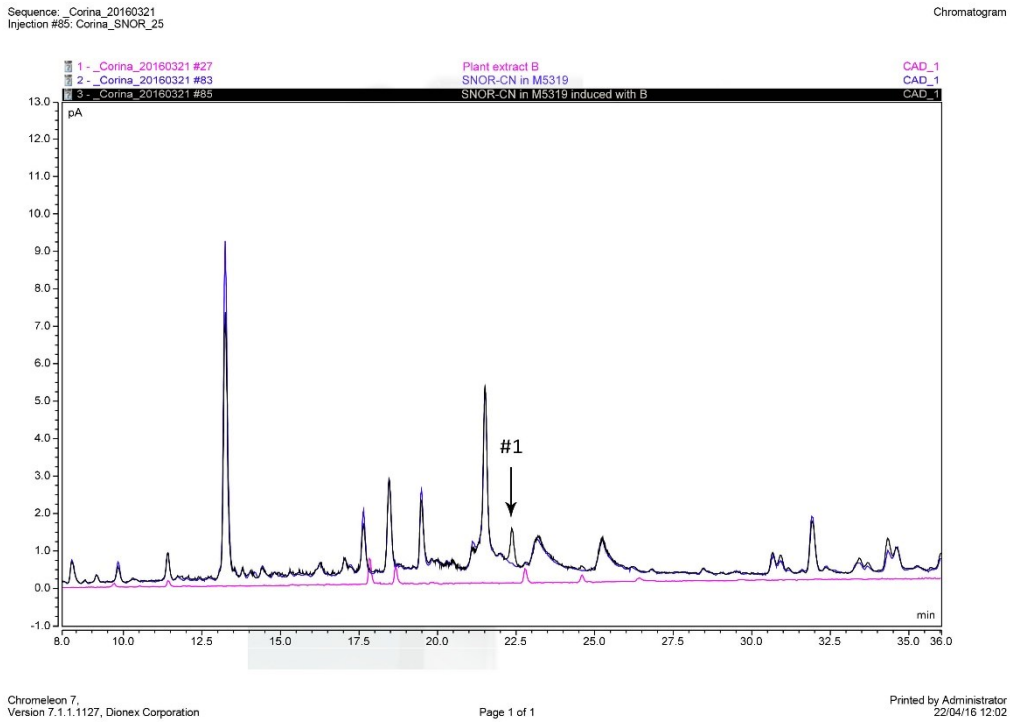

Figure 38: Comparative HPLC-CAD analysis of SNOR M5319 BS (black), the corresponding non-induced culture extract SNOR M5319 XS (blue) and the diluted MeOH plant extract B (pink) yielded the newly emerged peak #1 (R_T 22.4), that was determined with MS.

Comparative analysis of extract SNOR M5319 GS

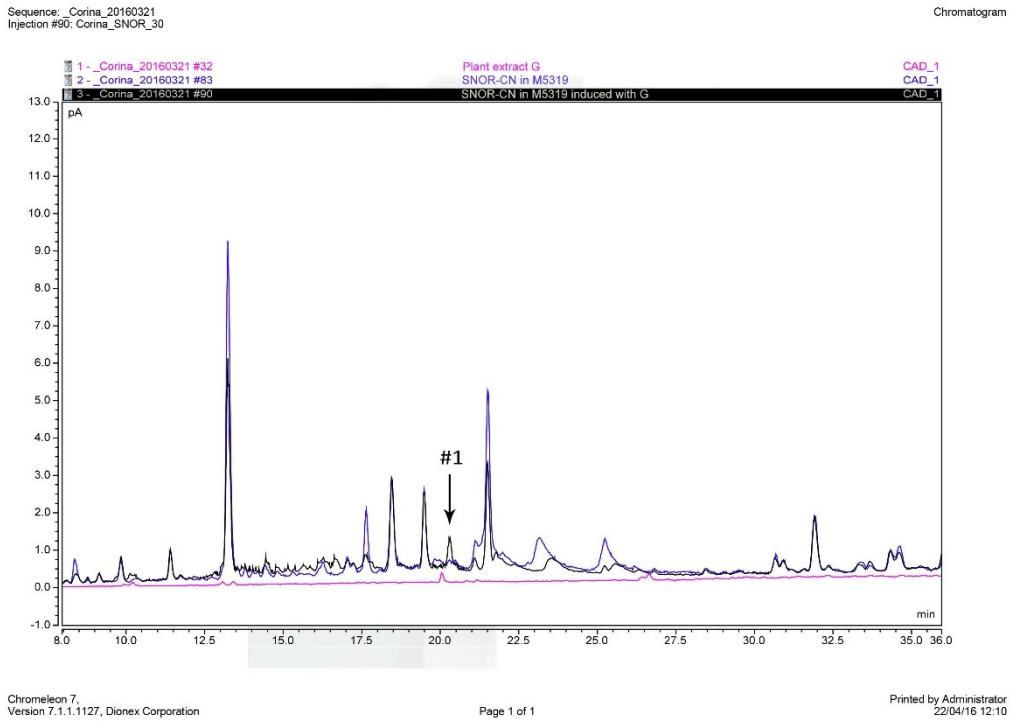

Figure 39: Comparative HPLC-CAD analysis of SNOR M5319 GS (black), the corresponding non-induced culture extract SNOR M5319 XS (blue) and the diluted MeOH plant extract G (pink) yielded the newly emerged peak #1 (R_T 20.4), that was determined with MS.

Comparative analysis of extract SNOR M5319 IS

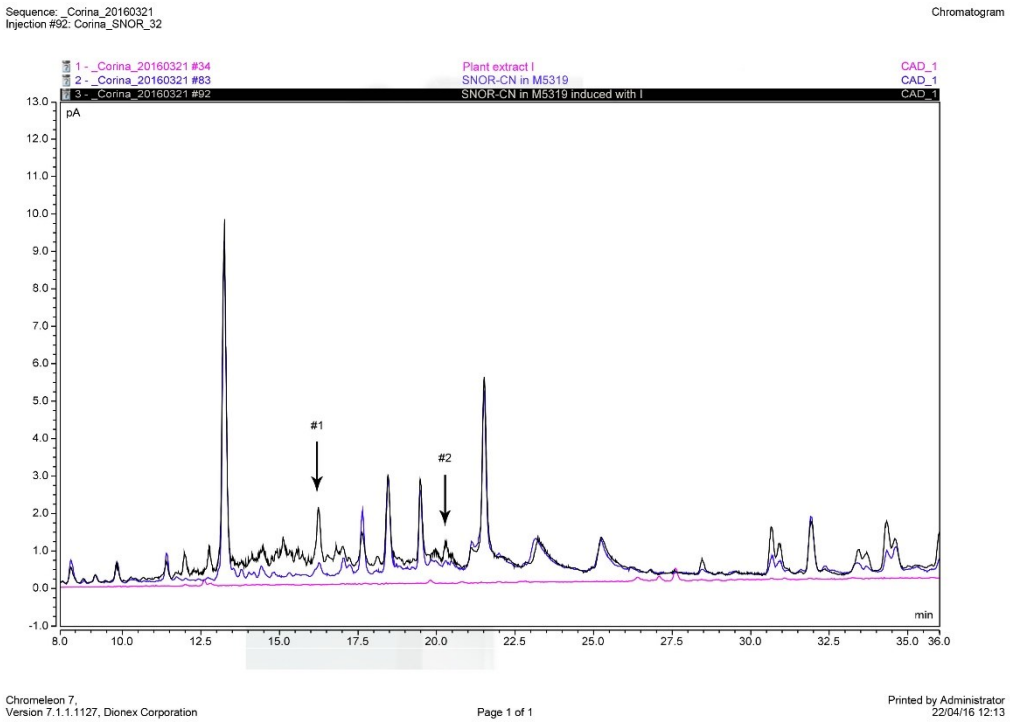

Figure 40: Comparative HPLC-CAD analysis of SNOR M5319 IS (black), the corresponding non-induced culture extract SNOR M5319 XS (blue) and the diluted MeOH plant extract I (pink) yielded the newly emerged peaks #1 (R_T 16.3) and #2 (R_T 20.4), that were determined with MS.

8.2 Vector Maps

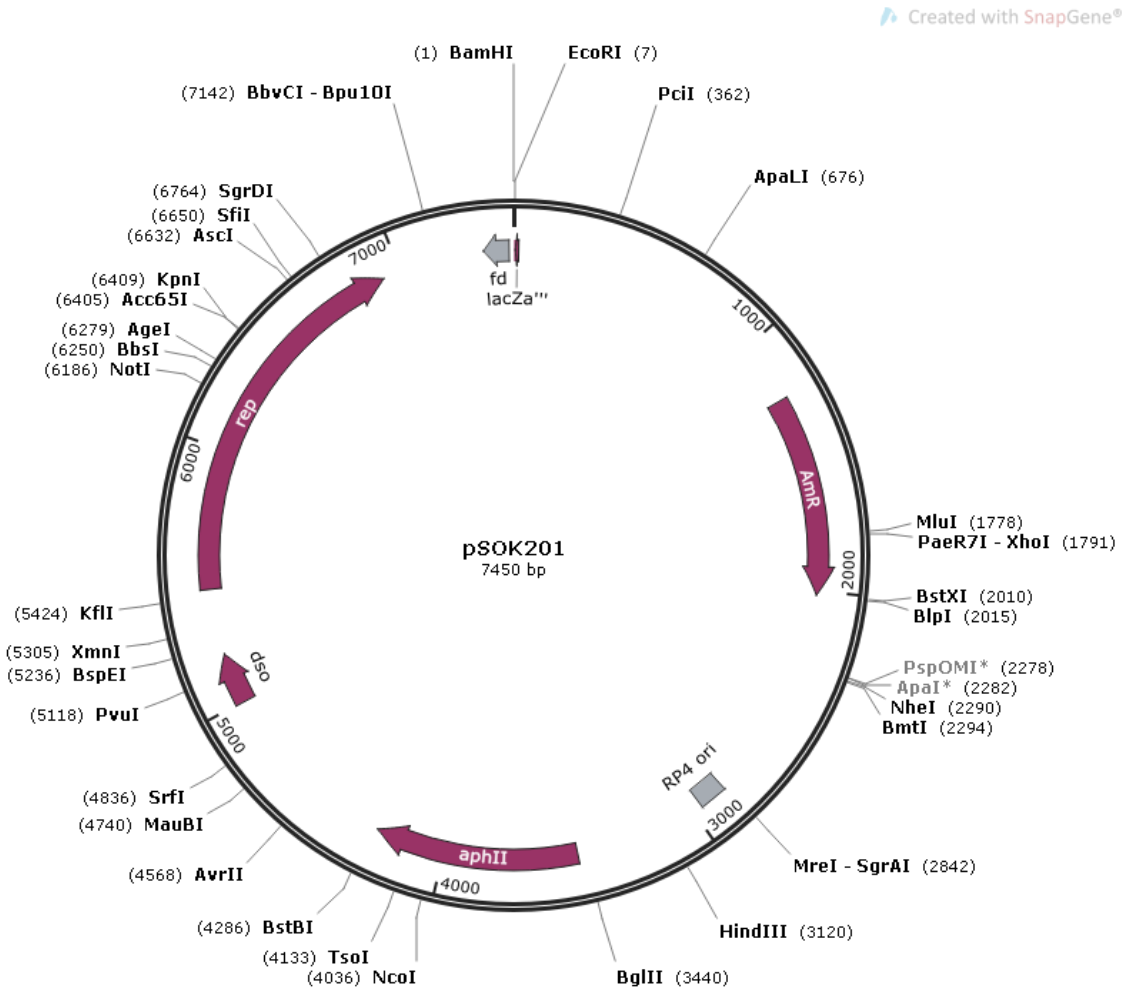

Figure 41: Plasmid map of pSOK201. Used to create the KO construct; Replication initioator protein gene, AmR, RP4 oriT, ColEI replication origin

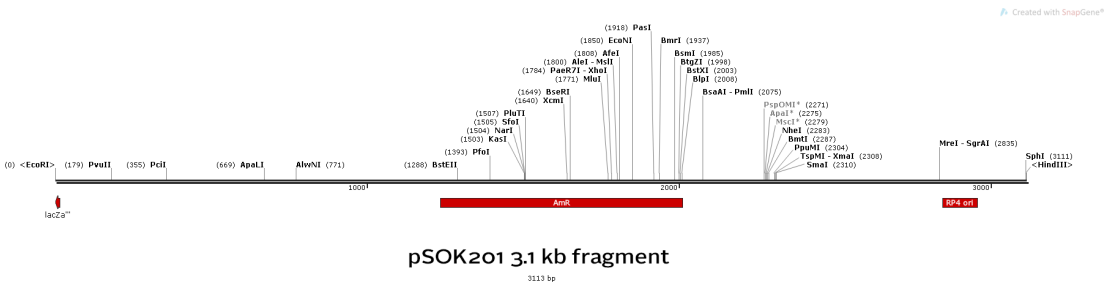

Figure 42: 3.1 kb fragment of pSOK201. Used for KO constructs; replication initiator protein gene, AmR , RP4 oriT; pSOK201 was cut with EcoRI and HindIII

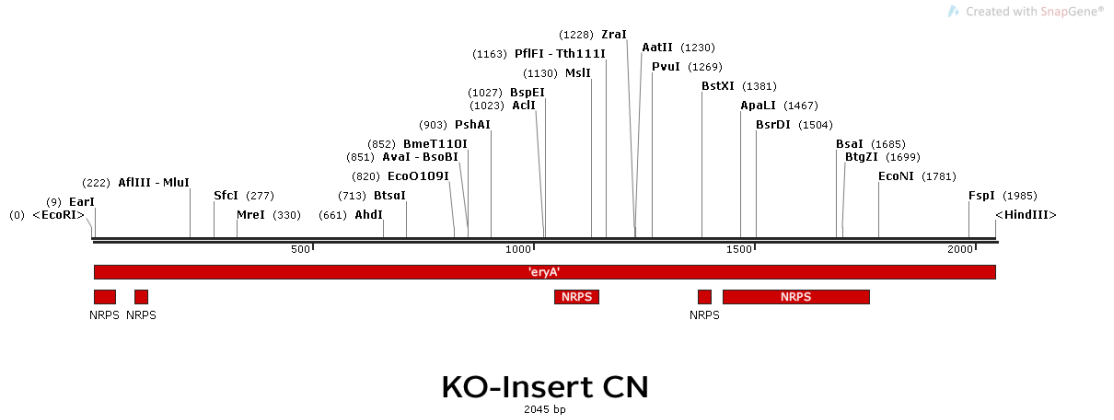

Figure 43: KO-insert CN. Designed with Clone Manager 6. Used for the production of the KO vector for SNOR NDA59

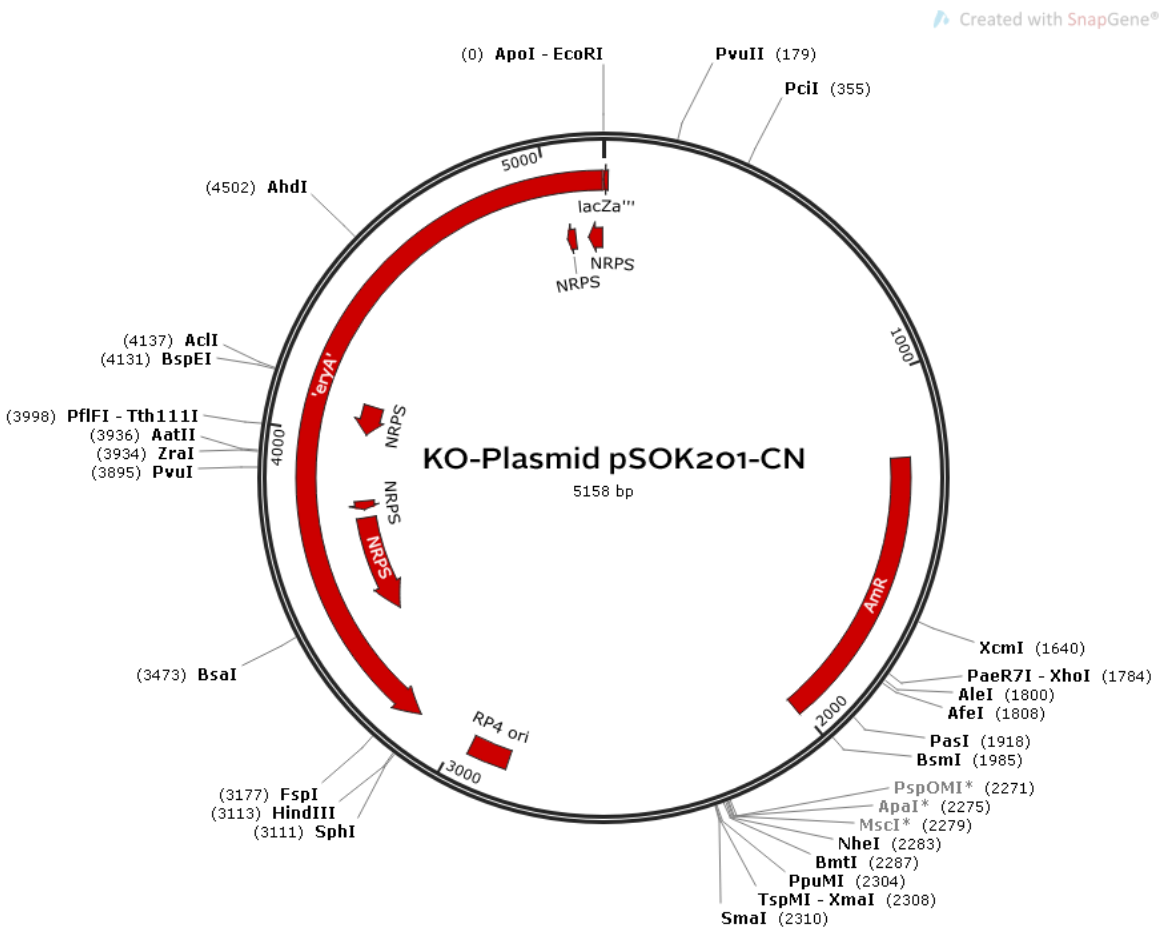

Figure 44: Plasmid map of the final KO-vector pSOK201-CN

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