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„Heterologous expression of terpene cyclases in genetically engineered bacterium“

verfasst von / submitted by

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Abbreviations

dH2O	distilled sterile water
EtOH	Ethanol
DMSO	Dimethyl sulfoxide
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
rpm	Revolutions per minute
pmol	picomol
dNTPs	Desoxyribonucleosidetriphosphates
pDNA	Plasmid DNA
PCR	Polymerase chain reaction
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
amu	Atomic mass units
SMs	Secondary metabolites
TS	Terpene synthase
PKS	Polyketide synthase
NRPS	Non-ribosomal peptide synthetase
bps	Base pairs
EDTA	Ethylenediaminetetraacetic acid
TRIS	tris(hydroxymethyl)aminomethane
DOXP	1-deoxy-D-xylulose 5-phosphate
MEP	2-C-methyl-D-erythritol 4-phosphate
IPP	isopentenyl pyrophosphate
DMAPP	dimethylallyl pyrophosphate
PEG	Polyethylene glycol

1 Abstract

Natural products, especially secondary metabolites from animals, plants and microorganisms, represent an important source for the discovery of new drugs.
So far, screening methods were common use in search of new lead compounds.
In times of the genomic era, where sequencing of whole genomes is available in an uncomplicated and fast way, it became clear, that many genes encoding secondary metabolites remain silent during laboratory conditions.
Heterologous expression is one approach to access the hidden potential of those cryptic genes.
Actinomycetes, most notably the genus *Streptomyces*, can be defined as one of the major producers of SMs.
Terpenoids are a class of SMs that is known to be antiinfective, antitumoral, antiviral and to possess even more beneficial properties which cause their widely use in medicine.
In this project, we tested genomes of marine actinomycetes for the presence of terpene synthase genes by the support of the bioinformatic tool antiSMASH. The analyses revealed 31 biosynthetic gene clusters which contained terpene synthase (TS) genes. According to their phylogeny, some TSs were suggested to synthesize new, previously undetected products.
Using a newly constructed vector, pAMX1, two terpene synthases were expressed in a heterologous *Streptomyces venezuelae* host. HPLC analyses showed differences in the spectra between the recombinant clones and the control. Further analyses could not be pursued due to lack of time but it is likely that with continuing research novel compounds might be detected.

Zusammenfassung

Naturstoffe, vor allem Sekundärmetaboliten aus Tieren, Pflanzen und Mikroorganismen stellen eine wichtige Quelle zur Entdeckung neuer Arzneistoffe dar. Bisher gebräuchliche Verfahren, um neue Leitstrukturen zu finden, sind beispielsweise Screeningmethoden.

Im genomischen Zeitalter ist es möglich, gesamte Genome innerhalb kurzer Zeit unkompliziert zu analysieren. Dadurch wurde klar, dass eine Vielzahl von Genen, welche in die Biosynthese von Sekundärmetaboliten involviert sind, unter Laborbedingungen still bleibt.

Ein Ansatz, um die biosynthetischen Produkte dieser stummen Gene trotzdem aufzuspüren, ist die heterologe Expression.

Der Großteil der Sekundärmetaboliten wird von Actinomyceten, insbesondere von der Gattung *Streptomyces*, produziert.

In diesem Projekt wurden einige Genome mariner Actinomyceten mittels des bioinformatischen Tools antiSMASH analysiert.

Die Analyse zeigte auf, dass 31 Gencluster Gene für Terpensynthasen beeinhalteten.

Aufgrund ihrer Phylogenie konnte man von einigen Terpensynthasen annehmen, dass sie neue, noch unentdeckte Verbindungen synthetisieren.

Mittels eines eigens konstruierten Vektors, pAMX1, wurden zwei Terpensynthasen in einem heterologen *Streptomyces venezuelae* Wirt exprimiert.

HPLC Analysen zeigten Unterschiede zwischen den Spektren der rekombinanten Klone und der Kontrollen. Aus zeitlichen Gründen war es bisher noch nicht möglich, die Versuche fortzuführen. Es ist aber wahrscheinlich, dass mit der Durchführung weiterer Tests neue Verbindungen gefunden werden können.

2 Aim of the work

To discover new terpenes by introducing terpene synthase (TS) genes into genetically engineered *Streptomyces venezuelae* was the goal in this diploma project.

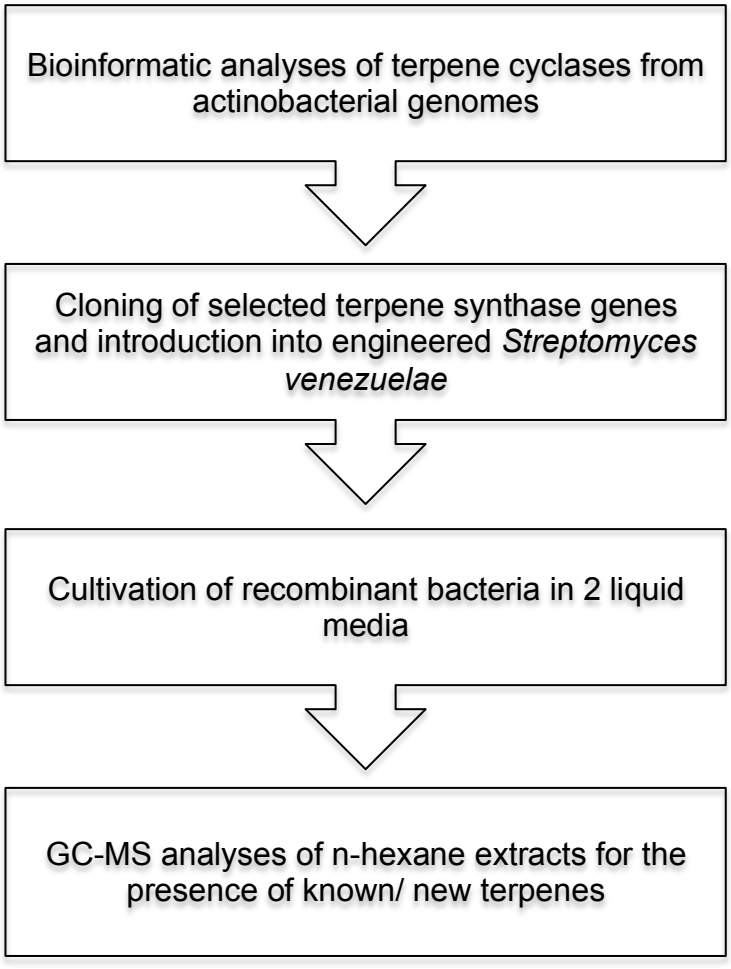
The idea was to use bioinformatic tools, mainly antiSMASH and MEGA to detect biosynthetic gene clusters containing terpene synthases with so far unknown products. For the bioinformatic analyses, we took marine actinobacterial genomes, because especially marine organisms seem to be a promising source for finding new natural products.

Phylogenetic analyses should demonstrate which genes are interesting to work with.

A suitable vector would be created to clone the selected TS genes into the engineered *Streptomyces venezuelae* JZ04 strain.

The recombinant bacteria were planned to be cultivated in two different liquid media.

N-hexane extracts should be analyzed by gas chromatography-mass spectrometry (GC-MS) to detect the produced terpenes. Overview of the working steps:



3 Introduction

3.1 Secondary metabolites

Secondary metabolites (SMs) are substances produced by plants, fungi and microorganisms. (Vaishnav, Demain, 2010) Unlike components of the primary metabolism, such as amino acids, nucleotides, lipids and carbohydrates, SMs are not essential for the growth of the microorganism. Nevertheless, their biosynthetic pathways are linked to the network of primary metabolism. (Barrios-González et al. 2003)
Chemical compounds from important pathways of the primary metabolism provide the raw material for the production of SMs. (Komatsu et al., 2010)
SMs are supposed to have a survival function for the producing organism, for instance the defense against other bacteria, fungi, plants, insects etc.. (Demain, Fang, 2000)

In fact, secondary metabolites play a very important role in human health because of their biological activities. They represent about two-thirds of all therapeutically used compounds. (Gomez-Escribano, Bibb, 2014)
Microbial metabolites can be antibacterial, antifungal, antiprotozoal, antitumoral, antiviral, immunosuppressive, immunomodulatory, antiinflammatory, hypocholesteremic and even more. (Bérdy, 2005)

SMs are not only used in medicine, but also in cosmetics, agriculture and industry. (O'Connor, 2015) Some of them act as pesticides and herbicides. (Bérdy, 2005)
Well-known examples of SMs are shown in Figure 1.

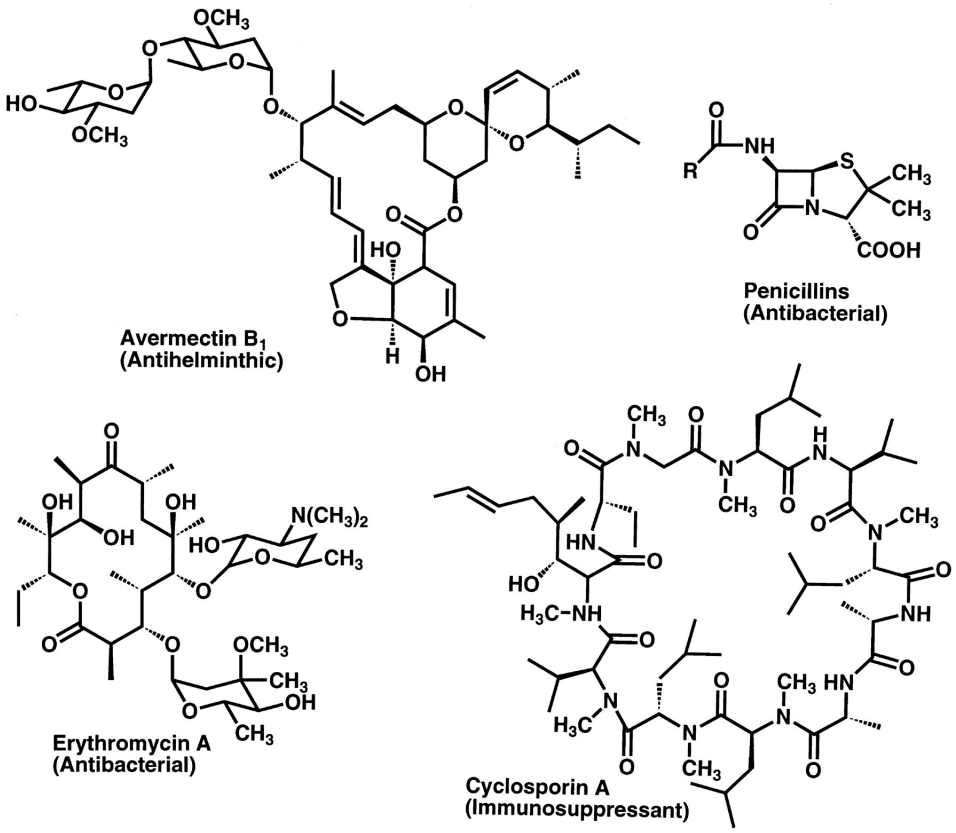


Figure 1: Examples of secondary metabolites (Cane et al., 1998)

3.1.1 Antibiotics

SMs cover a wide range of bioactive compounds, even though antibiotics are the most common ones. (Barrios-González et al. 2003)

Penicillins, Cephalosporins, Tetracyclines, Aminoglycosides, Chloramphenicol, Macrolides and Glycopeptides are the most important antibiotic groups among others. (Demain, 2009)

Antibiotics can be classified according to their modes of action. Four key mechanisms are: the inhibition of the bacterical cell wall synthesis, the DNA synthesis, the protein synthesis or the folate synthesis (Fig. 2). (Kaufman, 2011)

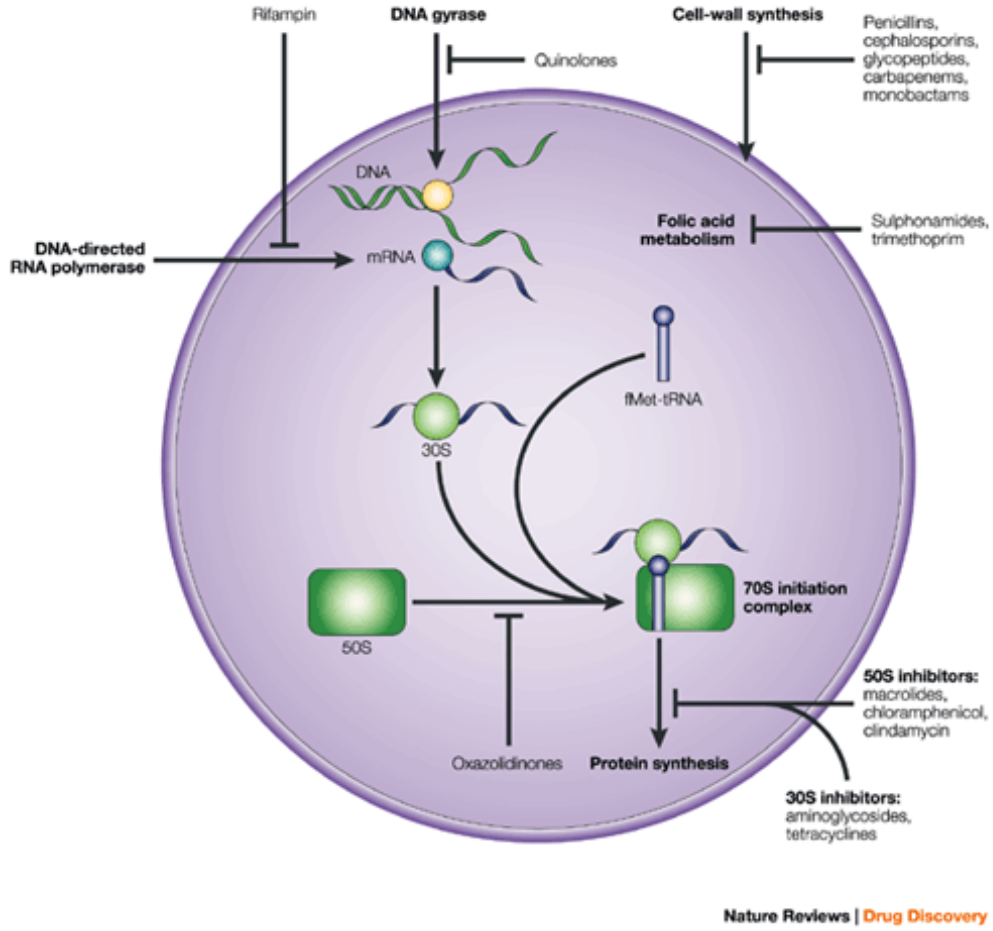


Figure 2: Targets of different antibiotic classes (Coates et al., 2002)

The breakthrough in the history of antibiotic discovery goes back to 1928, when Alexander Fleming accidentially detected Penicillin.

He found an inhibiting areola on his Petri dish that contained *Staphylococcus* bacteria. *Penicillium notatum*, a mold, was identified as the producer of a substance that inhibits bacterial growth.

Later, this first antibiotic was named Penicillin.

(http://www.acs.org/content/acs/en/education/whatischemistry/landmarks/flemingpenicillin.html, retrieved Oct. 4, 2016)

In 1939, Howard W. Florey, Ernst B. Chain and his assistants developed a process for the industrial production of Penicillin. In 1945, Fleming, Howard and Chain won the Nobel Prize in Physiology or Medicine. (Ligon, 2004)

Today, more than 90 percent of pathogenic *Staphylococcus aureus* show resistance against Penicillin.

(http://www.laboratory-journal.com/science/pharma-drug-discovery/antibiotic-resistance-facing-challenges-bacterial-infections, retrieved Oct. 4, 2016)
Resistant bacteria appear rapidly worldwide and put the efficacy of antibiotics at risk. This crisis has its origin in the overuse of antibiotics, the inappropriate prescribing of those drugs and the extensive agricultural use. (Ventola, 2015)

Looking at the timeline below (Fig. 3), it becomes clear that only a few years after the development of an antibiotic, cases of resistances against it were reported.
(It is not meant here that each antibiotic is useless against every pathogen)

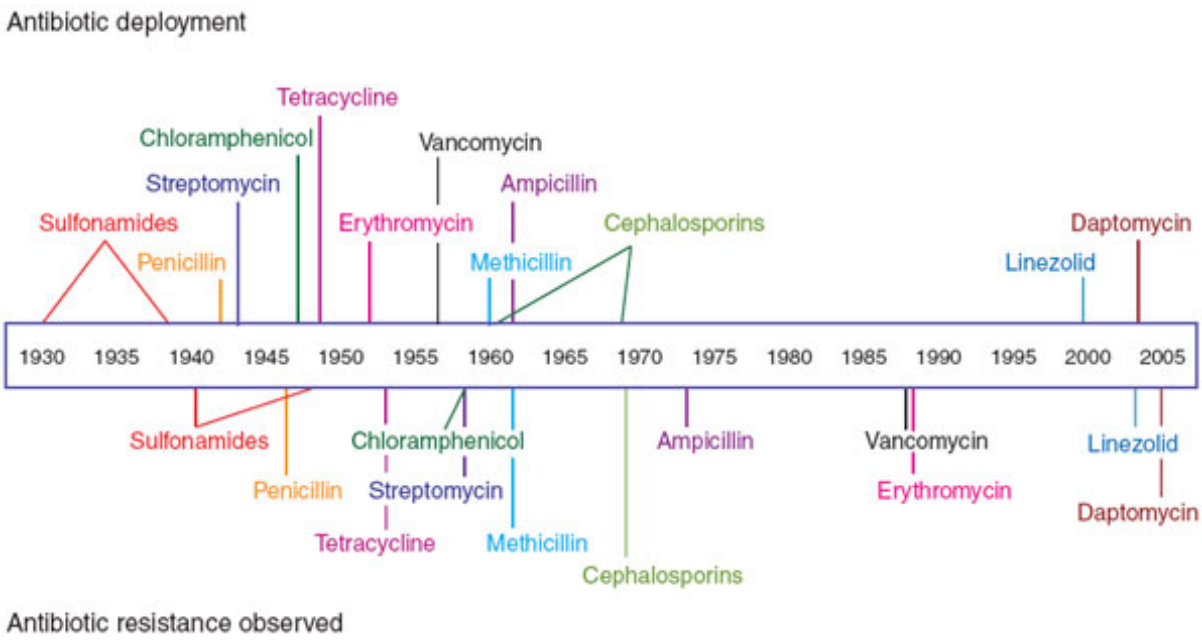


Figure 3: This figure shows a timeline of antibiotic resistance in relation to its development (Clatworthy et al., 2007)

Each year antimicrobial resistance causes many cases of death, and the problem is rising.
Scientists predict a higher mortality rate due to antimicrobial resistance (AMR) than due to cancer for the future (Fig. 4).

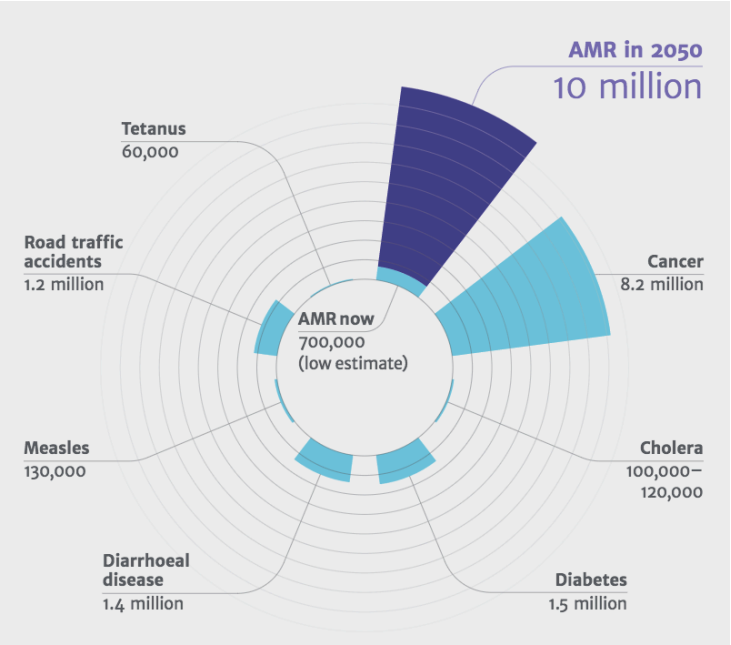


Figure 4: A prediction for the future concerning death cases due to antimicrobial resistance(https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf)

Since the 2000s only a few new antibiotics were discovered and developed into human drugs (Fig. 5).

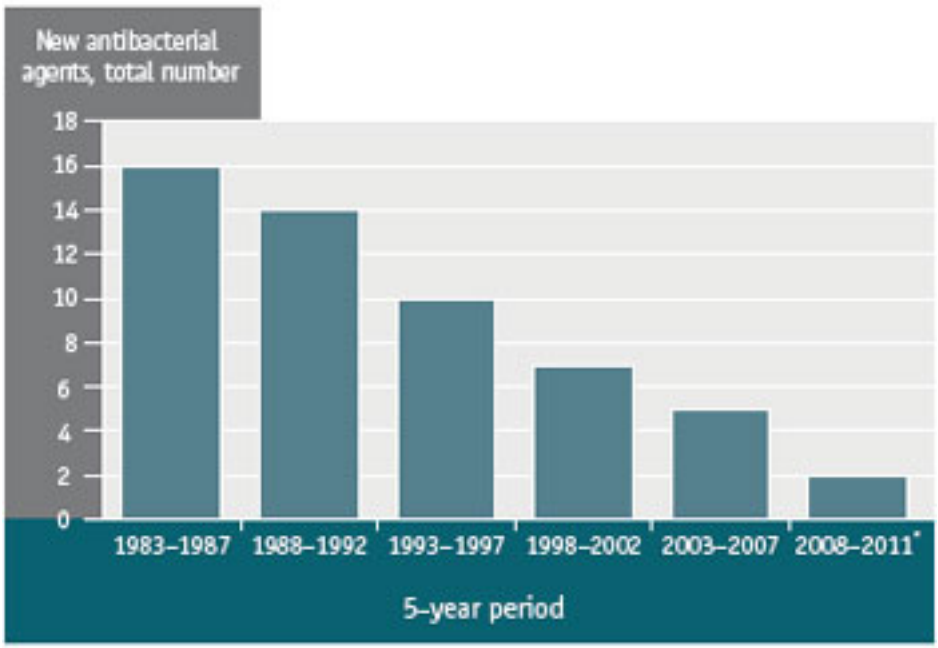


Figure 5: An overview of the discovery of antibiotics since the 2000s. (http://www.phac-aspc.gc.ca/cphorsphc-respcacsp/2013/resistance-eng.php, retrieved Nov. 5, 2016)

One of the reasons for this is the fact that the pharmaceutical industry is not economically interested to invest in the development of new antibiotics, because they are used for a short time by the patients and often are curative.
Assuming this information, it is obvious that there is a lack of new antimicrobial drugs (Ventola, 2015) and it is crucial for the future to develop new antimicrobial compounds.

3.2 Genomic era in the discovery of new bioactive compounds

When the first antibiotic biosynthetic genes were discovered in the 70s and 80s, this was a progress in understanding the genetic and biochemical background for their biosynthesis. (Ziemert et al., 2016)
In the last years, genomic methods developed rapidly and revealed the potential of organisms to produce wide more SMs than expected. (Breitling et al., 2013) For example, sequencing revealed that one *Streptomyces* sp. genome can contain 20-50 biosynthetic clusters for secondary metabolites, but it is known that a considerable part of SMs is not produced under laboratory conditions or synthesized in too little amounts to detect them. (Breitling et al., 2013; Bentley et al., 2002)
It is necessary to mention here that biosynthetic genes for secondary metabolites are clustered on the chromosome, and I will give more details about biosynthetic clusters a bit later on.
Putative gene clusters which are not expressed under laboratory conditions and have an unknown product are called cryptic or silent gene clusters. (Gomez-Escribano, Bibb, 2014)
Since the first complete genome of the microorganism *Haemophilus influenzae* was published in 1995, numerous genomes were being sequenced. (Bode, Müller, 2005)
These days thousands of sequenced genomes are available, a complete bacterial genome can be sequenced in only a few hours. (Ziemert et al., 2016)
As it is shown in Figure 6, by 2014 more than 30,000 sequenced bacterial genomes were available.

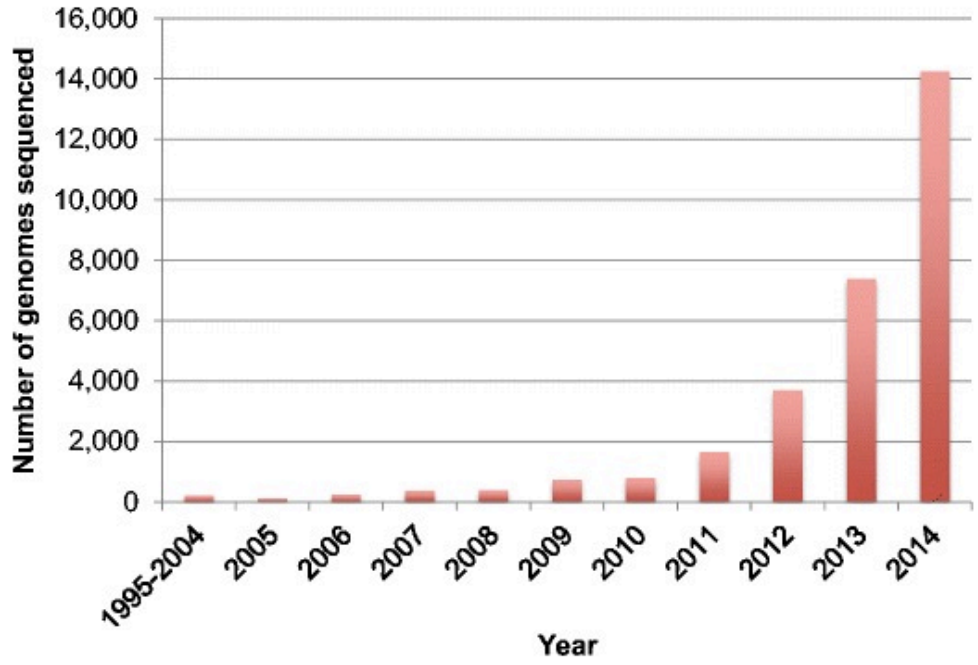


Figure 6: This figure represents numbers of bacterial and archaeal sequenced genomes per year (submitted to NCBI) (Land et al., 2015)

Today most of the new metabolites are prognosticated by the use of bioinformatic analyses of such presumable secondary metabolite gene clusters in sequenced genomes. (Breitling et al., 2013) To predict and isolate natural products based on the genetic information is the classical idea of genome mining.
Several bioinformatic tools make it possible for researchers to easily receive genetic data by computational procedures. The first reported tool that automatically minds gene clusters was DECEIPHER® , developed in 2001. Several other tools followed, such as BAGEL, CLUSEAN and antiSMASH, the latter was used in this diploma project. Those programs screen genomes and assess the outcoming results using profiles of already known biosynthetic enzymes. (Ziemert et al., 2016)

3.2.1 Expression of genes in heterologous hosts

To uncover the „hidden“ metabolic products of a gene cluster, one important stategy is to find out the presence of cryptic gene clusters with the support of bioinformatics and afterwards the expression of the interesting genes in a heterologous host. (Gomez-Escribano, Bibb, 2014)

Genome sequencing and bioinformatics uncovered 262 clearly predicted terpene synthases. Many of those predicted terpene synthase genes seem to be silent in the original producing organism, but their biochemical function could be identified by expressing those genes in a heterologous *Streptomyces* host. (Yamada et al., 2015)
In 2015, Yamada et al. found 13 new terpene structures by heterologous expression in a *Streptomyces* host and detection via GC-MS. Some of the new terpenes have never been reported before. For instance, two closely related diterpenes, hydropyrene and hydropyrenol were synthesized by a heterologous *Streptomyces* host containing the *sclav_p0756* gene, while the original strain, *Streptomyces clavuligerus* ATCC 26074 didn't produce the compounds under any laboratory conditions.
Both structures are unique. No reports on their further development have been published so far. (Yamada et al., 2015)

3.3 Biosynthesis of SMs in bacteria

As mentioned, secondary metabolites cover a variety of chemical structures.
Their synthesis is managed by many enzymes. Polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) can be defined as two major classes of enzymes concerning the secondary metabolite production. (Ichikawa et al., 2012)
To ensure that enzymes, which are responsible for the biosynthesis of the scaffolds and their modifications, work in a coordinated manner, the biosynthetic genes are arranged in clusters in the organism’s genome. (Zotchev, 2008)
As it is mentioned earlier, the genomes of actinomycetes contain more gene clusters encoding enzymes, than actually biosynthesized and detected natural products. (Doroghazi, Metcalf, 2013)
For example, the analysis of the genome of *Streptomyces coelicolor* revealed that this bacterial genome has over 20 gene clusters that encode secondary metabolites, while only 5 chemically distinct compounds have been isolated from this organism back then. (Bentley et al., 2002)
A typical antibiotic biosynthetic gene cluster is organized as follows:
The biosynthesis of a scaffold (a molecule skeleton) of an antibiotic is ensured by genes that encode enzymes such as PKSs, NRPSs, TSs or others.

Genes encoding scaffold modification enzymes, such as hydroxylases, methyltransferases etc. are situated in the surroundings.
Usually, regulatory genes can be also present in a cluster. They are responsible for the regulation of the expression of biosynthetic genes.
To ensure resistance to its own antibiotic, there are genes which encode the enzymes for inactivation of the antibiotic, modification of its target in the microorganism or efflux of the produced compound out of the cell. (Zotchev, 2008)

A schematic of the antibiotic biosynthesis gene cluster is shown in Figure 7.

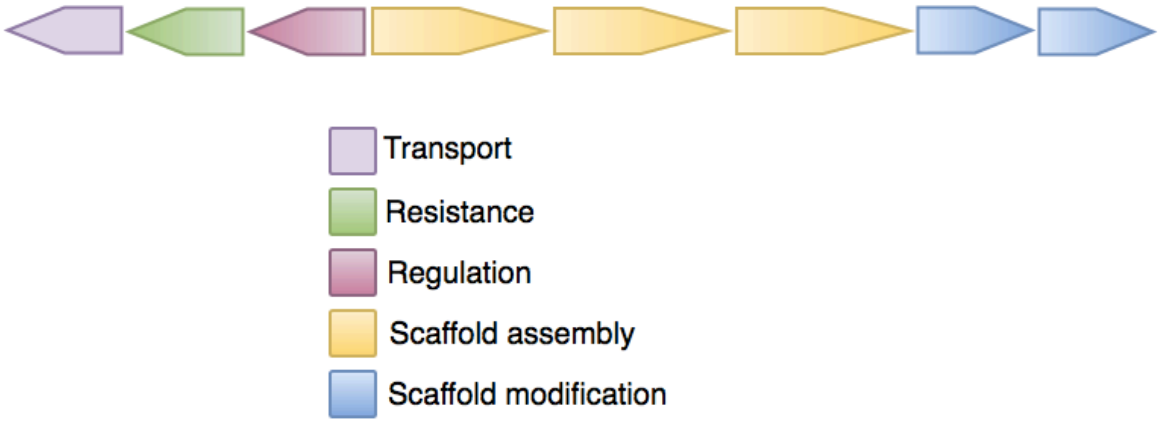


Figure 7: Schematic of an antibiotic biosynthesis gene cluster (Zotchev, 2008)

3.3.1 Biosynthesis of SMs on the example of Erythromycin

Major groups of secondary metabolites are polyketides and non-ribosomal peptides. They are synthesized by adding small monomer units, such as acetic or malonic acid to linear oligomers by multifunctional enzymes (for ex., PKSs). (Cane et al., 1998)
A clinically relevant example for a polyketide is the macrolide antibiotic Erythromycin, produced by the bacterium *Saccharopolyspora erythraea.* (Oliynyk et al., 2007)
Erythromycin is synthesized from acyl CoA precursors by a PKS. (Shen, 2003)
6-deoxyerythromycin B synthase (DEBS) ,a type I PKS, synthesizes the macrolide ring of Erythromycin. Three proteins, DEBS 1,2 and 3, are organized in 6 modules.

Each module contains at least three different functional domains: a ketosynthase (KS), an acyltransferase (AT) and an acyl carrier protein (ACP). (Khosla et al., 2007) These 3 enzymatic activities are recognized as a minimal PKS.

The acyltransferase loads the correct extender unit onto the acyl carrier protein in the front of DEBS1. ACP is a protein on which the growing carbon chain is attached, while KS is responsible for the Claisen condensation, linking the new extender unit with the growing polyketide chain. Each condensation step is accompanied by loss of carbon dioxide. In addition to minimal PKS, there can be other activities, as, for example, DEBS3, has a thioesterase activitiy (TE) that catalyzes release of the polyketide chain from PKS and cyclisation of the intermediate heptaketide to 6-DEB (Fig. 8). (Staunton, Weissmann, 2001)

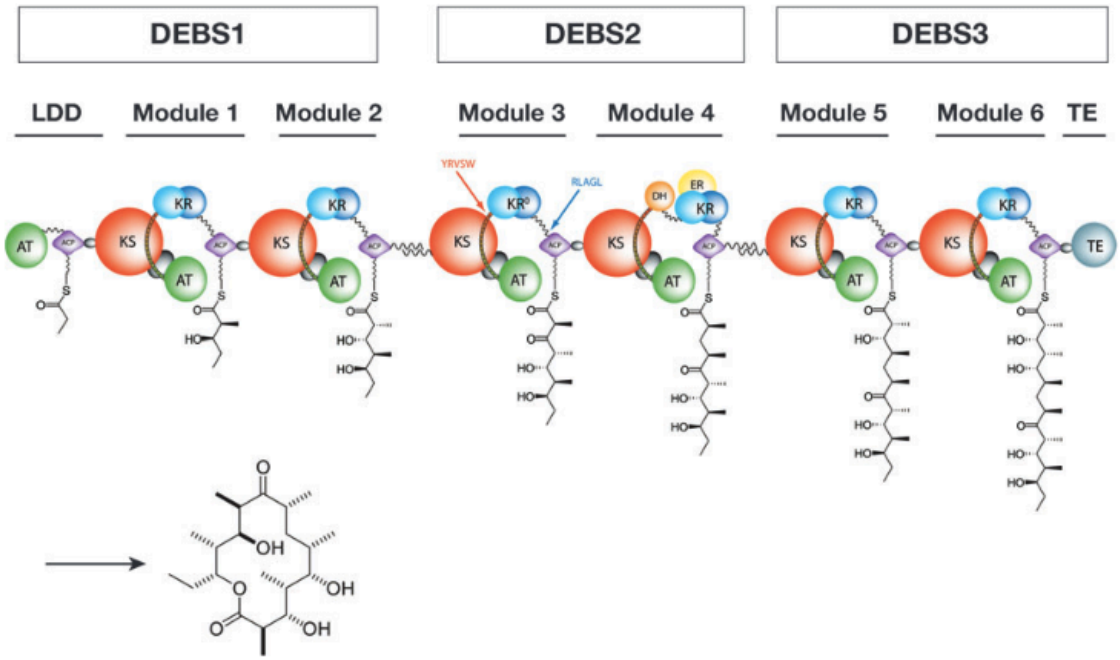


Figure 8: Example for a polyketide synthase. 6-deoxyerythromycin B synthase, DEBS, synthesizes the macrolide ring of Erythromycin (Khosla et al., 2007)

Besides polyketides and non-ribosomal peptides, many other secondary metabolite compound classes are known, such as terpenes, aminoglycosides, aminocoumarins, lantibiotics, and many more. (Medema et al., 2011)

This project brings terpenes into focus. Further information about this class of SMs is provided later.

3.4 Actinomycetes as producers of secondary metabolites

Actinomycetes are regarded among the most efficient producers of SMs.
More than a million natural compounds were described, of which over 200,000 apparently have bioactive properties.
About 22,000 of those components are produced by microbes and nearly half of them come from actinomycetes (Fig. 9). (Solecka et al., 2012)

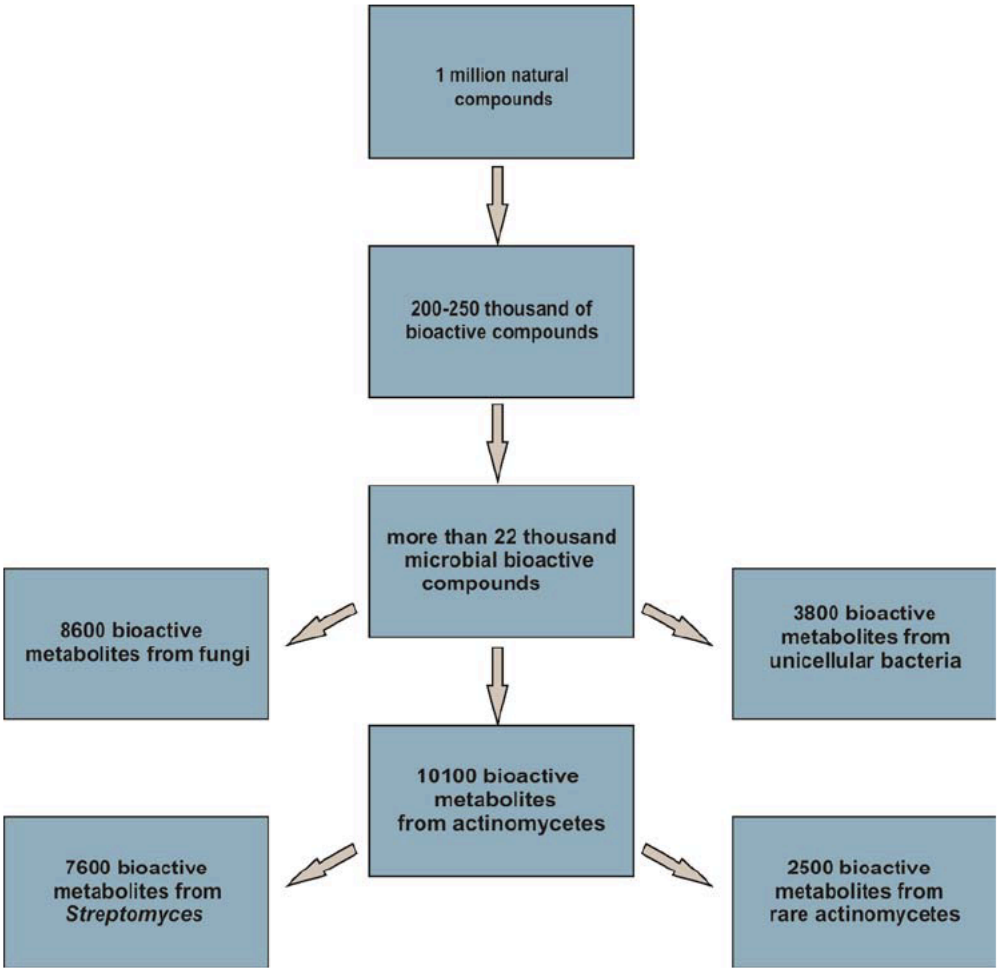


Figure 9: Overview of the origin of natural products (Solecka et al., 2012)

Especially the genera *Streptomyces, Saccharopolyspora, Amycolatopsis, Micromonospora and Actinoplanes* produce widely used compounds. (Solanki et al., 2008)
Actinomycetes belong to one of the biggest bacterial phyla.
They are GC-rich, gram-positive, filamentous bacteria.

Their growth distinguishes itself due to a combination of tip expansion and branching of the hyphae, which named them after the greek words for ray (aktis) and fungi (mykes). (Das et al., 2008)

Many actinobacteria produce spores. With some exceptions, the major part lives aerobic. Actinobacteria can be found in both aquatic or terrestrial environment (for example the genus *Streptomyces*). Some of them live in symbiosis with a plant (for example *Frankia* spp.) and others are found in the intestinal flora (for example *Bifidobacterium* spp.). (Barka et al., 2016)

The life cycle of these bacteria is extraordinary, for example the one of well-studied *Streptomyces* sp. (Fig. 10).

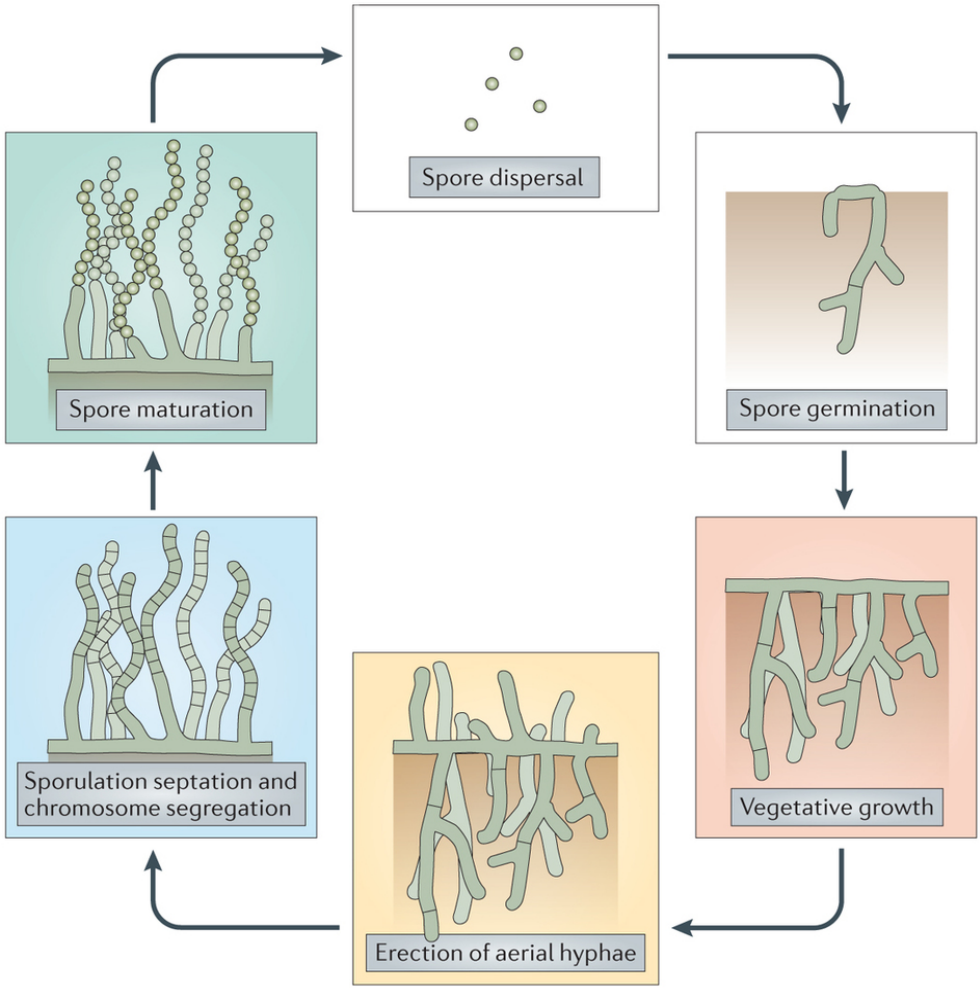


Figure 10: Schematic life cycle of streptomycetes (Bush et. al., 2015)

Under favorable conditions a spore germinates and grows to a hypha which also branches out.
A mycelial network of vegetative hyphae appears.
When nutrients are at low level, aerial hyphae develop.
With the duplication of the genome, cross walls are formed and a thick cell wall is build. The cells differentiate into spores that stay in a resting state until they germinate again in a suitable environment.
(http://www.pharmazeutische-zeitung.de/index.php?id=62994, retrieved Sep. 30, 2016)
Environment changes and nutrient starvation lead bacteria to antibiotic production. (Liu et al., 2013) The largest part of antibiotics is produced at a stage of developing aerial mycelium. (Barka et al., 2016)

3.4.1 Antibiotics produced by the genus *Streptomyces*

The representatives of the genus *Streptomyces* produce the major part (about 75 %) of microbial bioactive secondary metabolites among actinomycetes. (Solecka et al., 2012)
Examples of well-known antibiotics produced by *Streptomyces* are shown in Table 1.

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

Antibiotic	Producing organism	Chemical class	Target
Chloramphenicol	*S.venezuelae*	-	proteine synthesis
Chlortetracycline	*S.aureofaciens*	Tetracycline	protein synthesis
Clavulanic acid	*S.clavuligerus*	ß-lactam	ß-lactamase inhibitor
Daptomycin	*S.roseosporus*	Lipopeptide	
Kanamycin	*S.kanamyceticus*	Aminoglycoside	proteine synthesis

Lincomycin	*S.lincolnensis*	Sugar-amide	protein synthesis
Neomycin	*S.fradiae*	Aminoglycoside	protein synthesis
Novobiocin	*S.niveus*	Coumerin glycoside	DNA gyrase
Oleandomycin	*S.antibioticus*	Makrolide	protein synthesis
Oxytetracycline	*S.rimosus*	Tetracycline	protein synthesis
Spiramycin	*S.ambofaciens*	Macrolide	protein synthesis
Streptomycin	*S.griseus*	Aminogylcoside	protein synthesis
Tetracycline	*S.aureofaciens*	Tetracycline	protein synthesis
Tobramycine	*S.tenebrarius*	Aminoglycoside	protein synthesis

Most antibiotics are produced species-specific. For example, the antibiotic-producing *Streptomyces* bacterium, that lives in symbiosis with plants, can protect the plants against pathogens. (de Lima Procópio et al., 2012)

3.4.2 Marine actinomycetes

Terrestrial members of actinomycetes have been studied since the 1950s.
Due to the re-discovery of the same compounds found in actinomycetes that live in terrestrial environment, scientists discover the marine environment as new sources for finding new natural products. It is easily imaginable that marine bacteria are more difficult to handle concerning their cultivation. (Zotchev, 2012) Nevertheless it is possible to isolate marine actinomycetes that produce interesting bioactive novel compounds.

For example, in 2003, a new cyclic γ-lactam-β-lactone containing compound, Salinosporamide A, was discovered from *Salinospora*, a marine actinomycete. (Feling et al., 2003) Salinosporamide A has great potential for the inhibition of the

chymotrypsin-like proteolytic activitiy of the mammalian 20S proteasome, which is a major target in tumor therapy. (Jensen et al., 2005)
Recently, Salinosporamide A (Marizomib) is clinically tested due to its anticancer activitiy.
(https://clinicaltrials.gov/ct2/results?term=marizomib&recr=Open&rslt=&type=&cond=&intr=&outc=&spons=&lead=&id=&state1=&cntry1=&state2=&cntry2=&state3=&cntry3=&locn=&gndr=&rcv_s=&rcv_e=&lup_s=&lup_e=, retrieved March 19, 2017)

The chemical structure of Salinosporamide A is represented on the Figure 11.

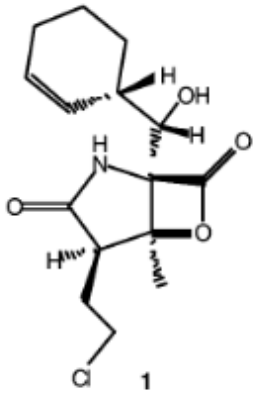


Figure 11: Salinosporamide A, a new compound that was discovered from a marine actinomycete. (Feling et al., 2003)

3.5 Terpenes

Terpenes, also called isoprenoids, are the most various group of low molecular mass natural products.
The initial precursor of all terpenes is dimethyl allyl diphosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP). (Wendt, Schulz, 1998)
There are two pathways for the biosynthesis of IPP/DMAPP, the mevalonate pathway and the MEP (2-C-methyl-D-erythritol 4-phosphate) pathway, which is also called the DOXP (1-deoxy-D-xylulose 5-phosphate) pathway (Fig. 12).

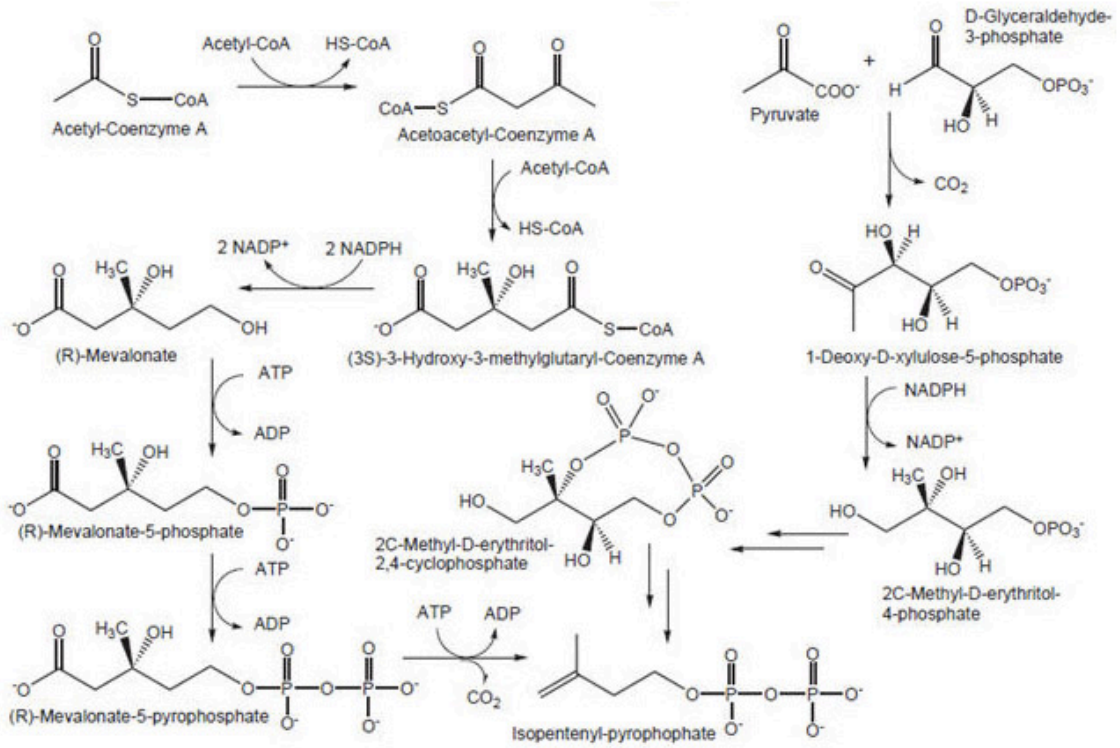


Figure 12: Two different pathways of the isoprenoid biosynthesis, the mevalonate and the DOXP pathway (http://www.sigmaaldrich.com/life-science/metabolomics/learning-center/isoprene-synthesis.html, retrieved Dec. 5, 2016)

In prokaryotes, the common pathway is the MEP pathway, while the mevalonate one is primarily used by plants.
(http://www.sigmaaldrich.com/life-science/metabolomics/learning-center/isoprene-synthesis.html, retrieved Dec. 5, 2016)
Many primary cyclic monoterpene, sesquiterpene and diterpene hydrocarbons and alcohols are synthesized from precursors such as geranyl diphosphate (GPP), farnesyl diphoshphate (FPP) and geranylgeranyl diphosphate (GGPP). (Yamada et al., 2015) (Fig. 13)

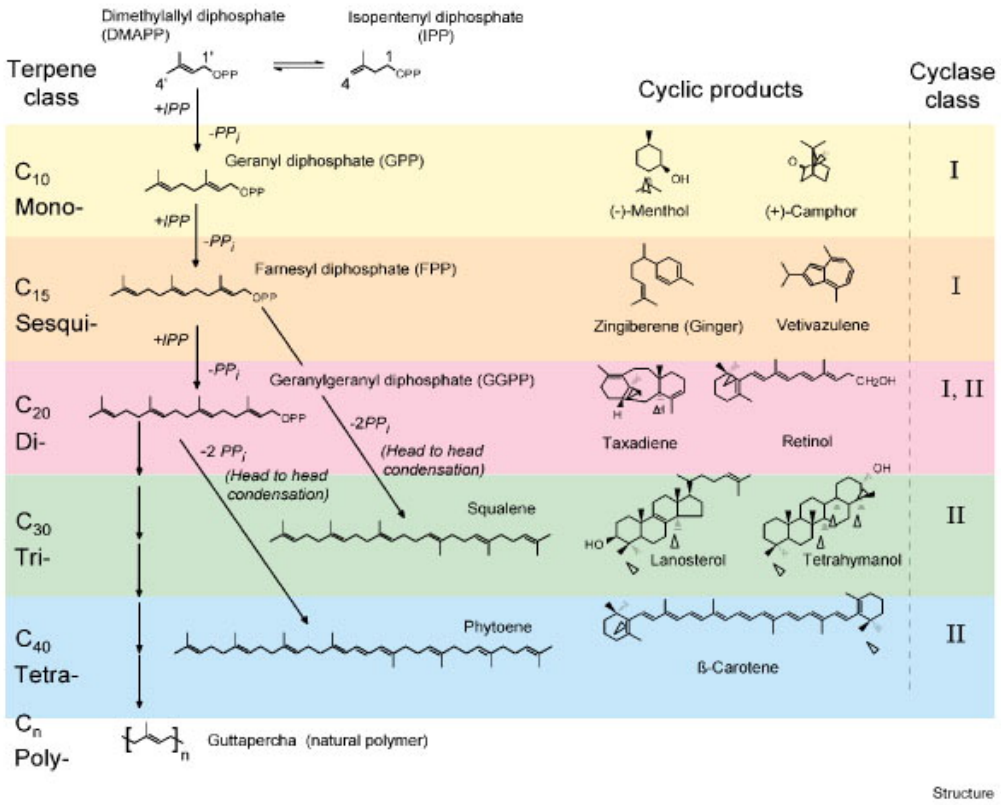


Figure 13: Overview of terpene classes and their precursors (Wendt, Schulz, 1998)

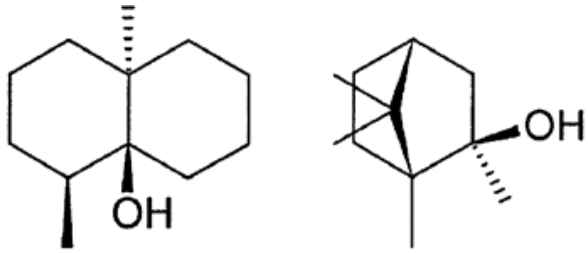
"Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer, and also to have antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, antiinflammatory, and immunomodulatory properties“. (Thoppil, Bishayee, 2011)

The spectrum includes well-established compounds such as Artemisinin, an antimalarial drug, the odorant menthol, the chemotherapeutic agent Taxol© and very many more. (Withers, Keasling, 2007)

In general, terpenes are known to be produced by plants and fungi.

In the last years it became clear that bacteria also produce terpenes, especially the genus *Streptomyces* or other actinomycetes. (Cane, Ikeda, 2012)

Geosmin, and 2-methylisoborneol (Fig. 14) are two terpenes with an earthy, musty smell often produced by bacteria. (Jüttner, Watson, 2007)



(-)-Geosmin (-)-2-Methylisoborneol

Figure 14: Examples for two terpenes that are often produced by bacteria: geosmin and 2-methylisoborneol (Jüttner, Watson, 2007)

Only a few microbial terpenes were found using classical methods, such as structure- or activity guided screenings.
Most of the new terpenes have been discovered by new techniques such as "genome mining". (Cane, Ikeda, 2012)

3.5.1 Streptomyces venezuelae

Streptomyces venezuelae is known as a producer of the antibiotic Chloramphenicol. (He et al., 2001) In comparison to other *Streptomyces* species*, S.venezuelae* has a rapid growth rate. It is easy to manipulate genetically and there is no mycelium clumping when it grows. These advantages make it a favourable candidate to work with.

Phelan et al. (2015) used an engineered *S.venezuelae* strain to establish the production of bisabolene, a terpene that can be used in the production of biofuel.
Bisabolene is normally not synthesized by bacteria, so the bisabolene synthase gene codon-optimized for *Streptomyces* was synthesized based on the amino acid sequence of the enzyme from the plant *Abeis grandis* (grand fir).
To increase the production, two genes encoding presumed competitors for the precursors, the geosmin synthase and the 2-methylisoborneol synthase, were deleted in this strain.
Interestingly, there was no significant increase of bisabolene production.

Next, the gene for the farnesyl pyrophosphate synthase (FPPS), that encodes an enzyme that synthesizes the key precursor in the terpene production, was overexpressed.

This genetic manipulation was successful, an increase of bisabolene production was proven. Compared to other engineered *S.venezuelae* variants, *S.venezuelae* JZ04 produced the highest amount of bisabolene (Fig. 15). (Phelan et al., 2015)

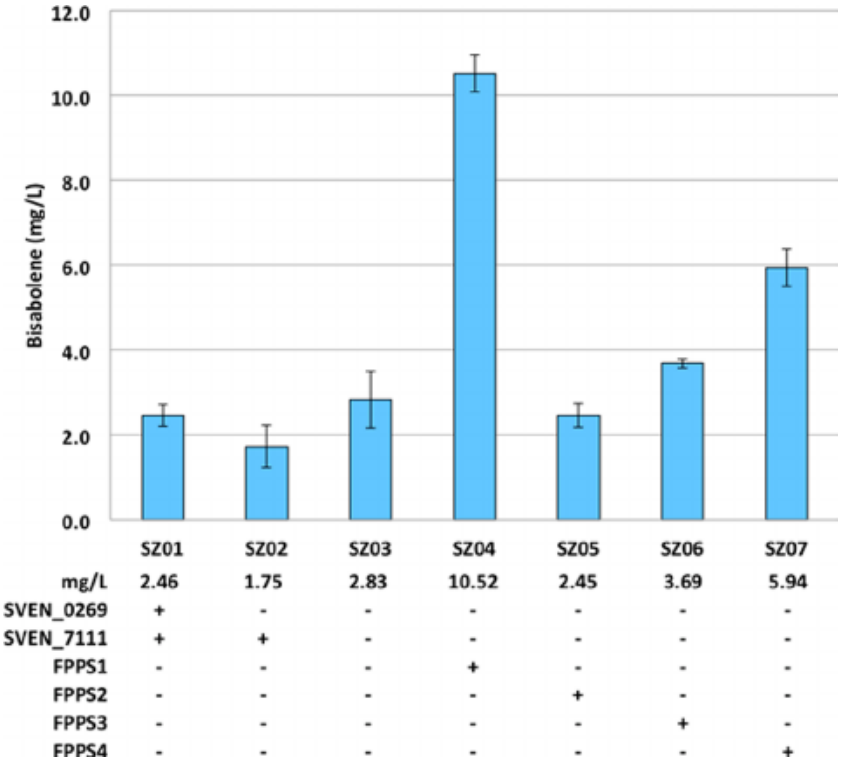


Figure 15: Streptomyces venezuelae variants and the amounts of bisabolene they produce. S.venezuelae JZ04 produces the highest amount of the biofuel precursor bisabolene (Phelan et al., 2015)

Using genetically engineered *Streptomyces venezuelae JZ04*, promised benefits in this project.

4 Results

4.1 Identification of gene clusters with antiSMASH

This project started with the detection of gene clusters encoding SMs using the software antiSMASH.
12 genome files (FASTA format) of marine actinomycetes were analyzed by antiSMASH in order to find genes that code for terpene synthases that do not have a known product so far.
As already explained in the introduction, marine organisms differ from terrestrial ones, and therefore might produce different metablic products. In the last years, several new metabolites that show for example antibacterial, anticancer, antiinflammatory, antifungal, antimalarial activities were isolated from marine actinomycetes. (Lam, 2006)

Actinomycetes in this project were isolated from *Antho dichotoma* (ADI), *Geodia barretti* (GBA) and *Phakelia ventilabrum* (PVA), marine sponges from Trondheim fjord in Norway, and were provided by Dr. Olga Sekurova and Prof. Sergey Zotchev.
It was thought that sponges themselves produce secondary metabolites, now we know that SMs are synthesized by the bacteria that are associated to the sponges.

Further information about antiSMASH are found in „Methods“.
An example of antiSMASH results is given in the Appendix.
The results showed that following gene clusters contain terpene synthases (Table 2):

Table 2: Bacterial genomes and the location of their terpene synthase genes

Bacterial strain (genome)	Location of TS genes
ADI 9118	cluster 16, 25
ADI 9224	cluster 6, 10, 17, 23
ADI 9302	cluster 2, 11, 27
ADI 9516	cluster 7,16, 29
ADI 9517	cluster 1, 8, 30
ADI 9602	cluster 2, 6, 13, 23

ADI 9615	cluster 20, 21
ADI 9707	cluster 17
ADI 9810	cluster 7, 26, 28
ADI 9812	cluster 20, 21
GBA 9401	cluster 11
PVA 9101	cluster 6, 31, 38

4.2 Phylogenetic tree

The next step in this work was to compare the protein sequences of the terpene synthase genes, that appeared as a result of antiSMASH, with several already identified terpene synthase genes' products in order to create a phylogenetic tree. (Yamada et al., 2015) The idea behind this phylogenetic analysis was to identify TSs that were as unrelated as possible to the already known terpene synthases. Such TSs would be of great interest for us since they promise new, unknown products.

Table 3 shows the function of the genes, that were already described by scientists, and their host microorganisms. (Yamada et al., 2015)

SAV: *Streptomyces avermitilis*
SCLAV: *Streptomyces clavuligerus*
SLT: *Streptomyces lactacystinaeus*
STSU_20912, ND90_0354: heterologous *Streptomyces* hosts

Table 3: Terpen synthases genes and their verified functions that were described before (Yamada et al., 2015)

Terpene synthase gene	Function
SAV_2998	pentalenene synthase
SAV_2163	geosmin synthase

SAV_3032	epi isozizaene synthase
SAV_76	avermitilol synthase
SCLAV_p0982	1,8-cineole
SGR_6065	epi-cubenol, a-muurolene, cadinadienes
SCLAV_p0068	(+)-T-muurolol synthase
SCLAV_p0765	hydropyrenol, hydropyrene, isoelisabethatriene B
SCLAV_p1169	clavulatriene A+B, prenyl-β-elemene, prenylgermacrene B
SCLAV_p1407	cucumin
SLT18_1880	cucumin
STSU_20912	tsukubadiene, odyverdienes A+B
ND90_0354	tsukubadiene, odyverdienes A+B

Using the software MEGA, a phylogenetic tree (Fig. 16) was build. (http://www.megasoftware.net/).

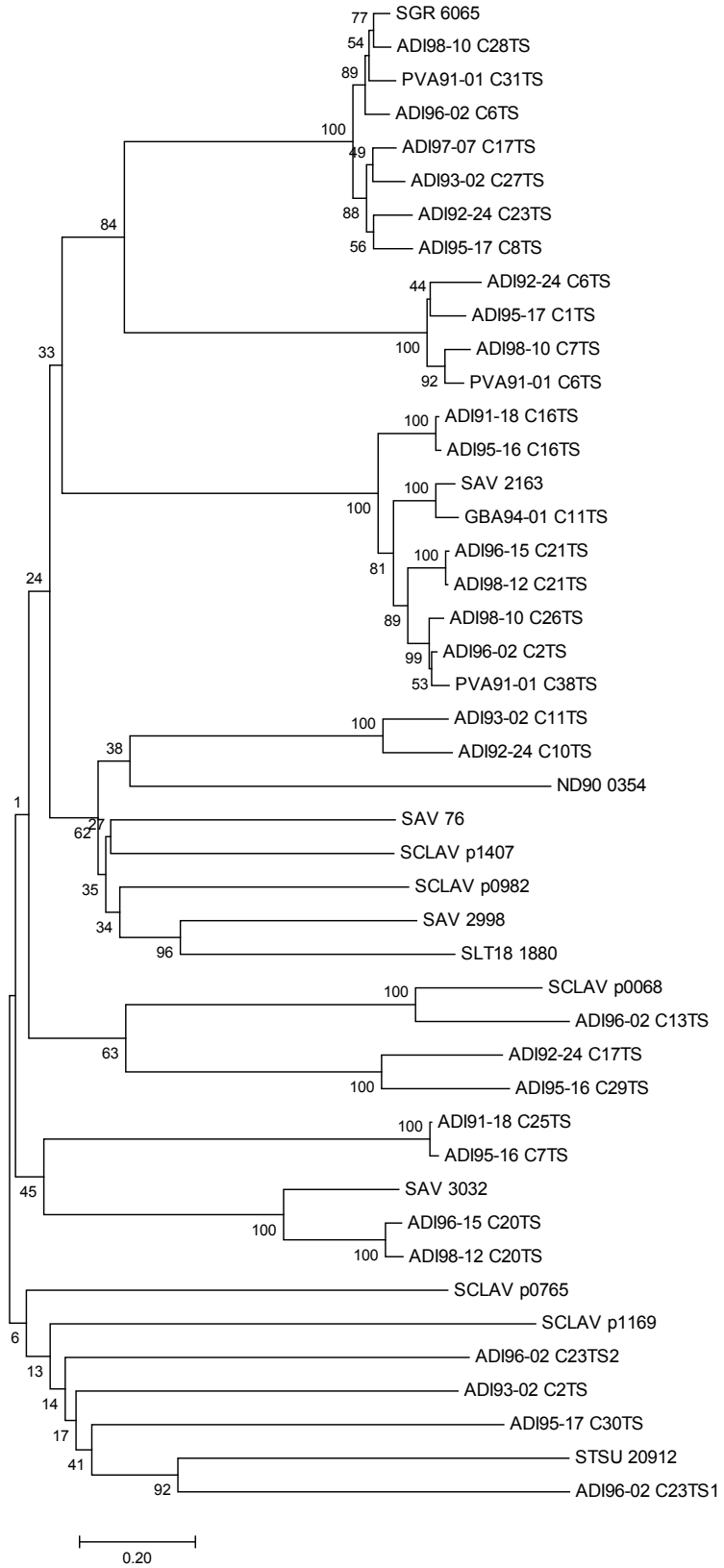


Figure 16: This phylogenetic tree was build with reference to known terpene synthases with described function in comparison to terpene synthases that were results of the antiSMASH analysis

Due to the phylogenetic analysis seven terpene synthase genes were expected to be interesting due to their position in the phylogenetic tree. They were not too close related to terpene synthases that have already well-known products. Using the BLAST software (Basic Local Alignment Search Tool) the function of those terpene synthases was looked up (Fig. 17).

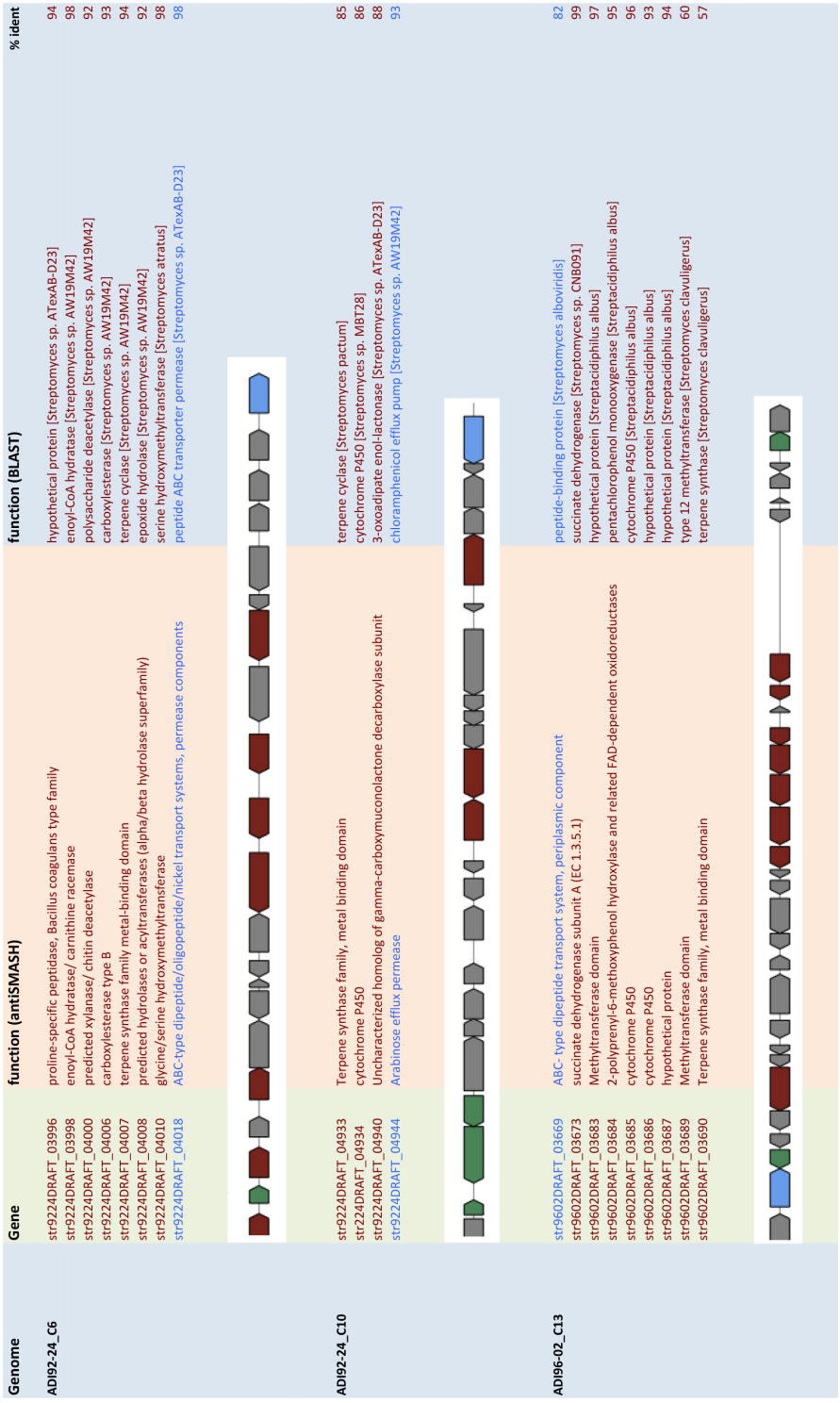


Figure 17/1: Organization of genetic loci of several terpene synthase genes chosen due to the phylogenetic analysis

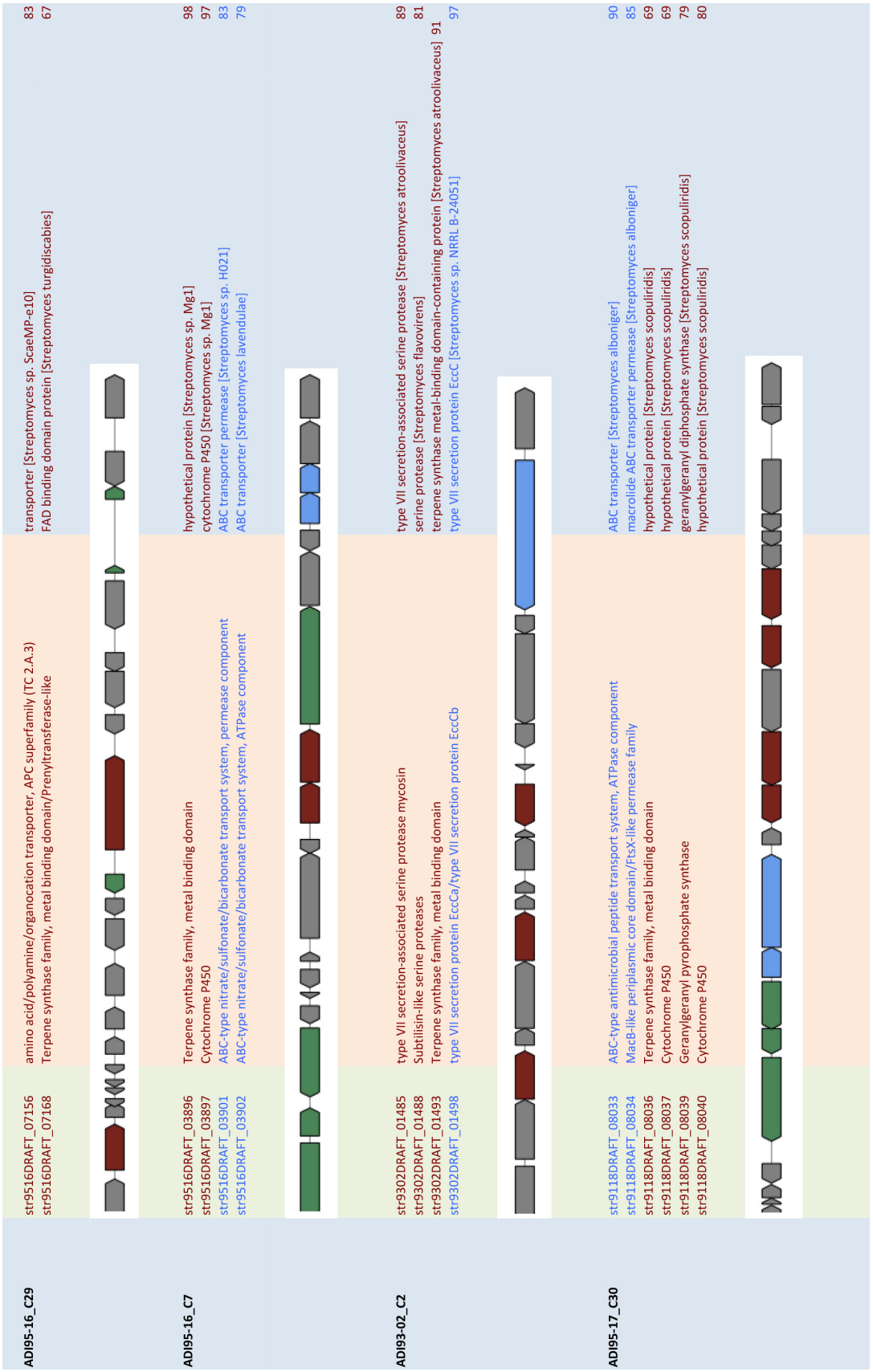


Figure 17/2: Organization of genetic loci of several terpene synthase genes chosen due to the phylogenetic analysis

Table 4 shows five bacterial strains that we chose to work with and their corresponding gene clusters that contain terpene synthases.

Table 4: Bacterial genomes we work with in this project and their corresponding terpene synthase gene clusters

Bacterial strain	Location TS gene of interest
ADI 9517	cluster 30
ADI 9302	cluster 2
ADI 9224	cluster 6
ADI 9602	cluster 13
ADI 9516	cluster 29

These genes were planned to be cloned into a heterologous *Streptomyces venezuelae* host JZ04, that was already successfully used for the production of the biofuel precursor, a terpene bisabolene. (Phelan et al., 2015)
A special vector was used to conduct this plan.
The vector pUC57::B4-OrolR-Ets-pks3 served as a source for the promoter B4 (Siegl et al., 2013) and the operator (OrolR). Only the B4-OrolR part was taken from this plasmid.
The vector was provided by Jaime Felipe Guerrero Garzón, MSc.
First, the promoter and operator were subloned into pGEM11 (Ampicillin resistance) because a special cutting site (NotI) was needed for subcloning into pKC1218H (Hygromycin resistance).
pKC1218H is a shuttle vector, that can replicate in both *E.coli* and *Streptomyces* and is used for intergeneric conjugation from *E.coli* to *Streptomyces*.
The finally constructed vector, containing a B4 promoter and an OroIR operator in pCK1218H, was called pAMX1.

4.3 Construction of vector pAMX1

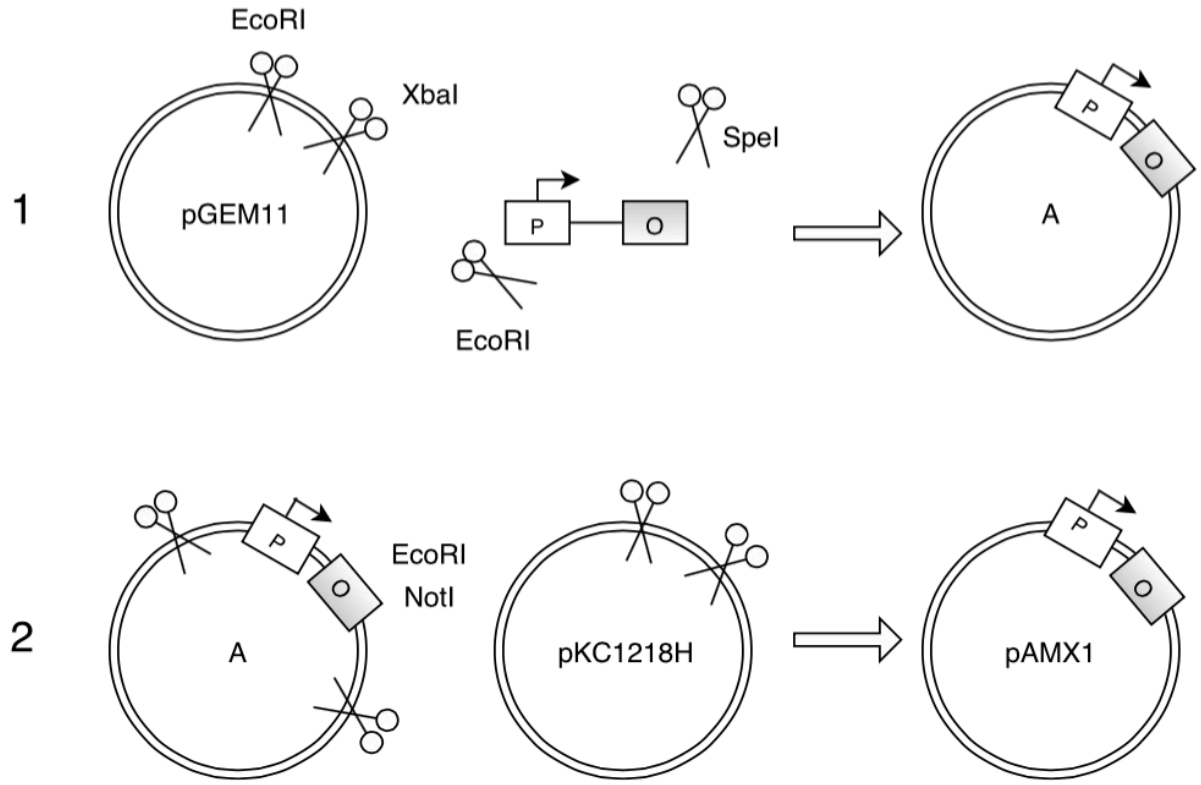


Figure 18: Schematic presentation of the vector pAMX1 construction (P...promoter; O...operator; description in the text)

The vector, containing promoter and operator in pGEM11 is called „A“ in the following (Fig. 18, point 1).

The plans for cloning were made by the use of the Clone Manager 9 software.
pUC57::B4-OroIR-Ets-pks3 was cut by the enzymes EcoRI and SpeI in order to introduce the promoter B4 and the operator OroIR into pGEM11.
pGEM11 was cut by EcoRI and XbaI (XbaI is compatible with SpeI).

The work continued with ligation and transformation into *E.coli* DH5 α competent cells and plating the transformation mix onto selective L-Agar media containing Ampicillin (100 µg/ml).

An overnight culture on the shaking incubator at 37 °C was made using LB medium with Ampicillin (100 µg/ml) in sterile tubes. 8 colonies were picked up with the toothpick and added to the tubes.

From this overnight culture the plasmid DNA was isolated (see "Materials"). The clone, containing the recombinant plasmid, was confirmed by running a restriction analysis: „A“ was cut with NdeI and NotI.

As a control, vector pGEM11 was cut with the same enzymes.

All the plasmids checked contained the insert, which is obvious when compared to the "empty" vector pGEM11 cut with the same enzymes (Fig. 19).

Size fragments „A“: 814 bps, 2466 bps

Size fragments pGEM11: 757 bps, 2466 bps

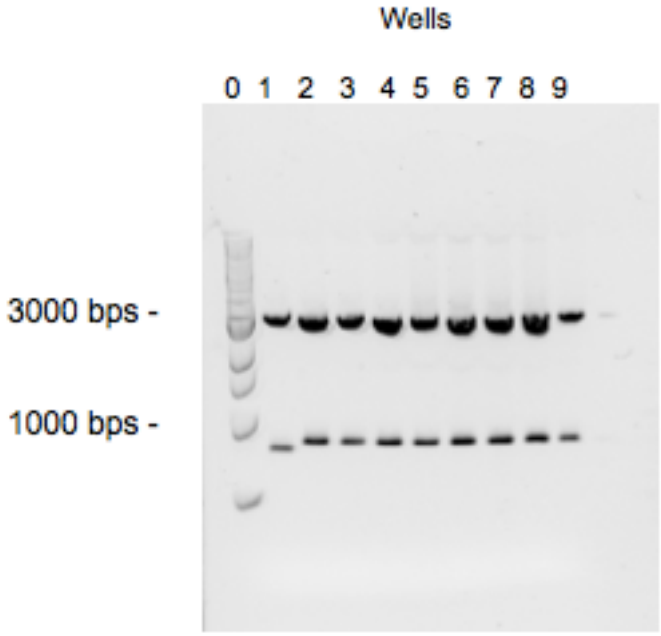


Figure 19: Construction of the vector "A" was successful. The expected fragments (vectors cut with NdeI and NotI) are 757 bps and 2466 bps in case of pGEM11, 814 bps and 2466 bps in case of vector "A". The difference is clearly visible in all samples.

well 0: 1kb DNA ladder, well 1:control (pGEM11), well 2-9: "A"

Vector „A“ and pKC1218H were then cut with two enzymes, EcoRI and NotI, to get the sequence containing promoter and operator.

The following ligation allowed the cloning of the promoter B4 and operator OroIR into pKC1218H (Hygromycin resistance) (Fig. 18, point 2).

The ligation mix was transformed into *E.coli* DH5 α competent cells.
For an overnight culture 12 single colonies were picked. They were each added in a sterile tube containing LB medium and Hygromycin (100 µg/ml).
From an overnight culture the plasmid DNA of 12 samples was isolated.

To ensure that we have the right clone, this time the pDNA was cut with EcoRI and SphI. As a comparison, pKC1218H was cut with the same enzymes.
Figure 20 shows that the construction of the final vector, pAMX1, worked. Size fragments control (pKC1218H): 1157 bps, 4688 bps
Size fragments pAMX1: 1235 bps, 4688 bps

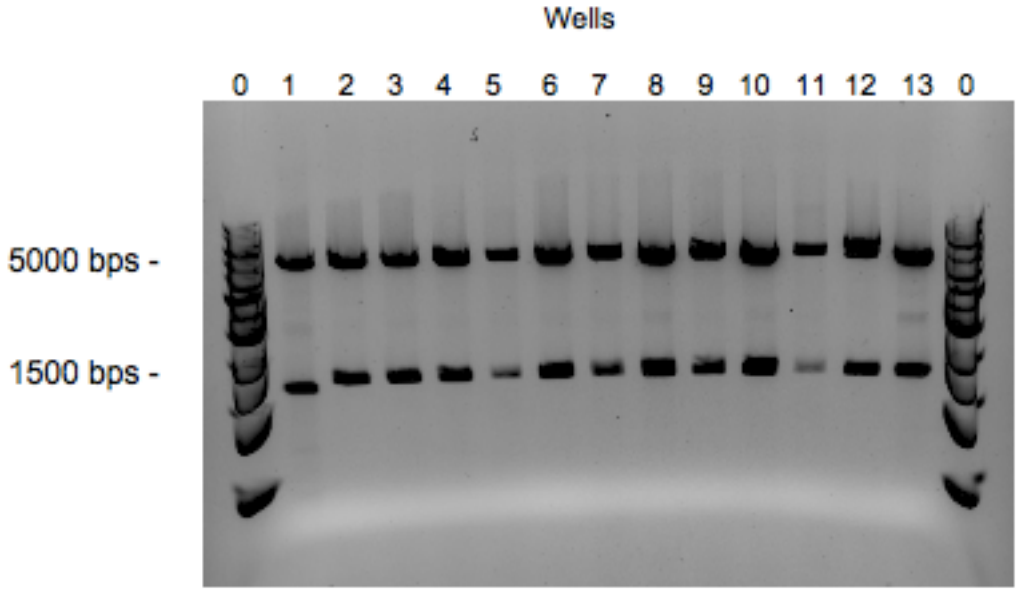


Figure 20: The picture shows the success in constructing the final vector for this work, pAMX1. The difference is clearly visible: in case of pKC1218H (control) the fragments are 1157 and 4688 bps, in case of pAMX1 1235 and 4688 bps (vectors cut with EcoRI and SphI)
well 0: 1kb DNA ladder, well 1: control (pKC1218H), well 2-13: pAMX1

pAMX1 was later used for the cloning of the terpene synthase genes.
The ligation mixes would be transformed first into E.coli DH5 α to isolate plasmids and find the ones, containing desired inserts, and afterwards into *E.coli* ET12567 competent cells (see later). *E.coli* ET12567 cells have the function to transfer vectors from *E.coli* to *Streptomyces*.

Detailled information about the transformation, the plasmid DNA isolation, the restriction digest and the ligation see „Methods".
Vector maps can be found in the Appendix.

4.4 Primer design

Primers for amplification of TS genes were designed by Prof. Zotchev using Clone Manager 9.

4.5 Amplification of genes for terpene synthases

The bacterial strains ADI 9302, ADI 9517, ADI 9224, ADI 9602 and ADI 9516
were grown on ISP4 medium. In some cases we also tried out IPS2 medium, but the bacterial strains growth was better on ISP4.
The genomic DNA was isolated from an overnight culture in order to run a PCR. Therefore, primers have been designed using the program Clone Manager 9.
The detailled process is described in „Methods".
Used kit and media recipes see „Materials".

A PCR was set up to get the terpene synthase genes that would be introduced into a heterologous *Streptomyces venezuelae* JZ04 host later (using vector pAMX1).
A gel electrophoresis showed the success of the PCR.
Figure 21 shows the PCR products before purification. Expected sizes:
ADI 9224 C6: 1225 bps, ADI 9302 C2: 1168 bps, ADI 9516 C29: 2740 bps,
ADI 9602 C13: 2048 bps

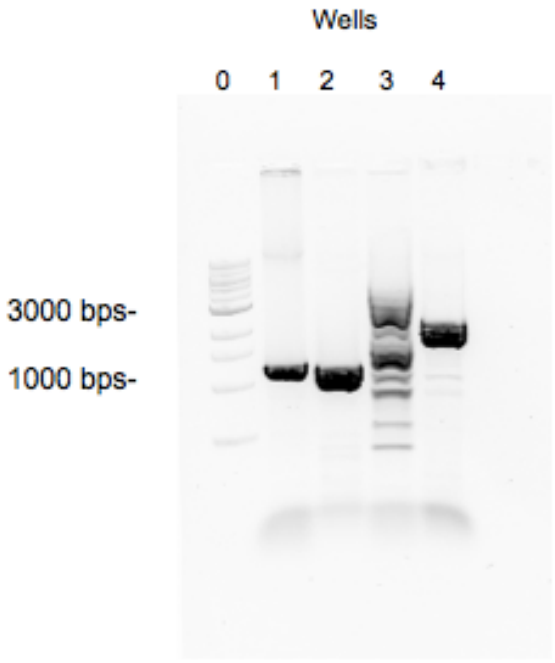


Figure 21: PCR products before purification; well 0: 1kb DNA ladder, well 1: 9224, well 2: 9302, well 3: 9516, well 4: 9602

The PCR products were cut out of the gel and purified with help of Zymoclean™ Gel DNA Recovery Kit. In the case of sample 3 only the upper part was cut out. Figure 22 shows the PCR products after their purification.

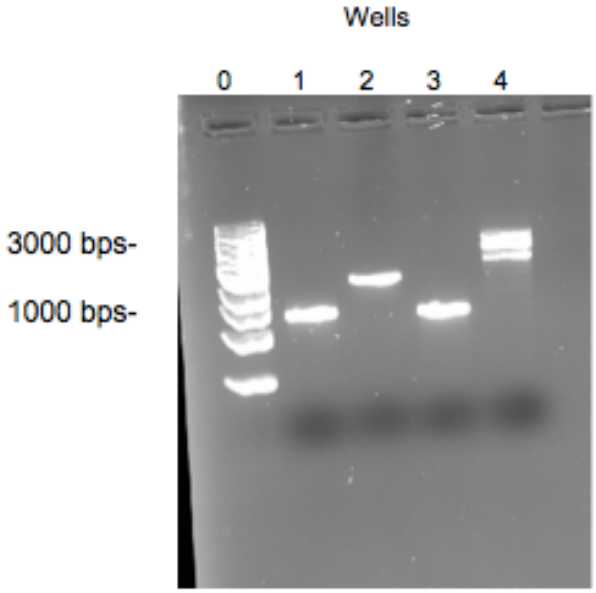


Figure 22: PCR products after purification
well 0: 1 kb DNA ladder, well 1: ADI 9224, well 2: ADI 9602, well 3: ADI 9302, well 4: ADI 9516

The settings of the PCR, used kits and gel electrophoresis are specified in „Materials" and "Methods“.

4.6 Cloning of terpene synthase genes into pAMX1

Two terpene synthase genes, 9516 C29 and 9602 C13, were directly cloned into pAMX1 (Table 5).

Table 5: Restriction digests that were performed to clone the terpene synthases 9516 C29 and 9602 C13 into pAMX1

Sample	Restriction enzymes
9516 C29 pAMX1 (control)	NsiI, HindIII PstI, HindIII
9602 C13 pAMX1 (control)	NotI. HindIII

Two ligation reactions were performed and then the ligation mixes were transformed into *E.coli* DH5 α competent cells.

The terpene synthase genes 9224 C6 and 9302 C2 were first cloned into pGEM3 to gain the restriction sites in order to be further subcloned into the conjugative vector (Table 6).

Table 6: Restriction digests to clone the terpen synthases 9224 C6 ad 9302 C2 into pGEM3

Sample	Restriction enzymes
9224 C6 pGEM3 (control)	SphI, HindIII
9302 C2 pGEM3 (control)	SphI, HindIII

The terpene synthase genes were ligated into pGEM3 and then transformed into *E.coli* DH5 α cells.

The transformation worked in all 4 cases. Overnight cultures were made picking colonies with a toothpick and using LB medium and Hygromycin (100 µg/ml) in case of 9516 C29 and 9602 C13 in pAMX1 or Ampicillin (100 µg/ml) in case of 9224 C6 and 9302 C2 in pGEM3.
The plasmid DNA was isolated from the overnight cultures.
Another restriction digest followed to check if all ligations worked and if we got the clones containing the desired inserts (Table 7).

Table 7: Restriction digest to check the cloning

Sample	Restriction enzymes
9224 C6 pGEM3 (control)	XbaI, HindIII
9302 C2 pGEM3 (control)	XbaI, HindIII
9516 C29 pAMX1 (control)	PstI, HindIII
9602 C13 pAMX1 (control)	NotI, HindIII

In two cases, the cloning was not successful.
The following steps in this project were made with the two clones p9302 C2 and p9602 C13, containing genes for terpene synthases.
Fragment 9302 C2 was cut out from the pGEM3 clone, purified, cut with appropriate restriction enzymes and ligated with pAMX1.
Figure 23 and 24 show that by the end two terpen synthases were successfully cloned into pAMX1.
Size of the expected fragments (cut with NotI, HindIII):
9602 in pAMX1: 2033 bps, 5889 bps
Size control (pAMX1): 34 bps, 5889 bps

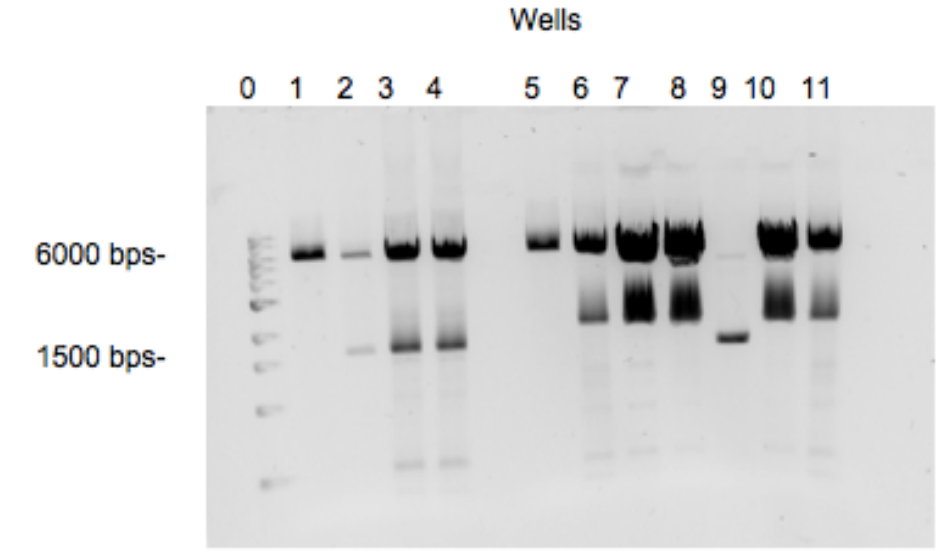


Figure 23: This gel picture shows that the terpene synthase 9602 was successfully cloned into pAMX1. Expected fragments (cut with NotI and HindIII): 2033 and 5889 bps in case of 9602 in pAMX1, 34 bps and 5889 bps in case of the control (empty pAMX1) well 0: 1kb DNA ladder, well 1: control (pAMX1), well 2-4: 9516 in pAMX1, well 5: control (pAMX1), well 6-11: 9602 in pAMX1

Size expected fragments (cut with XbaI and HindIII): 9302 in pAMX1: 1172 bps, 5902 bps. Size fragments control (pAMX1): 21 bps, 5902 bps

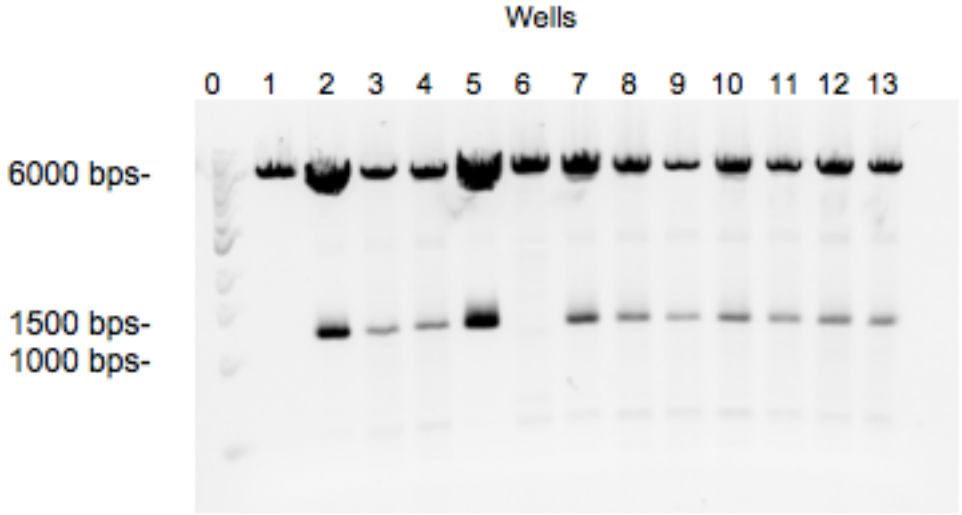


Figure 24: This gel picture shows the successful cloning of the terpene synthase 9302 into pAMX1. Expected fragments (cut with XbaI and HindIII): 1172 bps and 5902 bps in case of 9302 in pAMX1, 21 bps (which is too small to be visible on the gel) and 5902 bps in case of the control (pAMX1), well 0: 1 kb DNA ladder, well 1: control (pAMX1), well 2- 13: 9302 in pAMX1

The two constructs p9602 and p9302 in pAMX1 were transformed into *E.coli* ET12567 competent cells for conjugative transfer into *S.venezuelae.*

4.7 Conjugation into *Streptomyces venezuelae*

As it was already explained in the introduction, the recombinant *Streptomyces venezuelae* JZ04 strain was engineered to establish the production of the terpene bisabolene. In this strain, two genes encoding presumed competitors for the precursors of terpene biosynthesis, the geosmin synthase and the methylisoborneol synthase were deleted (Table 8). A high terpene precursor supply was achieved by overexpressing the FPPS. (Phelan et al., 2015) The strain was provided by Dr. Olga Sekurova and Prof. Sergey Zotchev.

Table 8: Genotype of Streptomyces venezuelae JZ04 (Phelan et al., 2015)

Strain	Genotype	Description
SZ04	ΔSVEN_0269; ΔSVEN_7111; pSV-coAgBis;:SVEN_0055::attP	T1-4D containing pSV-coAgBis and FPPS1 under control of ermEp* promoter inserted at the aatP chromosomal site

Two vectors, containing genes for terpene synthases, p9602 C13 and p9302 C2 were conjugated into *Streptomyces venezuelae JZ04.*
For the conjugation, *E.coli* ET12567 cells were grown on LA plates containing three antibiotics, Hygromycin (100 µg/ml), Kanamycin (20 µg/ml) and Chloramphenicol (25 µg/ml).
Hygromycin selects only cells containing the pKC1218H-related plasmid (pAMX1), Kanamycin and Chloramphenicol ensure the selection for helper plasmid pUZ2008 in *E.coli* ET12567 competent cells.

Streptomyces spores have been mixed with 2 X YT medium. The spores were heatshocked at 50 °C for germination, cooled down and mixed with *E.coli* ET12567 cells. The conjugation mix was briefly centrifuged at 4,000 rpm in order to

concentrate, the pellet was suspended in $^1/_2$ of the total volume of 2 X YT medium and spread on a plate with SFM medium (without antibiotics). An incubation time (room temperature) followed for about 16-18 hours.

A solution of dH_20, Nalidixic acid (30 µg/ml) and Hygromycin (100 µg/ml) was spread on each plate with a spatula. Nalidixic acid suppresses the growth of *E.coli* and Hygromycin selects the pAMX1 based plasmids which has a Hygromycin resistance gene.

The plates were incubated at 30 °C for 2-3 days.

Transconjugants appeared after a few days (Fig. 25, Fig. 26).

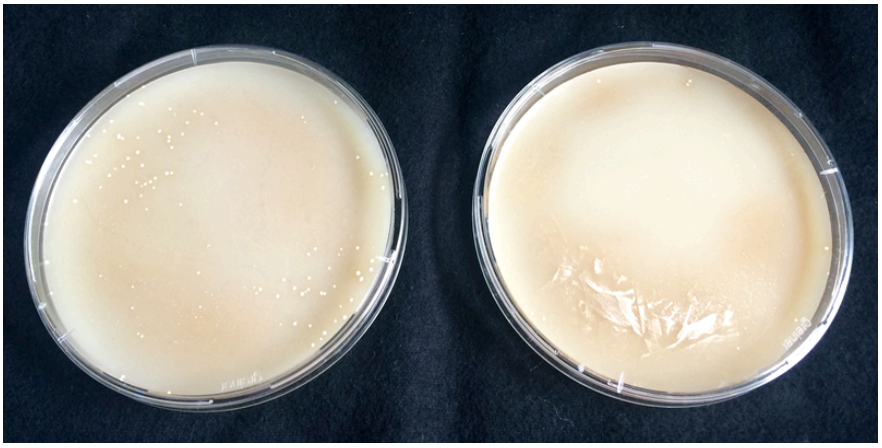


Figure 25: Transconjugants. To the left: control (pAMX1), to the right: clone 9602 C13 in pAMX1

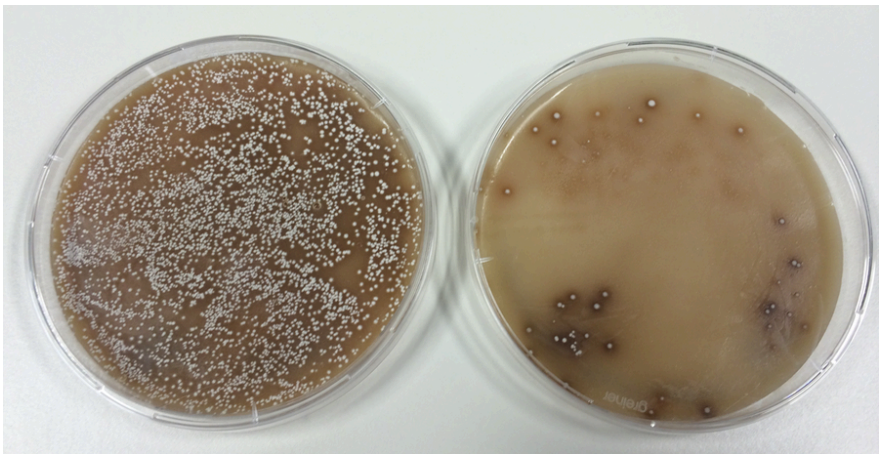


Figure 26: Transconjugants. To the left: control (pAMX1), to the right: clone 9302 C2 in pAMX1

Next, transconjugants were picked using a toothpick and transferred to a new plate containing SFM medium and the three antibiotics Hygromycin (100 µg/ml), Nalidixic acid (30 µg/ml) and Apramycin (50 µg/ml). Hygromycin ensures the selection of the pAMX1 plasmid, Nalidixic acid suppresses the growth of *E.coli*. Apramycin was used for the selection of *Streptomyces venezuelae* JZ04.
The plated bacteria were incubated at 30 °C until sporulated.
Figure 27 and 28 show colonies of transconjugants which are sporulated.

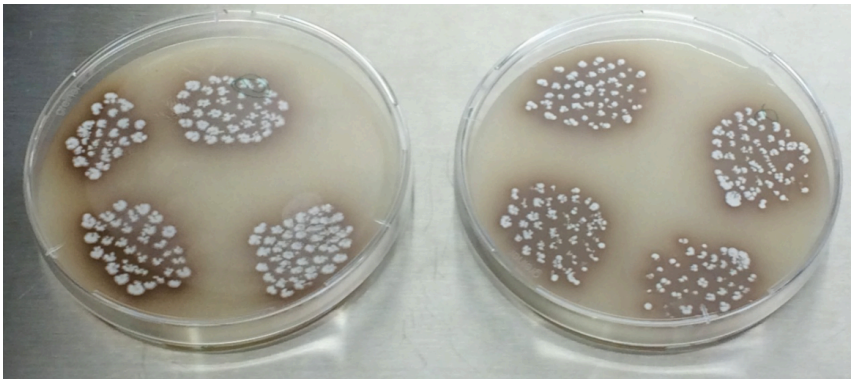


Figure 27: Transconjugants on the selective plates. To the left: control (pAMX1), to the right: clone 9602 in pAMX1

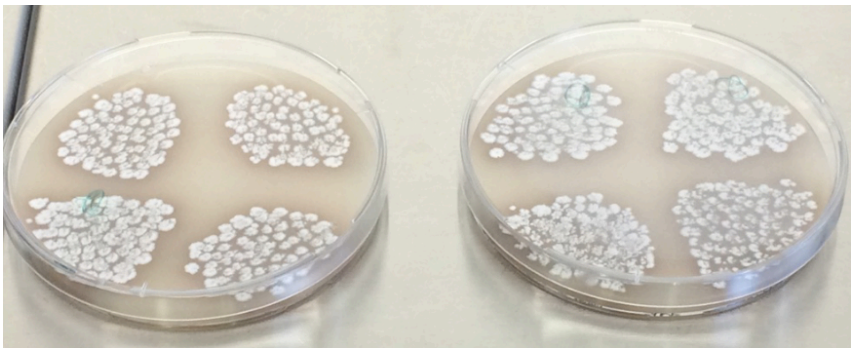


Figure 28: Transconjugants on the selective plates. To the left: control (pAMX1), to the right: clone 9302 in pAMX1

The next step was to transfer a single colony of transconjugants on a new plate with ISP4 medium and two antibiotics: Apramycin (50 µg/ml) and Hygromycin (100 µg/ml). This enabled the selection of only *Streptomyces venezuelae* JZ04 clones that contained conjugative vectors, and making the spore suspension of a recombinant strain for further storing at -80 °C.

The plates were incubated at 30 °C. When spores appeared (Fig. 29), a 20 % glycerol stock of spores was made and stored in a freezer until needed to start a fermentation culture.

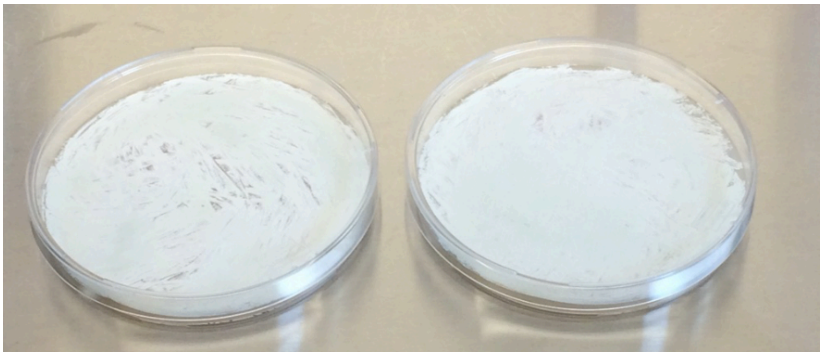


Figure 29: Sporulated cultures. Spores are visible as a greyish layer on top of S.venezuelae strains aerial mycelium. To the left: control (pAMX1), to the right: 9302 in pAMX1

4.8 Fermentation, Extraction and GC-MS analysis

4.8.1 Fermentation

A pre-culture for fermentation was made by inoculating the spore suspension of the recombinant strain (*Streptomyces venezuelae* containing the terpene synthase 9602 or 9302 in the expression pAMX1 vector) into TSB medium containing Hygromycin (100 µg/ ml). As a control, the *S.venezuelae* strain, containing only the empty vector pAMX1, was used for such a pre-culture. The pre-culture was grown in the shaking incubator at 30 °C over night.

The overnight cultures were used to start the fermentations in two different media, either PM4-1 with Hygromycin (100 µg/ml) or 5282 with Hygromycin (100 µg/ml).

Fermentation cultures were also made for the control.

The cultures (Fig. 30) were grown in the shaking incubator at 30 °C for 72 hours.

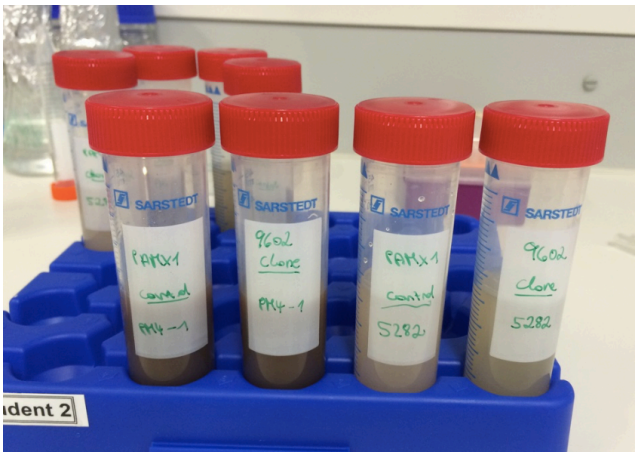


Figure 30: Fermentation cultures

The detailled process is described in „Methods“.

4.8.2 Extraction

After 72 hours, the fermentation cultures were divided in smaller flasks to centrifuge them for 5 minutes at 4,000 rpm. This ensured a seperation of the supernatant and the pellet.

The extraction from supernatant was performed with n-hexane by shaking the mixture at 30 °C for 30 minutes.

The extraction from pellet was first performed with methanol by also shaking the mixture at 30 °C for half an hour.

After following centrifugation at 4,000 rpm for 5 minutes another n-hexane extraction from the supernatant (shaking incubator 30 °C for 30 minutes) was made.

After the second centrifugations the upper phases from each sample were stored at -20 °C for the following GC-MS analysis.

4.8.3 GC-MS analysis

For the GC analyses, the n-hexane extracts were filled into the machine with a syringe. The blank sample (n-hexane) was mesured first because this was the solvent for the extracts. Figure 31 shows the chromatogram of n-hexane.

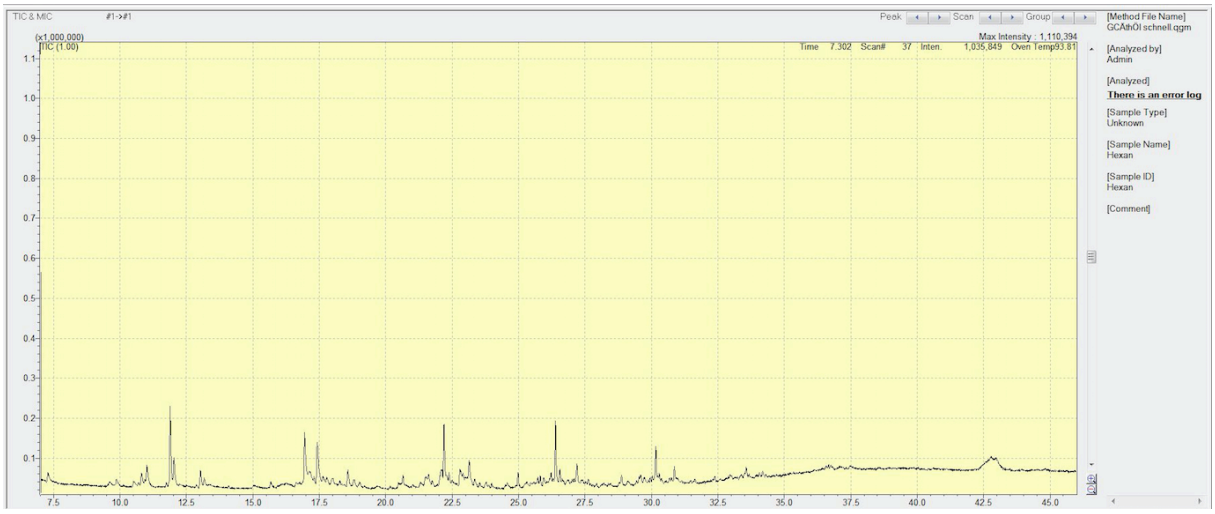


Figure 31: GC spectrum of n-hexane

Next, the spectra for the extracts of the supernatants and the pellets were measured for both: the control strain, containing the empty vector (pAMX1), and for strains containing terpene synthase genes overexpression vectors (9602 or 9302) (Fig. 32 and 33).

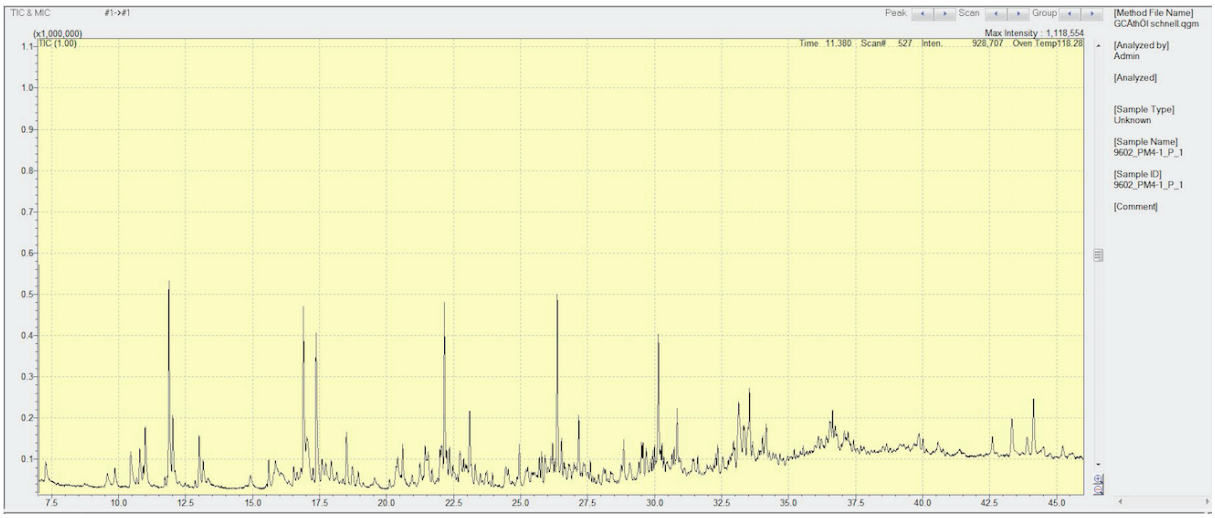


Figure 32: GC spectrum of 9602 in pAMX1 (PM4-1 medium, pellet)

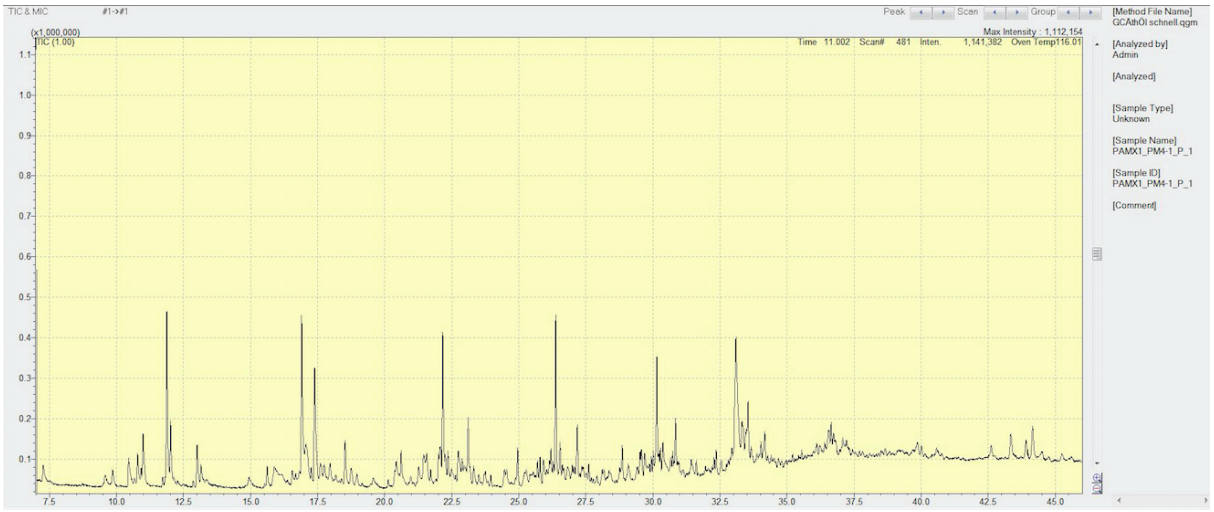


Figure 33: GC spectrum of pAMX1 (PM4-1 medium, pellet)

The spectra all looked very similar, so we decided to perform a HPLC analysis too.
Settings for the GC-MS analyses see "Methods".
More GC chromatograms are found in the Appendix.

4.8.4 HPLC

A preparation of the n-hexane extracts was made to perform a HPLC.
Known amounts of the extracts (500 µl) were each filled in Eppendorf tubes and were dried in the hood.
25 % of the volume (125 µl) dichlormethan was added to the dried samples.
Then methanol was added till the original amount of 500 µl (375 µl).
The mix was centrifuged for 4 minutes. Afterwards 200 µl of the supernatant were analyzed by HPLC. HPLC settings are found in "Methods".
Figure 34, 35 and 36 show some of the chromatograms that were overlayed in order to see differences first between the control (pAMX1) and the clone (9302 or 9602 in pAMX1) and second, between pellets and supernatants.
Also the spectrum of the blank sample (methanol) was overlayed with the other spectra.

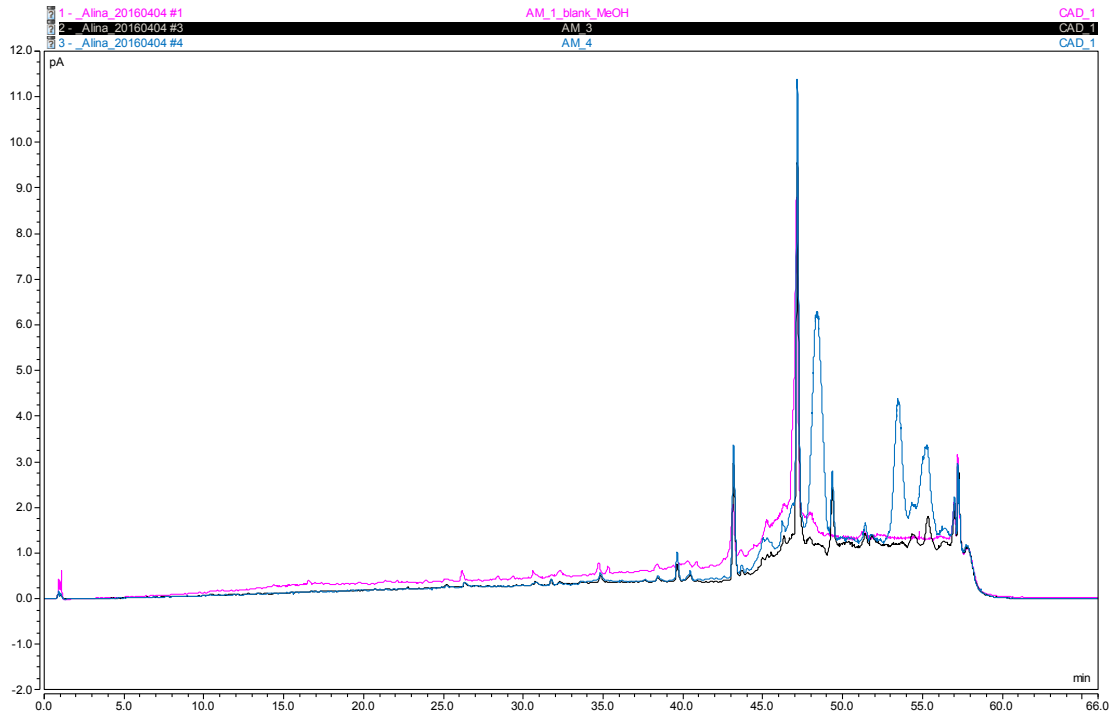


Figure 34: This spectrum shows:

pink line: blank sample (methanol)

black line: clone 9602 in pAMX1 (PM4-1 medium, pellet)

blue line: pAMX1 (PM4-1 medium, pellet)

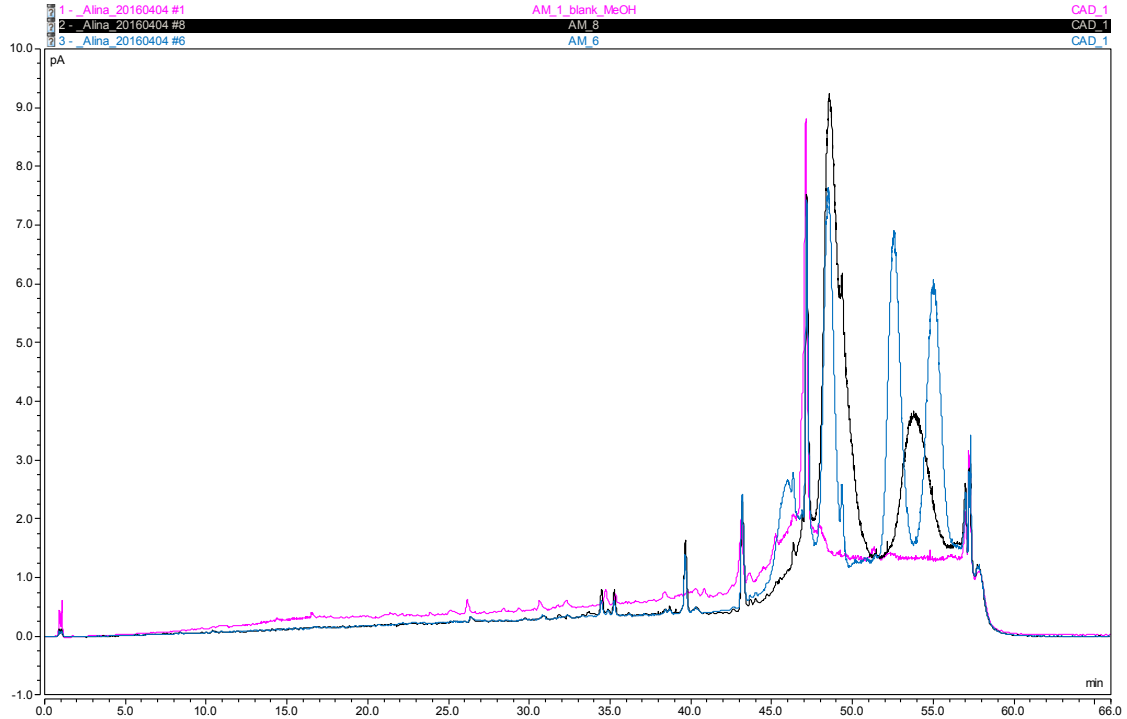


Figure 35: This spectrum shows:

pink line: blank sample (methanol)

black line: pAMX1 (5282 medium, supernatant)

blue line: clone 9602 in pAMX1 (5282 medium, supernatant)

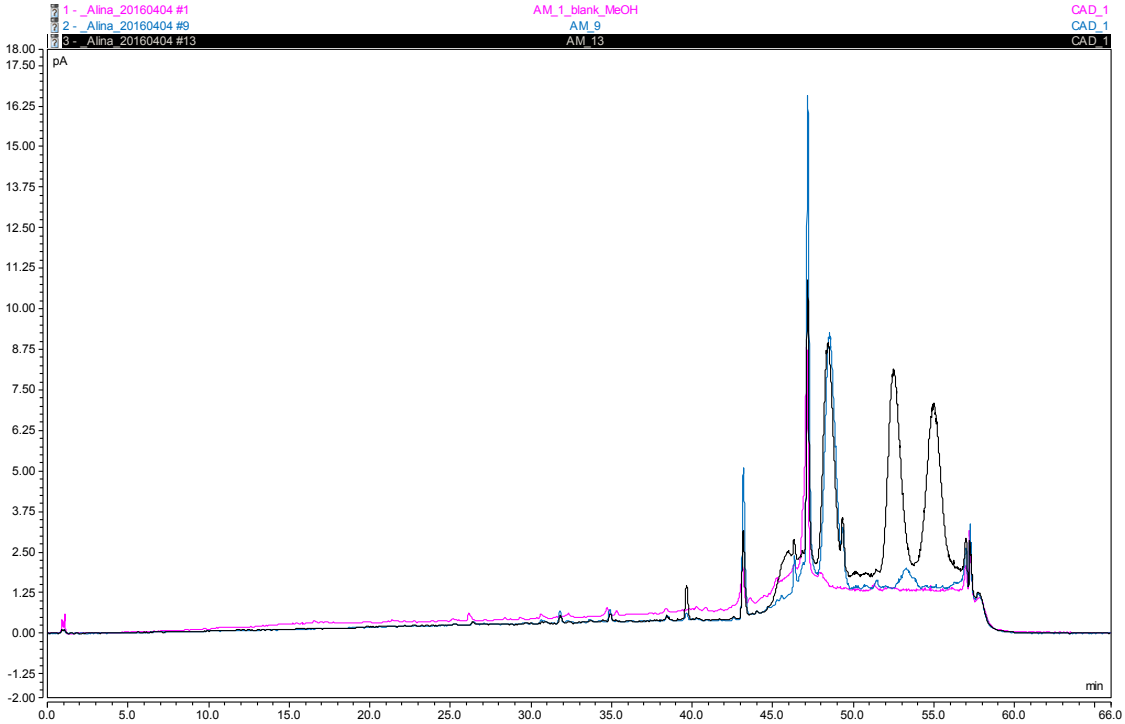


Figure 36: This spectrum shows:

pink line: blank sample (methanol)

black line: clone 9302 in pAMX1 (5282 medium, pellet)

blue line: pAMX1 (5282 medium, pellet)

More HPLC chromatograms are found in the Appendix.

5 Discussion/Outlook

The development in DNA sequencing technologies made it possible to sequence entire genomes in a fast and low-cost way.
Genome sequencing showed that microorganisms have potential to produce more secondary metabolites than originally expected, because only some of them are produced during fermentation. Many novel compounds might be discovered by exploring those cryptic biosynthetic pathways. (Ochi et al., 2014)
One of the strategies to exploit the full biosynthetic potential is the heterologous expression of a desired gene in a suitable host. (Gomez-Escribano, Bibb 2014)
Scientists detected 13 new terpene structures via heterologous expression using a *Streptomyces* host. Two compounds had not been reported before. (Yamada et al., 2015)
Terpenes are used in medicine and have different biological activities. Well-known examples would be Artemisinin, an anti-malarial drug, or Taxol®, which is used in the treatment of cancer. (Withers, Keasling, 2007)

In this project, the idea was to express terpene synthase genes in a heterologous *Streptomyces* host hoping to find new terpenes.
Today, bioinformatic tools are widely used for analysing genomes. antiSMASH for example, aligns regions of a gene cluster with related ones from a database. (Medema et al., 2011)

Our work started with the analysis of genomes from marine actinomycetes using the above-mentioned online program antiSMASH.
With the construction of a phylogenetic tree using the software MEGA we found out, that some of the genomes contain terpene synthase genes which might be unique.
These terpene synthase genes were expressed in a heterologous *Streptomyces* host. For this purpose, a special expression vector, pAMX1 was constructed. pAMX1, a modified pKC1218H plasmid, contains a strong constitutive promoter and has properties to be transferred from *E.coli* to *Streptomyces* by intergeneric conjugation.
The terpene synthase genes were amplified by PCR and cloned into pAMX1 under control of the strong promotor B4 (ermE*p-based).

The conjugations of the plamids from *E.coli* to *Streptomyces* were successful. Fermentation cultures of control and overexpressing strains were made.

After seperating pellet and supernatant, both components were extracted with n-hexane, in case of the supernatant first with methanol and afterwards with n-hexane.

Finally, we analysed the extracts by GC-MS.

All GC chromatograms looked similar, this problem might occured because of the plastic vessels in which the extracts were stored. Maybe the solvents dispended chemicals from the plastic material. It is probably better to use glass vessels in the future.

Next, we decided to analyse the extracts by HPLC.

The spectra showed differences between the control and the overexpression strains, which indicated a production of compounds. Due to lack of time, further analyses could not be pursued so far. We believe that there is much potential for finding new compounds if more experiments would be conducted.

6 Materials

6.1 Media

L-Agar (LA)

Agar	10 g
Difco™ bacto tryptone	10 g
NaCl	5 g
distilled water	up to 1000 ml

L-Broth (LB)

Difco™ bacto tryptone	10 g
Difco yeast extract	5 g
NaCl	5 g
distilled water	up to 1000 ml

Difco ™ ISP Medium 2 (ISP2)

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

pH 7,2

Difco ™ ISP Medium 4 (ISP4)

Soluble starch	10 g
Dipotassium Phosphate	1 g
Magnesium Sulfate	1 g
Sodium Chloride	1 g
Ammonium Sulfate	2 g
Calcium Carbonate	2 g
Ferrous Sulfate	1 mg
Manganous Chloride	1 mg

Zinc Sulfate	1 mg
Agar	20 g
dH_2O	1000 ml

pH 7,2

TSB

CASON Bouillione	30 g
distilled water	1000 ml

PM4-1

Glucose	15 g
Soy meal	15 g
Corn steep solids (Sigma)	5 g
$CaCO_3$	2 g
TMS-1 solution	6 ml
distilled water	up to 1000 ml

Preparation:
The glucose solution was prepared and sterilized at first.
5 g of corn steep solids were dissolved in 890 ml of dH_2O.
The solution was split in 3 flasks, each containing 297 ml.
5 g of soy meal and 0,7 g of $CaCO_3$ were poured in each flask and mixed.
After sterilization at 121 °C for 20 minutes 33 ml of sterile glucose solution and 2 ml of TMS-1 solution were added to each flask.

5282

Glycerol	30 g
$NaNO_3$	2 g
NaCl	5 g
$CaCO_3$	1 g
$CoCl_2$ x 7 H_2O	1 mg
dH_2O	ad 1000 ml

pH 7,8

SFM

Mannitol	20 g
Soy flour	20 g
Agar	20 g
TMS-1 solution	1 ml
distilled water	1000 ml

2 X YT

Difco™ bacto tryptone	3,2 g
Difco™ bacto yeast extract	2 g
NaCl	1 g
distilled water	200 ml

6.2 Stock solutions and chemicals

Antibiotics:

Apramycin (AM):	100 mg/ml	in sterile water
Kanamycin (KAN):	40 mg/ml	in sterile water
Chloramphenicol (CML):	25 mg/ml	in 96 % EtOH
Hygromycin (HYG):	100 mg/ml	in sterile water
Nalidixic acid (NAL):	30 mg/ml	in 0,1 N NaOH
Ampicillin (AMP):	100 mg/ml	in sterile water

Sterile filtration
Storage on -20 °C

TMS-1 solution

$FeSO_4$ x 7 H_2O	5000 mg
$CuSO_4$ x 5 H_2O	390 mg
$ZnSO_4$ x 7 H_2O	440 mg
$MnSO_4$ x H_2O	150 mg

Na_2MoO_4 x 2 H_2O	10 mg
$CoCl_2$ x 6 H_2O	20 mg
HCl 1M	50 ml
dH_2O	up to 1000 ml

TSS buffer

PEG 8000	5 g
$MgCl_2$ x 6 H_2O	0,3 g
DMSO	2,5 ml
LB medium	up to 50 ml

Trace elements solution

$ZnSO_4$	40 mg
$FeCl_3$ x 6 H_2O	200 mg
CuCl x 2 H_2O	10 mg
MnCl x 4 H_2O	10 mg
$Na_2B_4O_7$ x 10 H_2O	10 mg
$(NH_4)_6Mo_7O_{24}$ x 4 H_2O	10 mg
dH_2O	1000 ml

TBE buffer

TRIS base 89 mM	10,8 g
Boric acid 89 mM	5,5 g
EDTA-Na_2 2 mM	0,7 g
distilled water	up to 1000 ml
pH 8	

Lysis buffer

TRIS HCl 200 mM pH 8	1 ml
EDTA 100 mM pH 8	1 ml
Triton X-100 1,2 %	120 µl
Lysozyme	200 mg
distilled water	up to 10 ml

0,8 % Agarose gel

Agarose	3,2 g
TBE buffer	up to 400 ml
used stain: GelRed™	20µl

1,5 % Agarose gel

Agarose	6 g
TBE buffer	up to 400 ml
used stain: GelRed™	20µl

Glycerol 20 %

Glycerol	200 ml
dH_2O	800 ml

6.3 Kits

Isolation of total DNA

QUIAGEN Dneasy Tissue Kit (Quiagen)

Isolation of pDNA

Wizard® Plus SV Minipreps DNA Purification System (Promega)

PCR kit

GC-rich PCR Kit (Sigma Aldrich)

Taq PCR Kit (New England Biolabs)

Q5- High- Fidelity 5x Master Mix (New England Biolabs)

DNA purification

DNA Clean & Concentrator ™-5-Kit (Zymo Research)

Gel recovery

Zymoclean ™ Gel DNA Recovery Kit (Zymo Research)

6.4 Bacterial strains and Plasmids

Used bacterial strains and plasmids are listed in Table 9.

Table 9: Plasmids and bacterial strains used in this project

Vector/ Bacterial strain	Antibiotic resistance	used for	Source/Reference
pUC57::B4-OrolR-Ets-pks3	Ampicillin	only promoter and operator part used	B4: Siegl et al., 2013; provided by Jaime Felipe Guerrero Garzón, MSc.
pGEM11	Ampicillin	introduction of promoter and operator; getting a NotI cutting site	Promega (USA)
pGEM3	Ampicillin	subcloning	Promega (USA)
pKC1218H	Hygromycin	construction of pAMX1	Dr. K. Engelhardt
pAMX1	Hygromycin	cloning TS genes	this work
E.coli DH5 α	-	transformation	Dr. D. Hanahan
E.coli ET12567	Kanamycin, Chloramphenicol	conjugation from *E.coli* to *Streptomyces*; contains helper plasmid pUZ8002	Flett, Mersinias et al., 1997

6.5 Primers

9302 C2

forward primer: 5’-GATCGCATGCCTTCCGGGTCTCAGAGGCATT-3’

reverse primer: 5’-GATCAAGCTTCCGGACGAAACGATGACGAAC-3’

9224 C6

forward primer: 5’-GATCGCATGCGCGATTCGATTCACGCATCC-3’

reverse primer: 5’-GTACAAGCTTGTCGTTGCCGGTTCAGCCATC-3’

9516 C29

forward primer: 5’-GATCATGCATTGGCCGTATGCGGCCAGAGC-3’

reverse primer: 5’-GTACAAGCTTCGGACACCACGCGCACGGAC-3’

9517 C30

forward primer: 5’-GTACTCTAGACGGAACCGCGACGACGGTTC-3’

reverse primer: 5’-GTACAAGCTTGCCTAGCACCTGGCCGGCAG-3’

9602 C13

forward primer: 5’-GATCGCGGCCGCCTTCGGATTCCTCAATCAGAG-3’

reverse primer: 5’-GTACAAGCTTCTGATCGTGGCGATCGACAC-3’

7 Methods

7.1 *In silico* methods

antiSMASH

AntiSMASH (antibiotics& secondary metabolite analysis shell) is a bioinformatic tool that allows the identification of biosynthetic gene clusters. (Medema et. al., 2011)
The program (https://antismash.secondarymetabolites.org/) was used for the identification of the gene clusters from marine actinomycetes genomes. When the whole genome sequences were loaded up, this showed that 31 gene clusters contain terpene synthase genes.

MEGA

The software MEGA (Molecular Evolutionary Genetics Analysis) includes facilities to make sequence alignments and create phylogenetic trees. (Tamura et al., 2013)
12 genome sequences from marine actinomycetes that code for terpene synthases with unknown products were compared with some genes encoding terpene synthases with products already known from the literature.
The phylogenetic tree is found in "Results".

Clone Manager

Clone Manager 9 is a software that simulates cloning and the use of restriction enzymes. It was used in this project to plan the cloning.

7.2 Storage of bacterial strains

Bacterial strains were stored at -80 °C using 20 % glycerol.

7.3 Preparation of media

Ingredients, except for agar (if needed), were solved in distilled water.
The mixture was split in flasks.
Dependent on the recipe, each flask contained the calculated amount of agar.
With the pH-meter the pH was adjusted in some cases.
All media were autoclaved at 121 °C for 20 minutes.
Media were stored at 4 °C.
Recipes are found in „Materials“.

7.4 Preparation of spore suspensions

Bacterial (*Streptomyces*) strains were grown until sporulated.
4,5 ml of glycerol were applied on the plates to collect the spores with a pipet.
The liquid was applied in a filter (syringe with cotton).
Spore suspensions were stored in cryo tubes at -80 °C.
They were used for the reproduction of the bacterial strains and later for the conjugation.

7.5 Preparation of overnight cultures of *E.coli* cells

A single colony of *E.coli* cells was picked with a toothpick and added to a specific amount of LB medium (for example 2 ml or 10 ml) and appropriate antibiotics.
The mixture was put on the shaking incubator (over night) at 37 °C.

7.6 Preparation of cell suspensions

Single colonies of *E.coli* strains were grown over night in the shaking incubator at 37 °C in 13 ml incubation tubes containing 2 ml of LB medium and appropriate antibiotics (if needed).
A little amount of overnight culture was put into 2 ml fresh LB media with appropriate antibiotics (if needed) and split in 2 Eppendorf tubes.
The tubes were centrifuged 4 minutes at 14,000 rpm.
The pellets were resuspended with 750 µl of 20 % glycerol and stored at -80 °C.

7.7 Preparation of antibiotic stock solutions

The antibiotics were weighed and solved in the required liquid (distilled water, 96 % ethanol, DMSO or 0,1 N NaOH).
The solutions were filtered using sterile filters.
Antibiotic stock solutions were stored at -20 °C in aliquots.
Antibiotics concentrations see „Materials“.

7.8 Preparation of competent cells

Prearrangements:

E.coli DH5 α cells were grown on plates with LA media.
E.coli ET12567 cells were grown on plates with LA, Kanamycin (20 µg/ml) and Chloramphenicol (25 µg/ml).
A single colony was picked with a toothpick and transferred to 10 ml of LB medium to make an overnight culture using the 37 °C shaking incubator.

For the preparation of competent cells, 0,5 ml of overnight culture was mixed with 50 ml of LB medium.
The flasks were put on the shaking incubator at 37 °C.
Every 30 minutes the OD 600_{nm} was measured.
An OD of approximately 0,4 was required to make sure to be in the exponential phase of bacterial growth.

From this point on we worked on ice.
The liquid was split in smaller amounts and incubated on ice for 10 minutes.
The work continued with centrifugation for 10 minutes at 3,000 rpm at 4 °C.
Each pellet was resuspended in 2,5 ml TSS buffer.
Competent cells were stored in Eppendorf tubes on -80 °C in 100 µl aliquots.

7.9 Isolation of plasmid DNA

The plasmid DNA was isolated by the use of the Wizard® Plus SV Minipreps DNA Purification System kit.
Working steps:
The bacteria containing the plasmid of interest were incubated in the shaking incubator over night at 37 °C.
On the next day, 1 ml of overnight culture per Eppendorf tube was centrifuged for 5 minutes.
Each pellet was resuspended with 250 µl of cell resuspension solution.
250 µl of cell lysis solution was added. The mix was inverted 4 times.
10 µl of alkaline protease solution was added and again the mix was inverted 4 times. Centrifugation at top speed followed for 10 minutes at room temperature.

A spin column was put into the collection tube. The cleared lysate was decanted into the spin column. Another centrifugation followed for 1 minute at top speed (room temperature). The flowthrough was discarded and the column was reinserted into the collection tube.

750 µl of wash solution containing ethanol was added. The tubes were centrifuged at top speed for 1 minute. The flowthrough was discarded and the tube again reinserted into the collection tube.
This working step was repeated with 250 µl of wash solution. The tubes were centrifuged at top speed (room temperature) for 2 minutes.

The spin column was transferred to a sterile Eppendorf tube. 100 µl of nuclease free water were added to the spin column. The tubes were centrifuged at top speed for 1 minute at room temperature.
The column was discarded and the DNA was stored at -20 °C.

7.10 Isolation of genomic DNA

The isolation of total DNA from *Streptomyces* in order to make a PCR was done by using the Dneasy Tissue kit (Quiagen)

Working steps:
Spores were added to 2 ml of TSB medium and incubated at 30 °C on a shaking incubator over night.
1 ml of the overnight culture was transferred into an Eppendorf tube. 0,5 ml sterile water was added. The mix was centrifuged at 13,000 rpm for 3 minutes.
The supernatant was discarded and the pellet was resuspended in 180 µl lysis buffer (see "Materials"). Lysozyme was added just before the experiment.
The mix was incubated at 37 °C for 15 minutes. Every 5 minutes the suspension was gently mixed by tipping the end of the tube.
25 µl of proteinase K was added, also 200 µl of AL buffer. The mix was pipetted up and down several times.
The mix was incubated at 55 °C for 30 minutes.
200 µl of 96 % ethanol was added. The mixture was applied on the column sitting on a collection tube. Centrifugation followed for 2 minutes at 13,000 rpm.
The column was transferred to a new tube. 500 µl of AW1 buffer was pipetted on the column and then the tube was centrifuged for 1 minute at 13,000 rpm.
The column was again transferred to a new tube. 500 µl of AW2 buffer was applied on the column. The tube was centrifuged for 3 minutes at 13,000 rpm.
The column was transferred to a new Eppendorf tube and 125 µl of preaheated AE (to 50 °C) buffer was filled in. The tube was incubated for 15 minutes at room temperature.
Centrifugation followed for 1 minute at 13,000 rpm. The column was discarded.
The DNA was afterwards checked by gel electrophoresis.
Storage at -20 °C.

7.11 Primer design

Primers were designed by Prof. Sergey Zotchev using the software
Clone Manager 9. The primers were ordered from Eurofins Genomics.

7.12 PCR

A PCR was performed for the terpene synthase genes that should later be cloned into *Streptomyces venezuelae*.
The isolated genomic DNA of the marine actinomycetes was used.

Prearrangements for the PCR:
The tubes containing dry pellets of the primers were spun down.
According to the concentration of the DNA, primers were diluted with a special amount of distilled water.
The concentration of the stock solution was 100 pmol.
5 µl of stock solution was mixed with 45 ml of distilled water to prepare a working solution (concentration 10 pmol). Storage at -20 °C.

PCR solution:

1 µl DNA
1 µl forward primer (working solution)
1 µl reverse primer (working solution)
8 µl buffer Q5
8 µl enhancer Q5
0,7 µl polymerase Q5
1 µl dNTPs mix (10 mM)
up to 40 µl water

The adjustment for the PCR is listed in Table 10.

Table 10: PCR setting used in this project

Process	Duration	Temperature	Number of cycles
Denaturation	1 minute	95 °C	1x
Denaturation	45 seconds	94 °C	

Annealing	45 seconds	60 °C	25x
Elongation	2 minutes 30 seconds	68 °C	
Final elongation	7 minutes	68 °C	1x
Storage		4 °C or -20 °C	

The PCR was set up for 4 samples: 9302, 9602, 9516, 9224.
The PCR products were checked by gel electrophoresis.
Gel pictures see „Results“.

7.13 Restriction digest

To make sure that we work with the correct plasmids, restriction enzymes were used to cut the DNA.
Appropriate endonucleases were chosen by the use of Clone Manager depending on the restriction sites of the plasmids.

For a restriction digest, a specific amount of distilled water (up to 20 µl), buffer CutSmart (for example 2 µl) , restriction enzymes (for example 0,8 µl each) and plasmid DNA (for example 4 µl) was mixed and incubated in a waterbath at 37 °C for a specified time (for example for 2 hours).
The plasmid fragments were checked via gel electrophoresis.

7.14 Gel electrophoresis

In this project gel electrophoresis was used to check the DNA after a restriction digest or a PCR.
According to the size of DNA fragments, either an 0,8 % agarose gel or an 1,5 % agarose gel was used. (see „Materials“)

First step was to pour the heated agarose gel into the electrophoresis chamber.
Depending on how many samples were checked, an 8-well or a 15-well comb was used.
The gel was cooled down for 20 minutes.
Afterwards TBE buffer was poured in the gel equipment.
The comb was taken out and samples were filled into the holes.
Used DNA-staining dye was GelRed®.
The gel ran for 40-60 minutes at 100 V.

At the end the gel was taken out to watch it under UV light in the GelDoc imager.
Some gel pictures are found in „Results“.

7.15 Gel DNA recovery

When for example PCR products had to bee cut out of the gel, it was placed inside the GelDoc imager. To see the DNA on the gel, UV light was used. The pieces of DNA were cut out using a scalpel.
Afterwards the DNA from the gel was purified in order to work with it furthermore.
For this procedure, Zymoclean™ Gel DNA Recovery Kit was used.

Protocol:
The excised gel slice was weighed and transferred to an Eppendorf tube.
3 volumes of ADB to each volume of agarose were added (for example, for 100 mg of agarose gel, 300 µl of ADB were added).
The tube with the gel piece was incubated at 50 °C until it melted.
The melted agarose solution was transferred to a Zymo-Spin™ column in a collection tube.
Centrifugation followed for 30-60 seconds. The flowthrough was discarded.
200 µl of DNA wash buffer was added to the column and centrifuged for 30 seconds.
This wash step was repeated.
Approximately 6 µl of DNA Elution buffer was applied directly on the column matrix.
The column was placed into an Eppendorf tube and centrifuged for 30-60 seconds for the elution of the DNA.
DNA was stored at -20 °C.

7.16 Ligation

Ligation was used for example for introducing a terpene synthase gene into the vector pAMX1.
Specific amounts of cut DNA were mixed with 2 µl of T4 DNA ligase buffer, 1 µl of T4 DNA ligase and distilled water up to 20 µl.
The mixture was put on an ice bath over night.

7.17 Transformation

In this project, either *E.coli* DH5 α or *E.coli* ET12567 competent cells were used.
For a transformation, competent cells were kept on ice.
1 µl of plasmid DNA was put to 100 µl of competent *E.coli* cells in an Eppendorf tube.
The mixture was put on ice for 15 to 30 minutes.
Afterwards, the cells were heat shocked for 40 to 60 seconds at 42 °C using a heating block.
After a short regeneration time on ice, 500 µl of LB medium was added to the tube and mixed.
The mix was put on the shaking incubator at 37 °C for 40 to 60 minutes.
100 µl of the transformation mix was plated out on a Petri dish containing LA medium.
Depending on the resistance gene of the plasmid, an appropriate antibiotic was also added. The Petri dishes were incubated at 37 °C over night. The next day it was checked if there was a bacterial growth that showed if the transformation worked.

7.18 Conjugation

In this project, *E.coli* ET12567 cells were used for conjugation.
After transformation of the correct plasmid into *E.coli* ET12567, the conjugative transfer to *Streptomyces venezuelae* JZ04 could start.
In this work two terpene synthase genes in vector pAMX1 (clone 9602 and 9302) were introduced into *Streptomyces* by conjugation.

Overnight cultures of *E.coli* ET12567 cells containing the plasmid of interest were prepared one day before the conjugation started. Therefore, ET12567 cells were spread out on selection plates with LA medium containing three antibiotics: Hygromycin (100 µg/ml), Kanamycin (20 µg/ml) and Chloramphenicol (25 µg/ml). These antibiotics selected only ET12567 cells that contained the right plasmid (pAMX1 has a Hygromycin resistance). The plates were incubated over night at 37 °C.
The next day, the clone that grew the best was scratched with a loop and transferred to 500 µl of 2 X YT medium.
50 µl of *Streptomyces* spores were put to 350 µl of 2 X YT medium. The mix was heatshocked at 50 °C for 5-10 minutes.

400 µl of heatshocked spores and 100 µl of ET12567 cells were mixed in an Eppendorf tube and centrifuged for 1 minute at 4,000 rpm.
250 µl of the supernatant was removed and the pellet was resuspended in 250 µl of the rest of the liquid.
The mix was spread out on a plate with SFM medium (without antibiotics).
The plates were incubated at room temperature for 16-18 hours with a foil because *Streptomyces venezuelae* JZ04 is a fast growing strain.

The next day, an antibiotic solution was prepared containing dH_2O, Nalidixic acid (30 µg/ml) and Hygromycin (100 µg/ml) and spread on each SFM plate with a spatula.
The plates were then incubated at 30 °C for 2-3 days.
Nalidixic acid suppresses the growth of *E.coli* and Hygromycin selects pAMX1, so after the incubation time, only *Streptomyces* cells containing the correct plasmid would grow on the plates.

Transconjugants could be seen after a few days (pictures see "Results").
Single colonies of the transconjugants were picked with a toothpick and transferred on a plate with SFM medium containing following antibiotics: Hygromycin (100 µg/ml), Nalidixic acid (30 µg/ml) and Apramycin (50 µg/ml). Apramycin ensured the selection of *Streptomyces venezuelae* JZ04.
The plates were incubated at 30 °C until spores appeared.

The best growing colony was taken with a loop and transferred to a new plate with ISP4 medium containing Apramycin (50 µg/ml) and Hygromycin (100 µg/ml).
After incubation at 30 °C for a few days spores were ready to prepare a glycerol stock that would be used for the extracts later.

7.19 Extraction

Fermentation culture:
When the transconjugants sporulated, spores were stored in glycerol.
An overnight culture was made using 500 µl of spore suspension and 15 ml of TSB medium with Hygromycin (100 µg/ml).
This mixture was put on the shaking incubator at 28 °C overnight.
Next day, 2,5 ml of overnight culture was put to 50 ml of either PM4-1 medium or 5282 medium each containing Hygromycin (100 µg/ml).
For 72 hours the cultures were placed onto the shaking incubator at 28 °C.

Extraction:
After 72 hours the cultures were centrifuged for 5 minutes at 4,000 rpm.
Each 50 ml culture was split in two bottles of each 25 ml.
The supernatant and the pellet were separated.

25 ml of supernatant was mixed with 2,5 ml n-hexane.
For 30 minutes the bottles stood on the shaking incubator at 30 °C which was the actual part of the extraction.
After centrifugation the upper phase was stored on -20 °C and taken for the GC-MS analysis.

The pellet was mixed with 10 ml methanol.
After 30 minutes on the shaking incubator at 30 °C (actual part of the extraction) the mix was centrifuged.
The supernatant was reextracted with 5 ml n-hexane on the shaking incubator at 30 °C for 30 minutes.
After another centrifugation the upper phase was taken for GC-MS
(storage at -20 °C).

7.20 GC-MS

The GC-MS was performed using following adjustments:
Equipment: Shimadzu GCMS-2010
Software: Shimadzu GCMS solution 4.11
Column: Zebron ZB-5, 60 m x 0,25 mm I.D., film thickness: 0,25 µm
Mobile phase: Helium 5.0, Flow 1,9 ml/ min, split proportion 1:5
Oven program: 50 °C- 270 °C with 6 °C/min
Injector: 270 °C
MS: Ion source 250°C, 70 eV
Scan: 40-500 amu/0,5 sec
Solvent cut time: 7 min

The order of the analysis is listed in Table 11.

Table 11: GC-MS analysis: order of the samples

Sample/Control	Medium	Supernatant (SN)/Pellet (P)
pAMX1	PM4-1	SN
9602	PM4-1	SN
pAMX1	PM4-1	P
9602	PM4-1	P
pAMX1	5282	SN
9602	5282	SN
pAMX1	5282	P
9602	5282	P

n-hexane only	-	-
9302	PM4-1	SN
9302	PM4-1	P
9302	5282	SN
9302	5282	P

7.21 HPLC

The n-hexane extracts were prepared for the HPLC.
500 µl of each extract was filled in an Epppendorf tube. The solvents were boiled away under the hood.
125 µl of dichlormethane was added to the tubes.
Also, 375 µl of methanol was added.
The centrifuged mix was filled in HPLC vessels in 200 µl aliquots.

The HPLC was made using following equipment and settings:
HPLC (containing pump, autosampler, column compartment, diode array detector): ULTIMATE 3000 RS (Dionex)
CAD (Charged Aerosol Detector): Corona Ultra RS (Thermo Fisher Scientific)
Column: Acclaim 120 C18 3 µm 120 Å, 2,1 x 150 mm (Dionex)
Mobile solvent: acetonitrile and 0,1 % formic acid
Gradient: in 45 minutes increase from 5 % acetonitrile to 95 % acetonitrile
10 minutes washing with 95 % acetonitrile, 10 minutes equilibration with 5 % acetonitrile
Flow rate: 0,5 ml/min
HPLC spectra are found in the Appendix. The order of the analysis is listed in Table 12.

Table 12: Order of the HPLC analysis

Sample/Control	Medium	Supernatant (SN)/Pellet (P)
methanol	-	-
9602	PM4-1	SN
9602	PM4-1	P
pAMX1	PM4-1	P
pAMX1	PM4-1	SN
9602	5282	SN
9602	5282	P
pAMX1	5282	SN
pAMX1	5282	P
9302	PM4-1	SN
9302	PM4-1	P
9302	5282	SN
9302	5282	P
n-hexane	-	-

8 References

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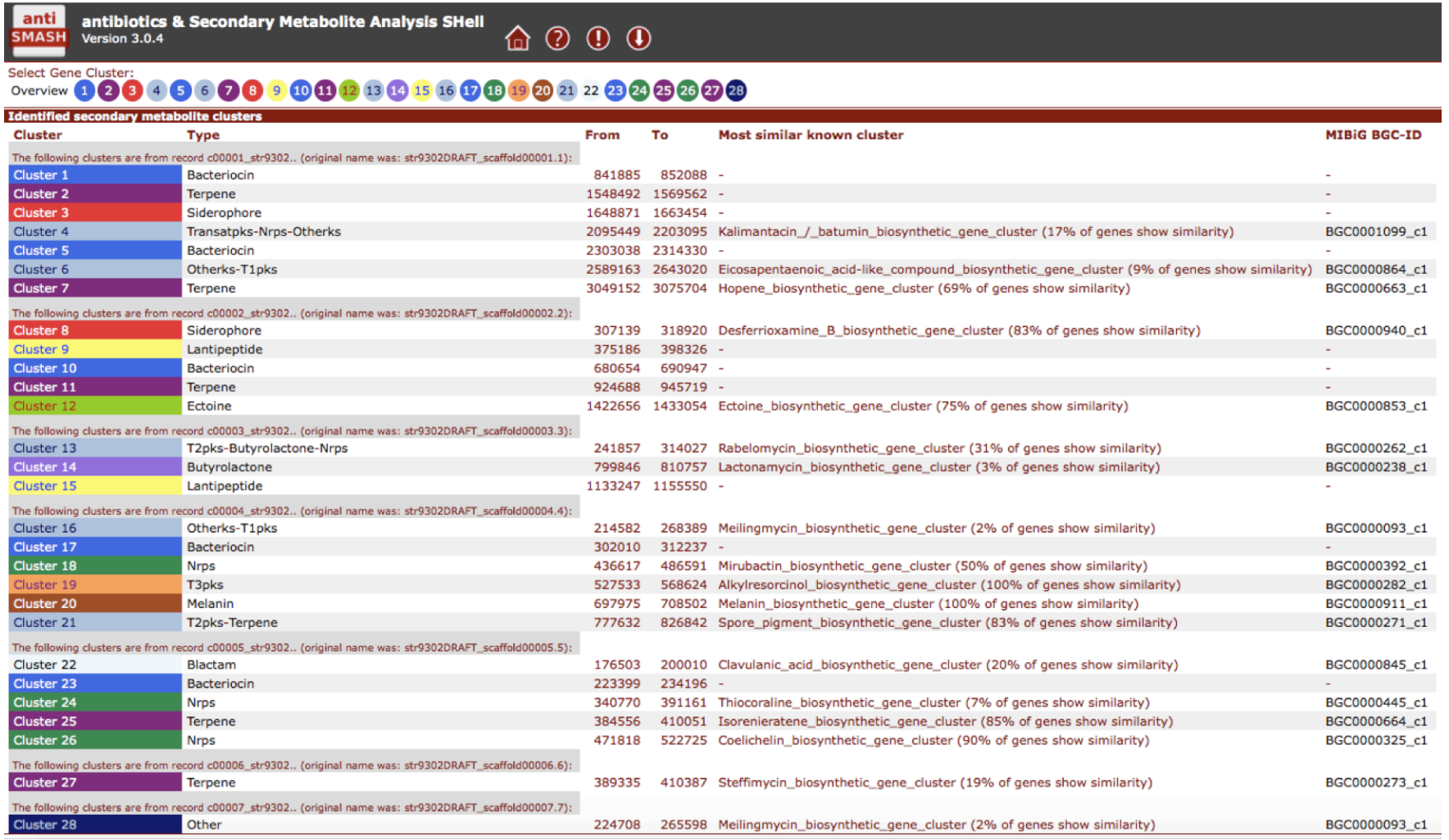

antiSMASH
antibiotics & Secondary Metabolite Analysis SHell
Version 3.0.4

Select Gene Cluster:
Overview 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

Identified secondary metabolite clusters

Cluster	Type	From	To	Most similar known cluster	MIBiG BGC-ID
The following clusters are from record c00001_str9302.. (original name was: str9302DRAFT_scaffold00001.1):					
Cluster 1	Bacteriocin	841885	852088	-	-
Cluster 2	Terpene	1548492	1569562	-	-
Cluster 3	Siderophore	1648871	1663454	-	-
Cluster 4	Transatpks-Nrps-Otherks	2095449	2203095	Kalimantacin_/_batumin_biosynthetic_gene_cluster (17% of genes show similarity)	BGC0001099_c1
Cluster 5	Bacteriocin	2303038	2314330	-	-
Cluster 6	Otherks-T1pks	2589163	2643020	Eicosapentaenoic_acid-like_compound_biosynthetic_gene_cluster (9% of genes show similarity)	BGC0000864_c1
Cluster 7	Terpene	3049152	3075704	Hopene_biosynthetic_gene_cluster (69% of genes show similarity)	BGC0000663_c1
The following clusters are from record c00002_str9302.. (original name was: str9302DRAFT_scaffold00002.2):					
Cluster 8	Siderophore	307139	318920	Desferrioxamine_B_biosynthetic_gene_cluster (83% of genes show similarity)	BGC0000940_c1
Cluster 9	Lantipeptide	375186	398326	-	-
Cluster 10	Bacteriocin	680654	690947	-	-
Cluster 11	Terpene	924688	945719	-	-
Cluster 12	Ectoine	1422656	1433054	Ectoine_biosynthetic_gene_cluster (75% of genes show similarity)	BGC0000853_c1
The following clusters are from record c00003_str9302.. (original name was: str9302DRAFT_scaffold00003.3):					
Cluster 13	T2pks-Butyrolactone-Nrps	241857	314027	Rabelomycin_biosynthetic_gene_cluster (31% of genes show similarity)	BGC0000262_c1
Cluster 14	Butyrolactone	799846	810757	Lactonamycin_biosynthetic_gene_cluster (3% of genes show similarity)	BGC0000238_c1
Cluster 15	Lantipeptide	1133247	1155550	-	-
The following clusters are from record c00004_str9302.. (original name was: str9302DRAFT_scaffold00004.4):					
Cluster 16	Otherks-T1pks	214582	268389	Meilingmycin_biosynthetic_gene_cluster (2% of genes show similarity)	BGC0000093_c1
Cluster 17	Bacteriocin	302010	312237	-	-
Cluster 18	Nrps	436617	486591	Mirubactin_biosynthetic_gene_cluster (50% of genes show similarity)	BGC0000392_c1
Cluster 19	T3pks	527533	568624	Alkylresorcinol_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000282_c1
Cluster 20	Melanin	697975	708502	Melanin_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000911_c1
Cluster 21	T2pks-Terpene	777632	826842	Spore_pigment_biosynthetic_gene_cluster (83% of genes show similarity)	BGC0000271_c1
The following clusters are from record c00005_str9302.. (original name was: str9302DRAFT_scaffold00005.5):					
Cluster 22	Blactam	176503	200010	Clavulanic_acid_biosynthetic_gene_cluster (20% of genes show similarity)	BGC0000845_c1
Cluster 23	Bacteriocin	223399	234196	-	-
Cluster 24	Nrps	340770	391161	Thiocoraline_biosynthetic_gene_cluster (7% of genes show similarity)	BGC0000445_c1
Cluster 25	Terpene	384556	410051	Isorenieratene_biosynthetic_gene_cluster (85% of genes show similarity)	BGC0000664_c1
Cluster 26	Nrps	471818	522725	Coelichelin_biosynthetic_gene_cluster (90% of genes show similarity)	BGC0000325_c1
The following clusters are from record c00006_str9302.. (original name was: str9302DRAFT_scaffold00006.6):					
Cluster 27	Terpene	389335	410387	Steffimycin_biosynthetic_gene_cluster (19% of genes show similarity)	BGC0000273_c1
The following clusters are from record c00007_str9302.. (original name was: str9302DRAFT_scaffold00007.7):					
Cluster 28	Other	224708	265598	Meilingmycin_biosynthetic_gene_cluster (2% of genes show similarity)	BGC0000093_c1

Figure 37: Example of an antiSMASH result

10.2 Vector maps

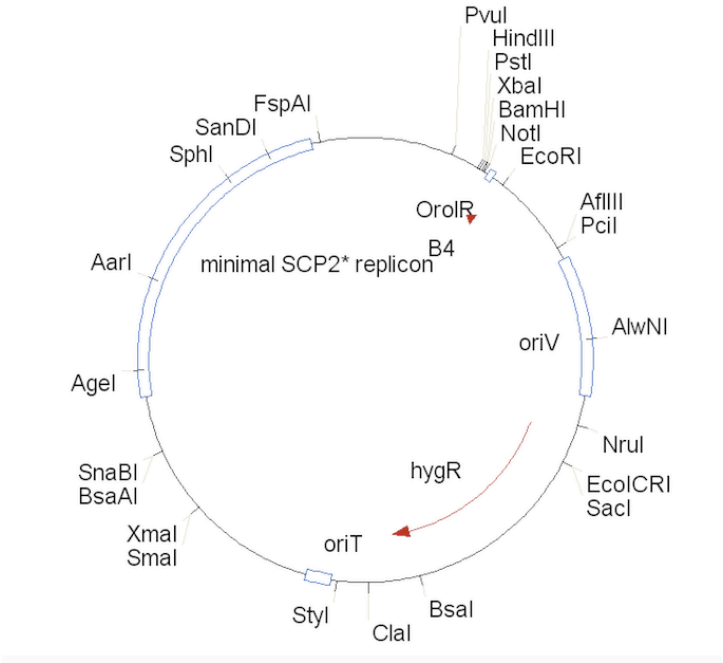

Figure 38: map of pAMX1: the vector contains a hygR gene (Hygromycin resistance), minimal SCP2 replicon (allows replication in Streptomyces), oriT, RP4 origin of transfer (for conjugation), B4 (promoter), OrolR (operator)

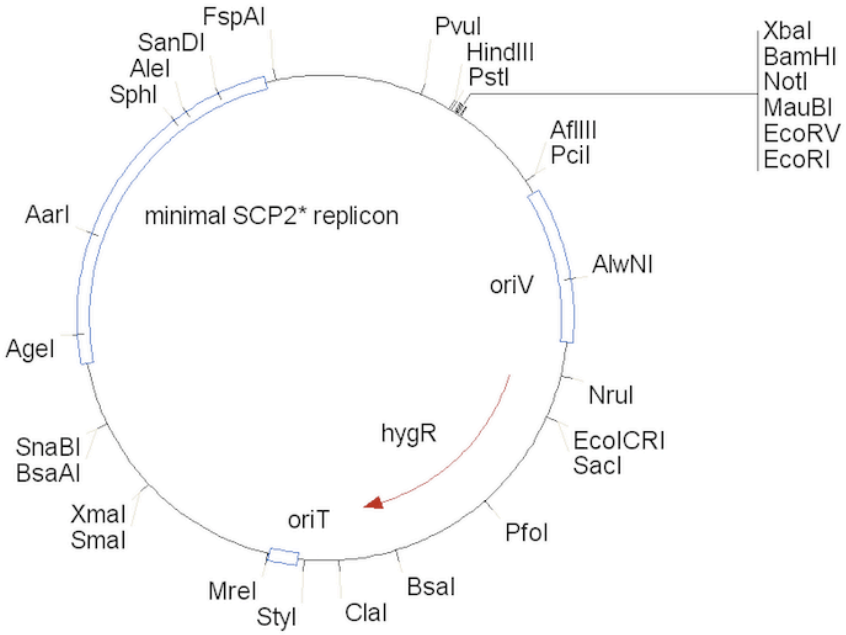

Figure 39: map of pKC1218H; the vector contains a hygR gene (Hygromycin resistance), minimal SCP2 replicon (allows replication in Streptomyces), oriT, RP4 origin of transfer (for conjugation), ori V (E.coli replication)

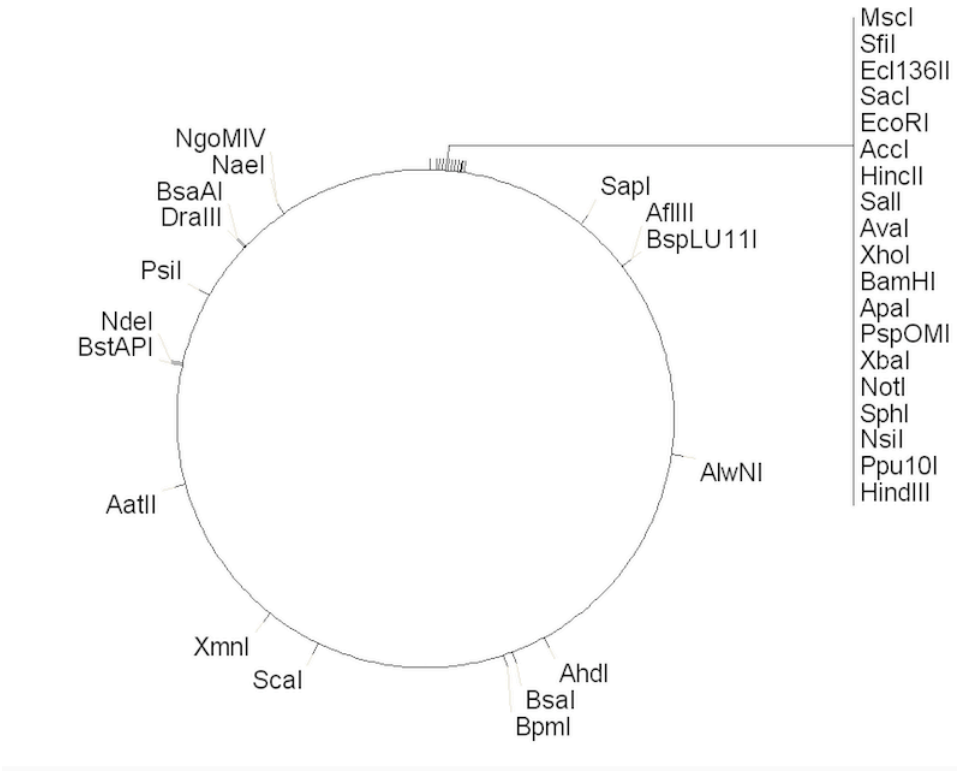

Figure 40: map of pGEM11Zf(+); general cloning vector containing a AmpR gene (Ampicillin resistance), f1 ori, T7 and SP6 promoters, lacZα (beta-galactosidase for color selection) (Promega, USA)

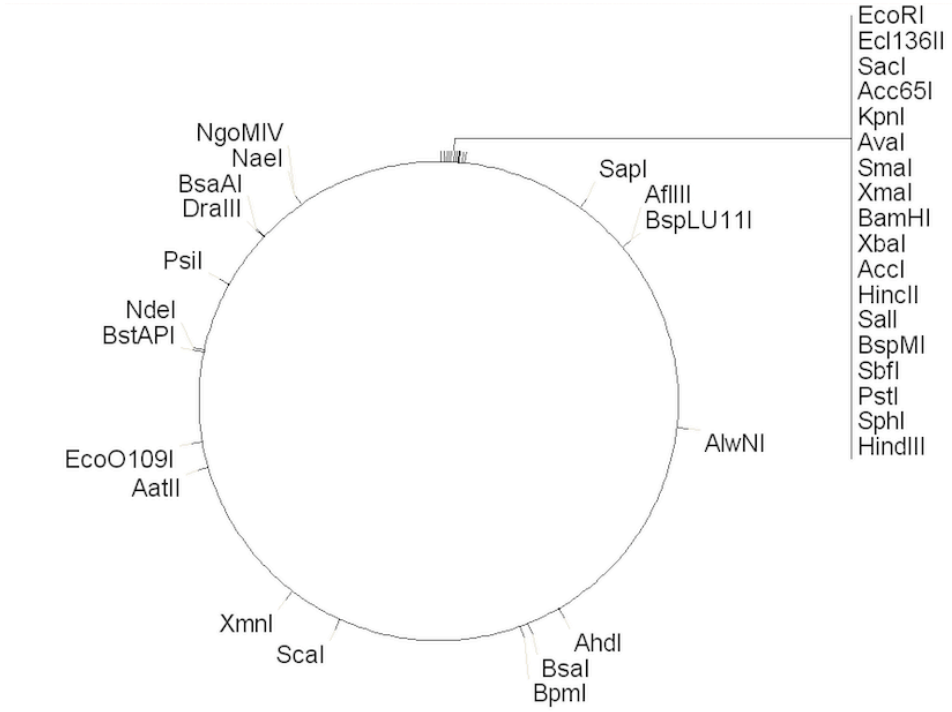

Figure 41: map of pGEM3Zf(+); general cloning vector containing a AmpR gene (Ampicillin resistance), f1 ori,T7, SP6 promoters, lacZα (beta-galactosidase for color selection) (Promega, USA)

10.3 GC spectra

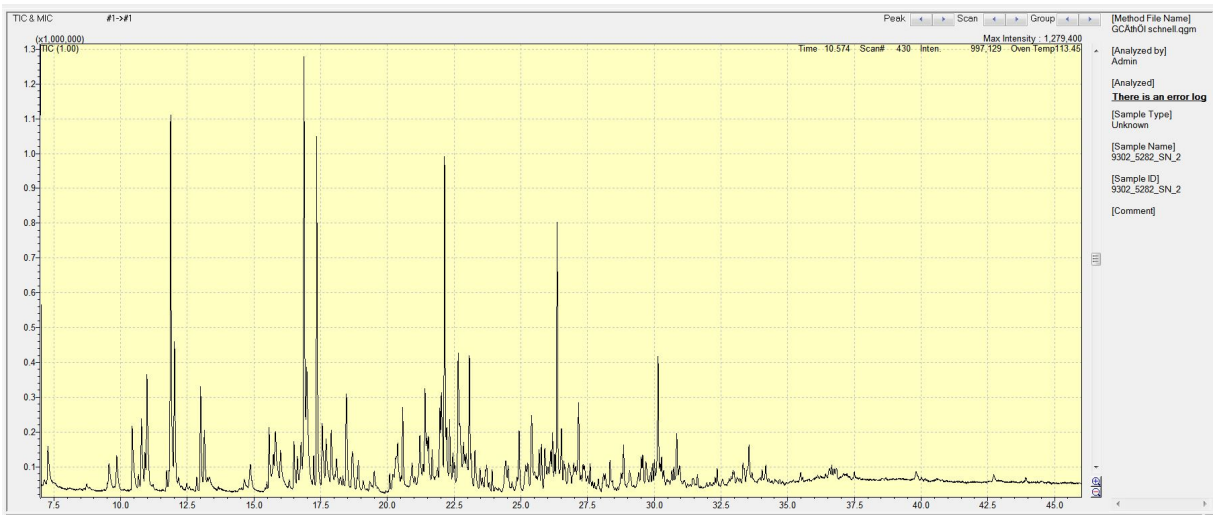

Figure 42: 9302 in pAMX1 (5282 medium, supernatant)

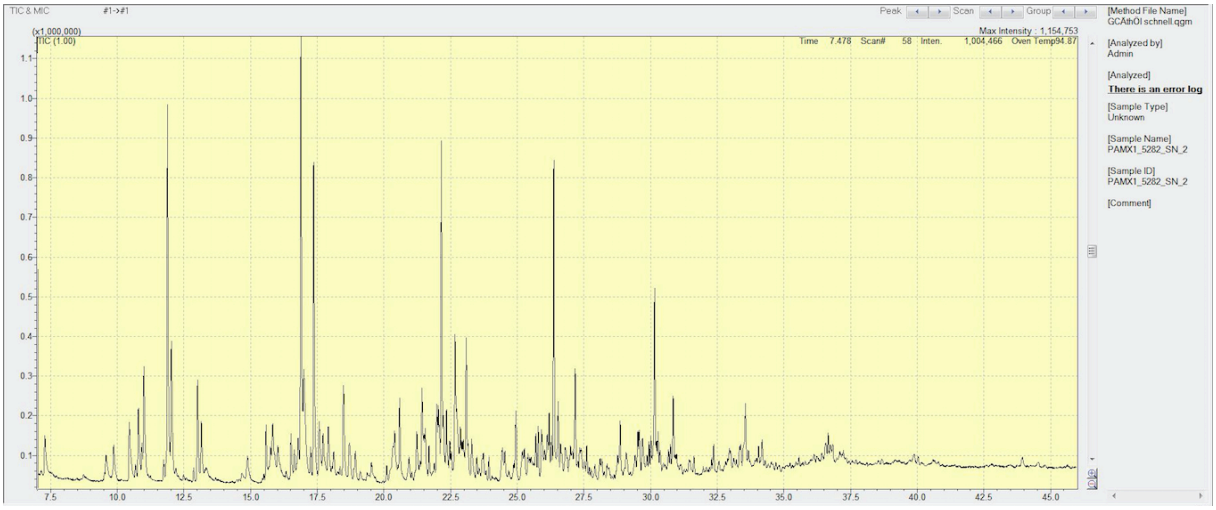

Figure 43: pAMX1 (5282 medium, supernatant)

10.4 HPLC spectra

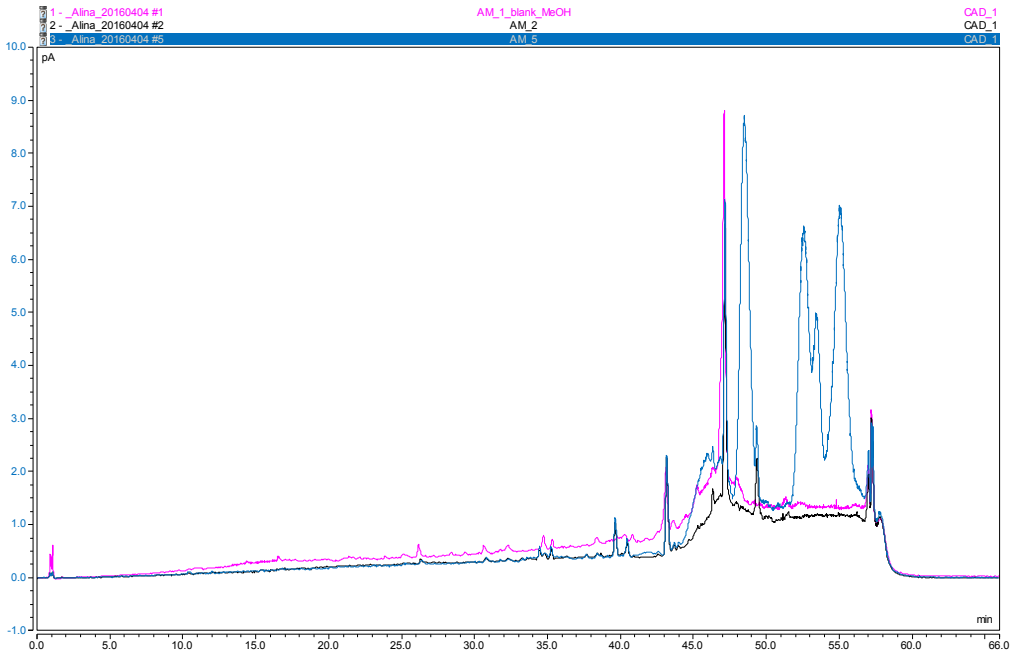

Figure 44: This spectrum shows:

pink line: blank sample (methanol)

black line: clone 9602 in pAMX1 (PM4-1 medium, supernatant)

blue line: pAMX1 (PM4-1 medium, supernatant)

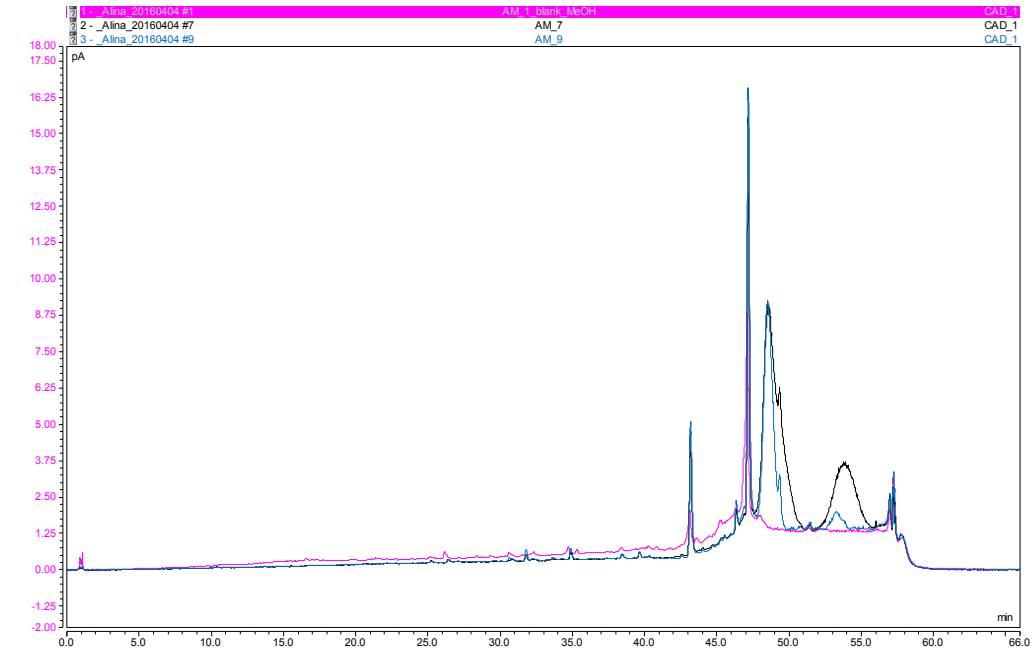

Figure 45: This spectrum shows:

pink line: blank sample (methanol)

black line: clone 9602 in pAMX1 (5282 medium, pellet)

blue line: pAMX1 (5282 medium, pellet)

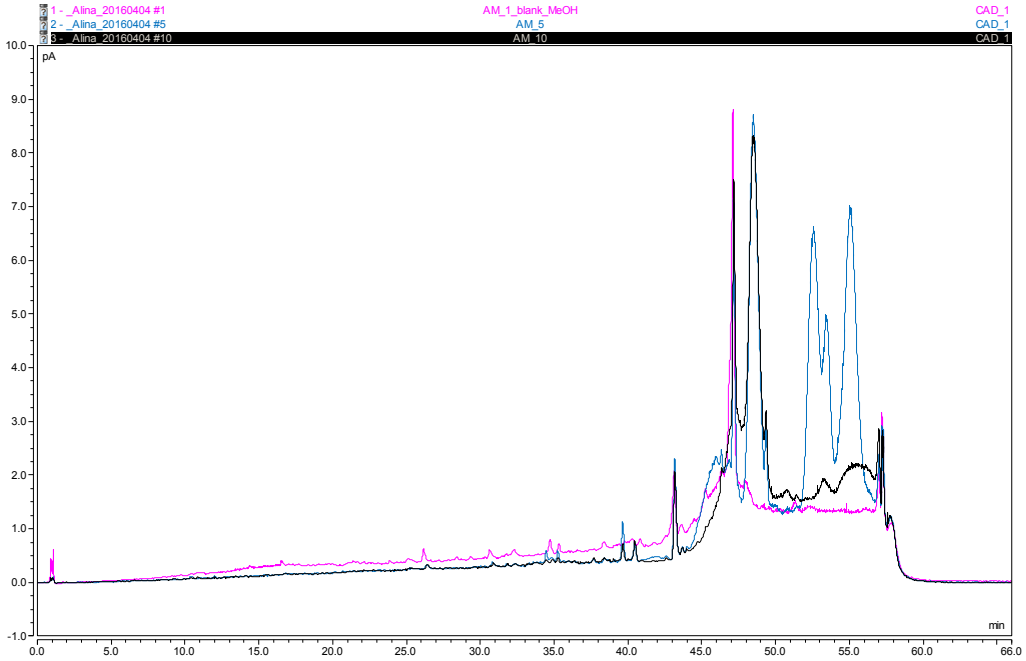

Figure 46: This spectrum shows:

pink line: blank sample (methanol)

black line: clone 9302 in pAMX1 (PM4-1 medium, supernatant)

blue line: pAMX1 (PM4-1 medium, supernatant)

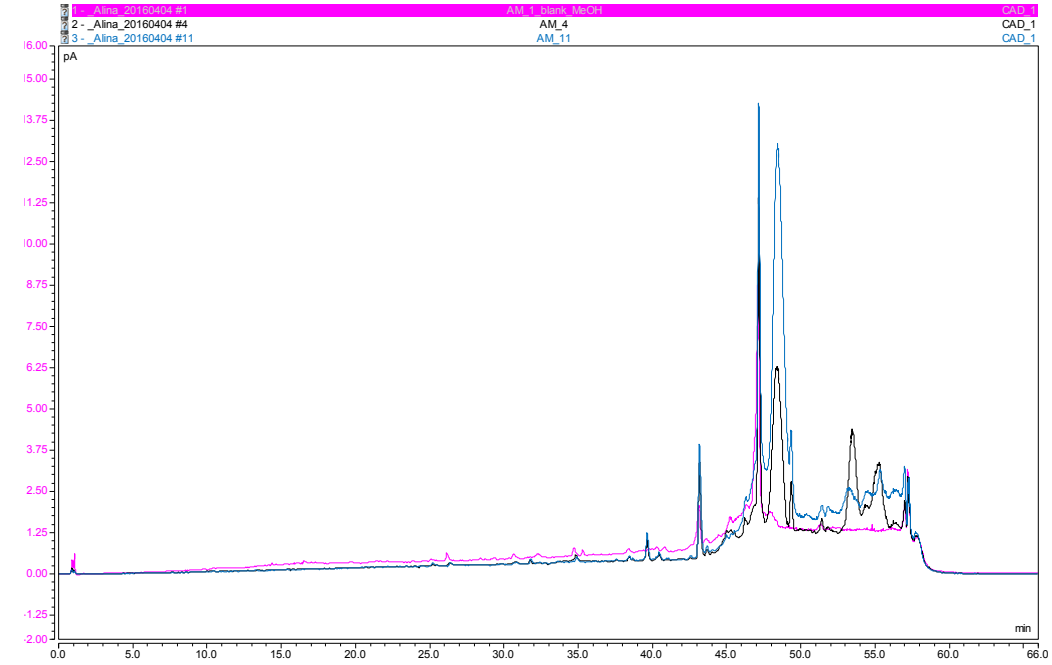

Figure 47: This spectrum shows:

pink line: blank sample (methanol)

black line: pAMX1 (PM4-1 medium, pellet)

blue line: clone 9302 in pAMX1 (PM4-1 medium, pellet)

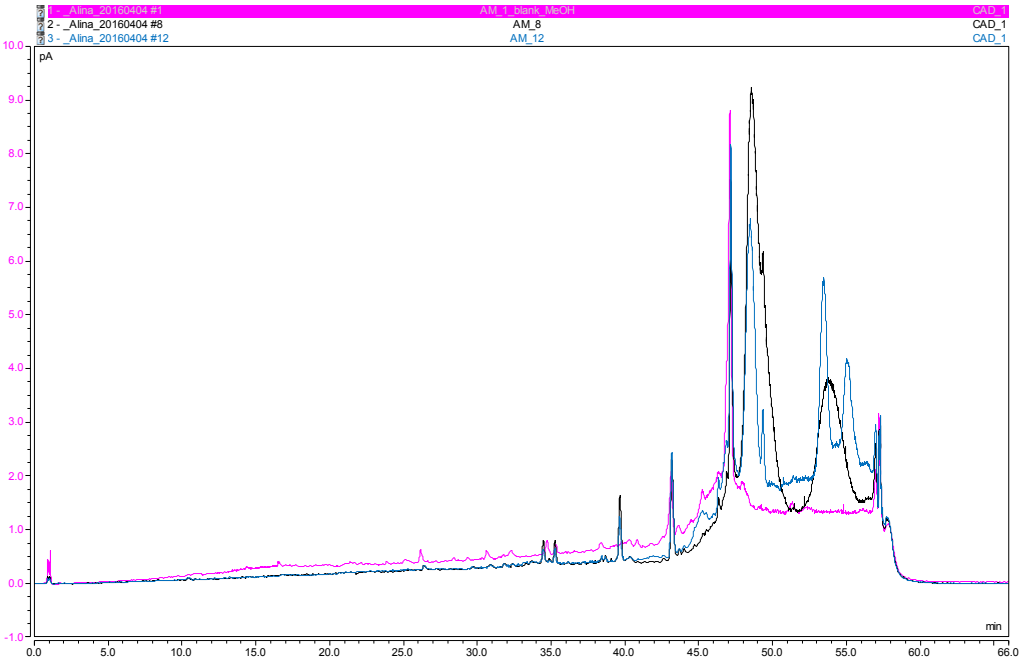

Figure 48: This spectrum shows:

pink line: blank sample (methanol)

black line: pAMX1 (5282 medium, supernatant)

blue line: clone 9302 in pAMX1 (5282 medium, supernatant)