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Exploration of the bacterium Streptomyces sp. S 4.7 for potentially novel antibacterial natural products

verfasst von | submitted by

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List of abbreviations

ACN	Acetonitrile
antiSMASH	Antibiotics and Secondary Metabolite Analysis Shell
BGC	Biosynthetic Gene Cluster
bp	Base pair
DNA	Desoxyribonucleic acid
gDNA	Genomic DNA
pDNA	Plasmid DNA
ddH2O	Double-destilled water
EtOAc	Ethyl acetat
HPLC	High-Performance Liquid Chromatography
HR-MS	High-Resolution Mass Spectrometry
kB	Kilo Base
KN	Knockout
LC-MS	Liquid Chromatography-Mass Spectrometry
MeOH	Methanol
NRP	Nonribosomal peptide
NRPS	Nonribosomal peptide synthetase
OE	Overexpression
OSMAC	One Strain Many Compounds
PCR	Polymerase Chain Reaction
PKS	Polyketide synthetase
rpm	Revolutions per minute
RT	Retention time
SARP	Streptomyces antibiotic regulatory protein
SM	Secondary metabolite
TF	Transciption factor
UV	Ultraviolet
WT	Wild type

1 Zusammenfassung

Die Arbeit untersuchte die Produktion sekundärer Metabolite in *Streptomyces* sp. S 4.7 mit dem Ziel, dessen Potenzial für die Entdeckung neuartiger antibakterieller Naturstoffe zu erforschen. Hierzu wurde eine Kombination aus klassischen Bioassay Screening und gezielten genetischen Manipulation des BGC 19, das für eine NRPS kodiert, angewendet.

Zur Untersuchung der Rolle von BGC 19 in der Metabolitenproduktion wurden Genaktivierungsexperimente durchgeführt, die auf das Gen S4.7_corr_29380 abzielten. Entgegen den Erwartungen zeigten die Knockout-Mutanten keine verringerte antibakterielle Aktivität, sondern in einigen Fällen sogar eine erhöhte Bioaktivität im Vergleich zum Wildtyp in Bioassays gegen *E. coli*. Die analytische HPLC-Analyse zeigte Veränderungen in den Metabolitenprofilen der Knockout-Stämme, einschließlich des Auftretens einer zuvor nicht detektierten Verbindung, welche im Wildtyp nicht vorhanden war. Dies deutet darauf hin, dass die Inaktivierung von BGC19 das metabolische Profil des Stammes verändert.

Weitere Experimente zeigten, dass die Produktion von Sekundärmetaboliten stark von den Kultivierungsbedingungen abhängt. Insbesondere führten Medien wie R5 und CSM+AW zu einer erhöhten antibakteriellen Aktivität, was mit dem OSMAC-Ansatz übereinstimmt. Darüber hinaus wurde in Bioassays ersichtlich, dass die Fraktionen nach einer Trennung mittels Flash-Chromatographie weniger bioaktiv waren. Das deutet darauf, dass die beobachtete antibakterielle Wirkung wahrscheinlich durch eine Kombination mehrerer Verbindungen oder synergistische Effekte und nicht durch einen einzelnen Metaboliten verursacht wird.

Die Überexpression von SARP-Regulatoren führte nicht zu einer konsistenten Erhöhung der Bioaktivität, und unabhängig erzeugte Mutanten zeigten variable Ergebnisse, was auf experimentelle Variabilität oder komplexe regulatorische Effekte hinweisen könnte.

Insgesamt zeigen die Ergebnisse, dass der Sekundärstoffwechsel von *Streptomyces* sp. S 4.7 hochkomplex ist und durch Wechselwirkungen zwischen Biosynthese-Genclustern sowie Umweltbedingungen beeinflusst wird. Diese Arbeit unterstreicht die Bedeutung der Kombination genetischer, analytischer und kultivierungsbasierter Ansätze, um das Potenzial von Actinomyceten für die Entdeckung neuartiger antibakterieller Naturstoffe besser zu verstehen und zu nutzen.

2 Abstract

This study investigated the secondary metabolite production of *Streptomyces* sp. S 4.7 to explore its potential for the discovery of novel antibacterial natural product. A combination of classical bioassay-guided screening and targeted genetic manipulation of biosynthetic gene cluster 19 (BGC19), encoding a non-ribosomal peptide synthetase (NRPS), was applied.

Gene inactivation experiments targeting S4.7_corr_29380 within BGC19 were performed to evaluate its role in metabolite production. Contrary to expectations, knockout mutants did not show reduced antibacterial activity and in some cases exhibited increased bioactivity compared to the wild-type strain in bioassays against *E. coli*. Analytical HPLC analysis revealed metabolic changes in the knockout strains, including the appearance of a previously undetected compound not present in the wild type. This suggests that disruption of BGC19 alters the metabolic profile of the strain.

Further experiments demonstrated that secondary metabolite production strongly depends on cultivation conditions. In particular, media such as R5 and CSM+AW resulted in increased antibacterial activity, consistent with the OSMAC approach. In addition, bioassay-guided fractionation revealed a decrease in activity after separation by flash chromatography, suggesting that the observed antibacterial effect is likely caused by a combination of compounds or synergistic interactions rather than a single metabolite.

Overexpression of SARP regulators did not lead to a consistent increase in bioactivity, and independently generated mutants showed variable results, indicating possible experimental variability or complex regulatory effects.

Overall, the results demonstrate that secondary metabolism in *Streptomyces* sp. S 4.7 is highly complex and influenced by interactions between BGCs and environmental conditions. This work showed the importance of combining genetic, analytical, and cultivation-based approaches to better understand and exploit the potential of actinomycetes for the discovery of novel antibacterial natural products.

3 Introduction

3.1 Antibiotics and their role in modern medicine

Antibiotics are defined as chemotherapeutic agents, that inhibit bacterial growth or induce bacterial cell death, while being selectively toxic to bacteria and not to human cells. This property is the basis of their clinical applicability, setting antibiotics apart from other antimicrobial substances [1]. Since antibiotics were introduced into medical practice in the mid-20th century, they have fundamentally transformed the treatment of bacterial infections, significantly reducing infection-related mortality [2]. In addition to treating infectious diseases, antibiotics have made it possible to safely perform advanced medical procedures such as major surgery, organ transplantation and cancer therapy, where patients are mostly vulnerable to bacterial infections due to immunosuppression or tissue damage [3].

The historical development of antibiotics is closely linked to the discovery of naturally occurring antimicrobial substances. The observation of the antibacterial activity of penicillin by Alexander Fleming in 1928 is commonly regarded as one of the key points in modern antibiotic research [3]. This period culminated in the ´golden age´ of antibiotic discovery, spanning around from the 1940s to the 1960s, during which numerous antibiotic classes were identified that are still in clinical use today (see Figure 1).

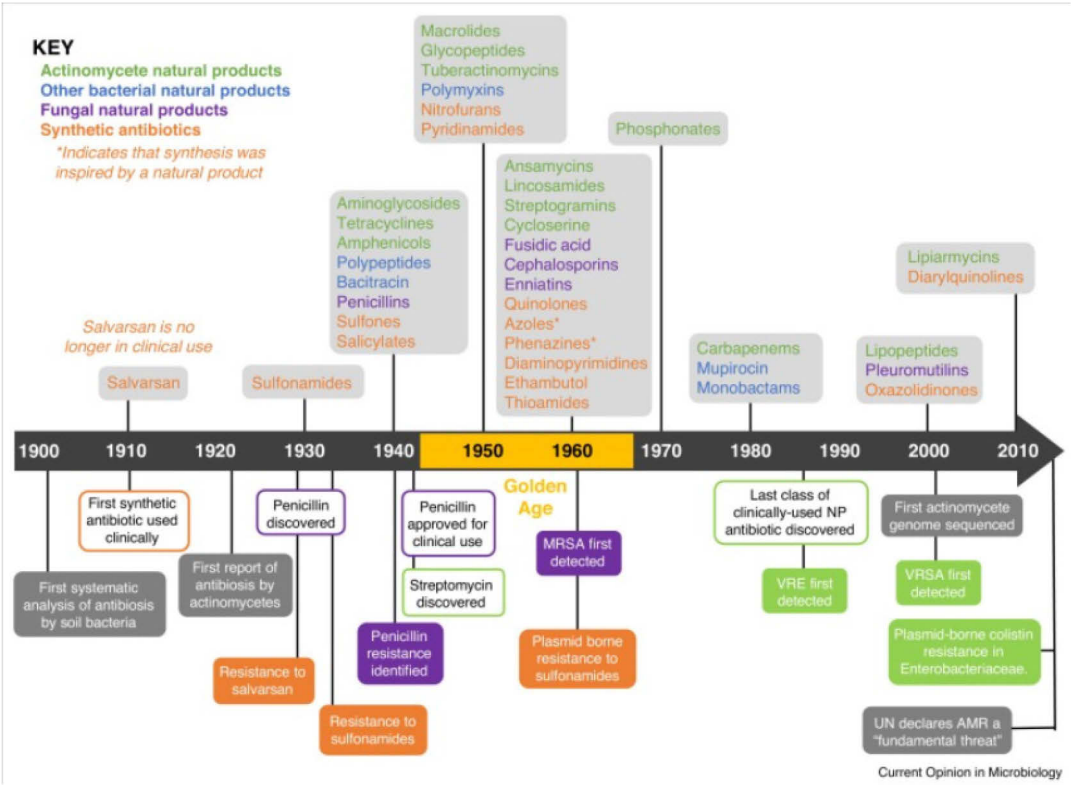


Figure 1 Timeline of new classes of antibiotic reaching the clinic [3].

The majority of antibiotics discovered during the golden age originate from natural products or are semi-synthetic derivatives thereof, which highlights the central role of microbial secondary metabolism in antibiotic development [3, 12]. These compounds exert their antibacterial effects by targeting essential cellular processes, such as cell wall biosynthesis, cell membrane function, protein synthesis, and nucleic acid metabolism [1,4]. An overview of the major molecular targets and mechanisms of action of different antibiotic classes is illustrated in Figure 2 [4].

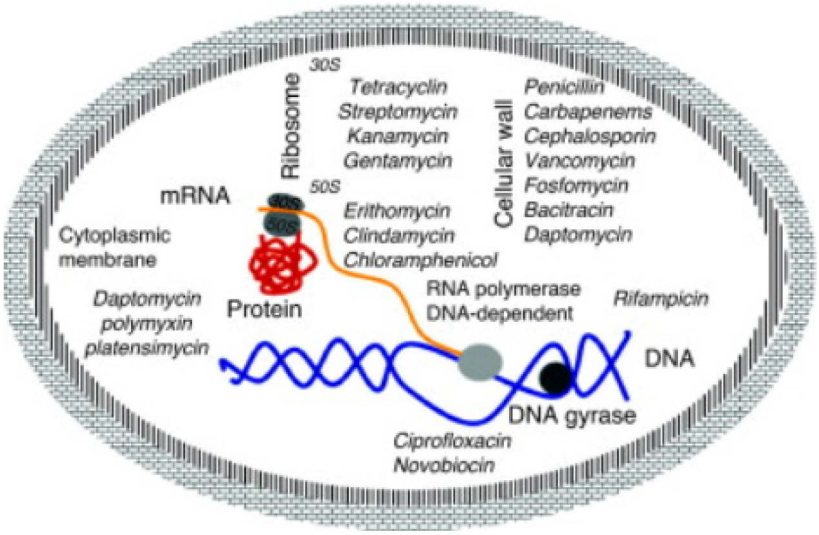


Figure 2 Overview of major cellular targets of antibiotics and their corresponding mechanisms of action in bacterial cells [4].

Despite their long-standing success, antibiotics are a limited resource, as the majority of clinically relevant classes were discovered several decades ago [2]. Nevertheless, they remain indispensable chemotherapeutic agents in contemporary medicine, particularly for vulnerable patient groups such as those undergoing cancer treatment [1]. Continued reliance on a limited number of antibiotic classes highlights the need for ongoing research to preserve existing antibiotics and identify new bioactive compounds [5].

3.2 Dangerous developments: antibiotic resistance

Shortly after the first antibiotics were introduced for clinical use, bacterial resistance was observed [6, 11]. One of the earliest examples was penicillin resistance, which is often caused by the production of β-lactamases that inactivate the antibiotic by hydrolysing its β-lactam ring [6]. Further molecular mechanisms that contribute to the development of antibiotic resistance in bacteria are schematically summarised in Figure 3.

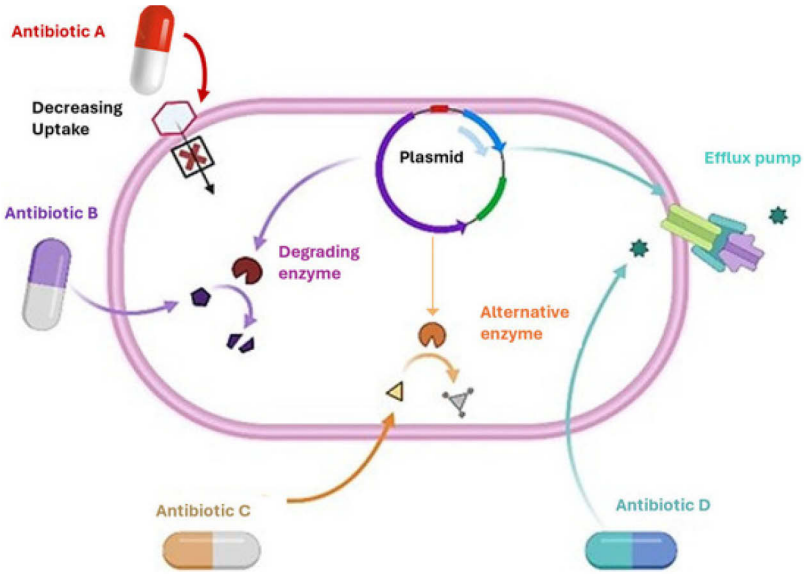


Figure 3 Major mechanisms of antibiotic resistance in bacteria, including decreased antibiotic uptake (Antibiotic A), enzymatic degradation (Antibiotic B), resistance-associated mutations (Antibiotic C) and active efflux pumps (Antibiotic D).) Adapted from Elshobary et al., 2025 [7].

In addition to these resistance mechanisms, bacteria can rapidly acquire resistance genes through horizontal gene transfer, which enables the spread of resistance among different species [8]. The growing prevalence of multidrug-resistant pathogens poses a significant threat to public health, mainly in hospital settings. Examples of clinically relevant pathogens include the ESKAPE group, which is responsible for many difficult-to-treat infections worldwide [9].

The extensive and often inappropriate use of antibiotics in human medicine, veterinary practice and agriculture has significantly enhanced the development of resistance [10, 11]. As a result, antibiotic resistance is regarded as one of the major challenges of modern medicine, highlighting the urgent need for more responsible use of existing antibiotics and the development of new antibacterial drugs [11].

3.3 Natural products as secondary metabolites

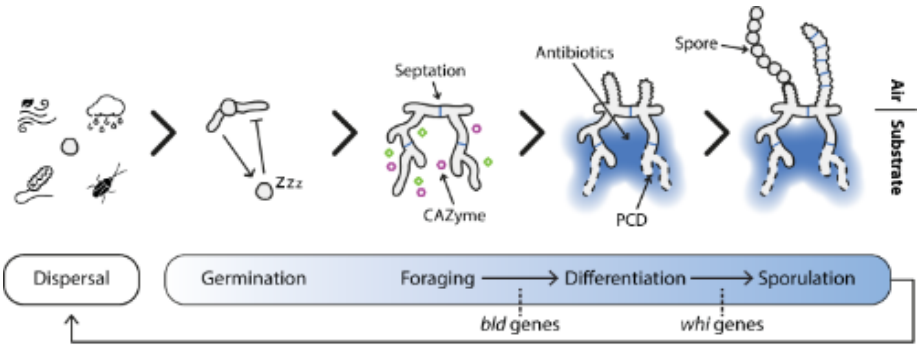
Natural products derived from microbial secondary metabolism have played a central role in antibiotic discovery, as a large proportion of clinically used antibiotics originate from or are based on naturally occurring compounds [12]. These secondary metabolites are not essential for microbial growth and are often produced during specific growth phases, such as the stationary phase, rather than during exponential growth [13, 14].

The ecological function of microbial secondary metabolites is closely linked to their role in natural habitats. By inhibiting competing microorganisms, these compounds provide a selective advantage in complex microbial communities, contributing to the acquisition and protection of limited nutrients [15]. Therefore, microorganisms are the primary source of naturally occurring antibiotic compounds, with bacteria and fungi being particularly important producers [12, 16].

Microbial natural products are characterised by a high degree of structural and chemical diversity, which is a key factor underlying their biological activity [12, 17]. Many of these compounds have complex molecular structures and are challenging to synthesise using purely synthetic methods [18]. This chemical diversity and structural complexity make natural products a valuable foundation for drug discovery, and they continue to play a crucial role in the development of new antibacterial agents [19].

3.4 Actinomycetes and *Streptomyces* as antibiotic producers

A wide variety of structurally diverse bioactive compounds are produced by *Actinomycetes*, including *Streptomyces* species, which are regarded as one of the most important sources of antibiotics [20, 4]. *Streptomyces* have a complex life cycle that is strongly influenced by environmental conditions and is closely connected to the production of secondary metabolites such as antibiotics (Figure 4). The life cycle begins with spore germination, followed by the formation of a branched vegetative mycelium that penetrates the substrate and secretes hydrolytic enzymes for nutrient acquisition. Upon nutrient limitation, developmental programs are initiated that lead to morphological differentiation and the formation of aerial hyphae. This transition is accompanied by extensive remodelling of the vegetative mycelium and is typically associated with the onset of secondary metabolite biosynthesis, including antibiotics. Ultimately, the aerial hyphae undergo synchronous septation and differentiate into chains of spores, ensuring survival and dispersal under adverse conditions [21].



Norte DM, et al. 2025
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Figure 4 Life cycle and developmental differentiation of *Streptomyces*. Spores germinate to form a vegetative mycelium that grows within the substrate and secretes enzymes for nutrient acquisition. When nutrients become limited, the bacteria start a developmental process that leads to the formation of aerial hyphae and the production of secondary metabolites, including antibiotics. The aerial mycelium later develops into chains of spores, which allow survival and spread. These developmental steps are controlled by genetic and regulatory networks. Adapted from [21].

Systematic screening of soil-dwelling *Actinomycetes*, particularly *Streptomyces,* has historically underpinned antibiotic discovery [23], with estimates suggesting that approximately 80 % of antibiotics are sourced from the genus *Streptomyces* [4]. To illustrate this point, several important drugs discovered from the genus *Streptomyces* should be mentioned, such as tetracycline, vancomycin, rifampicin, nystatin and daptomycin (Figure 5) [4]. Consequently, the search for novel natural products has progressively extended to unconventional and extreme environments such as marine ecosystems, plants, insects, animals and desert soils [22].

Figure 5 Antibiotics from *Streptomyces ssp. Adapted from [4].*

Selman Waksman made a major contribution to antibiotic discovery by systematically screening soil microorganisms for antibacterial activity [23]. This work resulted in the discovery of several significant antibiotics, including streptomycin and neomycin. Streptomycin was a breakthrough in the treatment of tuberculosis, demonstrating the immense therapeutic potential of metabolites produced by soil-dwelling bacteria [24]. These findings established *Actinomycetes* as a particularly rich source of bioactive secondary metabolites, laying the foundation for natural product-based antibiotic research [25, 12].

A more recent example illustrating the biosynthetic potential of *Actinomycetes* is *Streptomyces* sp. S4.7, which was isolated from the rhizosphere of the alpine plant *Leontopodium nivale*. This strain was reported to produce two structurally novel lipopeptides, Viennamycin A and Viennamycin B. Genome analysis revealed a dedicated biosynthetic gene cluster encoding a modular non-ribosomal peptide synthetase (NRPS) assembly line together with transport and tailoring enzymes. Interestingly, although crude extracts showed antibacterial activity, the purified compound displayed little to no bioactivity, suggesting a potentially atypical ecological or regulatory role. Furthermore, genome sequencing revealed numerous additional biosynthetic

gene clusters, emphasising the significant metabolic capacity of this organism and its importance in genome-guided natural product discovery [26].

3.5 Biosynthetic gene clusters (BGCs)

Biosynthetic gene clusters (BGCs) are regions of the genome that contain the genes required for the biosynthesis of secondary metabolites [27]. Many antibiotic biosynthetic gene clusters encode large multi-enzyme systems such as non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), which are responsible for the assembling of structurally complex natural products [28]. In general, NRPS pathways produce peptide-based natural products, whereas PKS pathways generate polyketide scaffolds, and many such products have antibiotic activity [39]. In *Actinomycetes*, genes involved in forming a specific natural product are usually found together within these clusters. BGCs are often organized in a modular architecture that comprises core biosynthetic genes, regulatory elements, transport-associated proteins and resistance genes. This coordinated gene arrangement enables efficient synthesis, modification and export of secondary metabolites [29]. A representative example of such an organization is the viennamycin biosynthetic gene cluster identified in *Streptomyces* sp. S4.7, which is schematically illustrated in Figure 6.

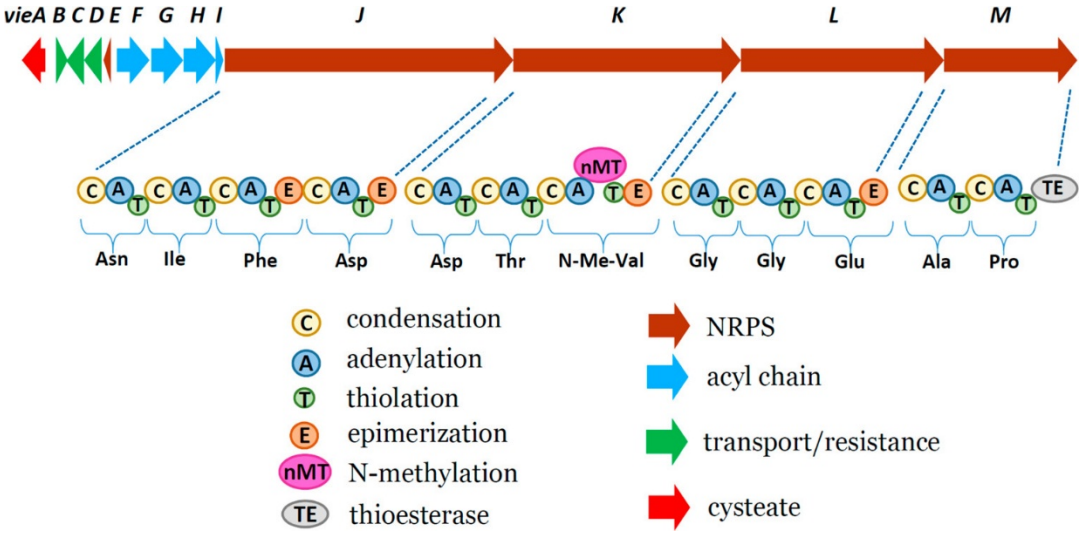

Figure 6 Overview of the organization of the viennamycin BGC in the genome of Streptomyces sp. S4.7 [26]

Advances in genome sequencing have revealed that microbial genomes harbour a considerably larger number of BGCs than was previously expected, including many cryptic clusters with novel bioactive products [30]. Many of these clusters are transcriptionally inactive or only weakly expressed under standard laboratory conditions, and are therefore referred to as silent gene clusters [31]. This suggests that the full potential of microbial biosynthetic pathways, particularly those of *Streptomyces* species, remains to be exploited.

The expression of BGCs is tightly controlled by complex regulatory networks. These include global regulators, as well as cluster-specific regulators that directly control the transcription of genes within a particular BGC [27]. In *Streptomyces,* SARP *(Streptomyces* antibiotic regulatory protein) family members represent an important class of pathway-specific regulators that play a key role in activation or enhancing the production of secondary metabolites [32]. Therefore, manipulation of such regulatory elements has emerged as a promising strategy for accessing novel natural products and increasing antibiotic yields.

3.6 Strategies for the discovery of novel antibiotics

The increasing prevalence of antibiotic resistance has highlighted the limitations of traditional antibiotic discovery approaches and has driven the development of new strategies to identify novel antibacterial compounds [33]. While early antibiotic discovery was mainly based on microbial screening approaches, modern research increasingly combines high-throughput screening with molecular, genomic and bioinformatic tools to access previously unexplored biosynthetic potential [3, 33, 34]. Different experimental and bioinformatic strategies have been developed to identify and activate biosynthetic gene clusters, thereby enabling access to previously undiscovered natural products (Figure 7) [5, 31].

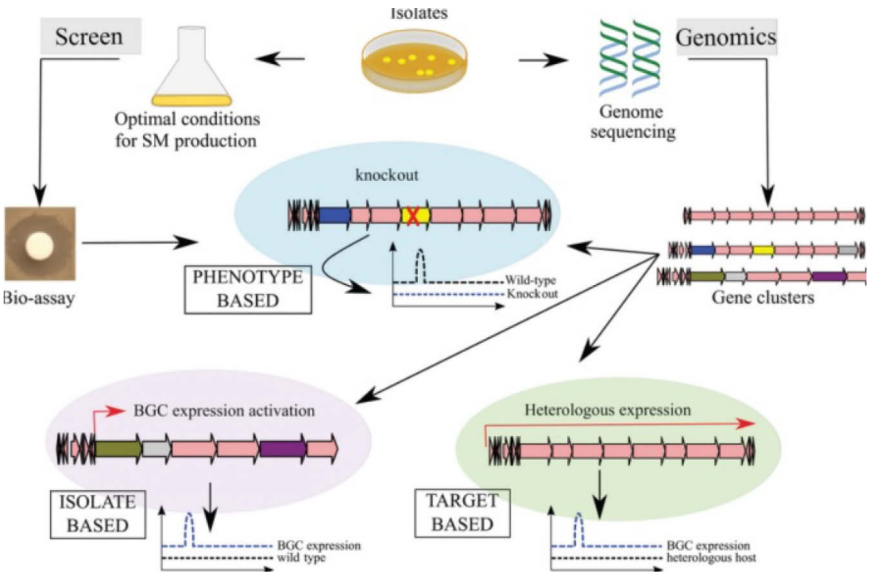

Figure 7 Schematic overview of phenotype-based and genome-guided strategies used for the identification and activation of biosynthetic gene clusters in bacteria, including bioassay screening, genome sequencing and heterologous expression approaches. Adapted from [5].

3.6.1 Classical bioprospecting

Classical bioprospecting involves isolating microorganisms from natural environments, followed by cultivation, and phenotypic screening for bioactive compounds, typically using bioassays to detect antibacterial activity. This approach was particularly successful during the golden age of antibiotic discovery, leading to the identification of many clinically important

antibiotics, particularly those derived from soil-dwelling actinomycetes [12, 35]. Figure 8 illustrates a typical workflow of bioassay-guided natural product discovery, including microbial cultivation, metabolite extraction and antibacterial screening.

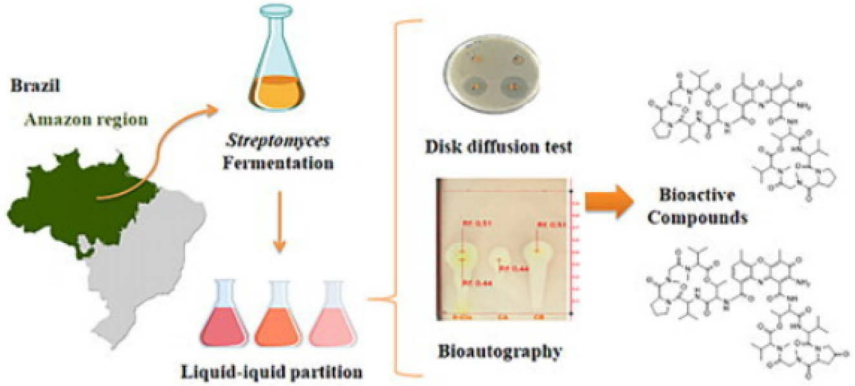

Figure 8 Schematic workflow of bioassay-guided natural product discovery from soil-derived *Streptomyces.* Environmental sampling is followed by microbial cultivation and fermentation, extraction and liquid-liquid partitioning of metabolites, and then antibacterial screening using disk diffusion and bioautography. Active fractions are further purified to identify bioactive compounds [36].

However, repeated screening of similar ecological niches has resulted in high rates of rediscovery of known compounds, which limits the efficiency of classical bioprospecting in the search for truly novel antibiotics [37, 38].

3.6.2 Genome mining

Genome mining has emerged as a powerful alternative to traditional screening methods. By analysing microbial genome sequences, it is possible to identify biosynthetic gene clusters encoding secondary metabolites in silico, regardless of whether the corresponding compounds are produced in a laboratory setting [39]. This strategy has revealed that many microorganisms harbour a far greater biosynthetic capacity than was previously thought. Genome mining therefore offers a rational basis for prioritising strains and gene clusters with the potential to produce novel antibiotics [40, 41].

3.6.3 Activation of silent gene clusters

To overcome the limited expression of silent or cryptic biosynthetic gene clusters, a variety of activation strategies have been developed [42]. These approaches can broadly be divided into genetic and cultivation-based methods. Genetic strategies aim at directly modifying the regulatory or structural elements of a gene cluster in order to stimulate transcription or enhance metabolite production. One of the most established techniques in actinomycete research is homologous recombination, which enables precise gene deletions, insertions or promoter replacements and is widely used for functional gene analysis. Closely related to this approach,

the introduction of strong or constructive promoters upstream of biosynthetic genes can significantly enhance transcriptional activity. Another powerful strategy is heterologous expression, in which entire biosynthetic gene clusters are transferred into well-characterised host organisms that provide favourable regulatory and metabolic conditions for compound production [43].

In addition to genetic manipulation, cultivation-strategies also play an important role. The OSMAC (One Strain-Many Compounds) approach involves systematically varying growth parameters, such as nutrient composition, temperature and salinity, in order to induce the production of diverse secondary metabolites from a single microbial strain [44]. These complementary strategies significantly increase the chemical diversity accessible from microorganisms and have become central tools in contemporary natural product discovery [42].

Based on these concepts, this thesis investigates the biosynthetic potential of a selected Streptomyces strain with a focus on a putative NRPS biosynthetic gene cluster identified by genome mining. The cluster was analysed by targeted gene inactivation, and pathway-specific regulation was examined by activating a SARP-type transcriptional regulator. Effects on secondary metabolite production and antimicrobial activity were assessed using bioassay-based screening.

4 Aim of the work

The increasing prevalence of antibiotic resistance highlights the urgent need for the discovery of novel bioactive compounds, particularly from well-known producer organisms such as *Streptomyces*.

Therefore, the aim of this work was to investigate the biosynthetic potential of *Streptomyces* sp. S4.7, with a particular focus on a NRP BGC 19 identified by antiSMASH 5.0.

To gain insight into the functional role of this gene cluster, targeted gene inactivation was performed and the resulting knockout mutant strain was analysed in comparison to the wild type. In addition, pathway-specific regulation was explored by overexpression of a SARP-type transcriptional regulator in order to assess its effects on secondary metabolite production.

The crude extracts from produced strains were purified and further examined using bioassay-guided approaches to evaluate changes in antimicrobial activity and to identify potentially bioactive compounds.

5 Materials

5.1 Bacteria Strains, Vectors, Plasmids and Primers

5.1.1 Bacteria strains

Table 1: Bacterial strains used in this work

Project organisms	**Characteristics**
Streptomyces sp. S 4.7 Wild type strain	Non-mutated form of the organism found in the environment
KN2, KN4, KN5, KN6 ; KN**7 (control)** and KN8 (control)	Knockout (deactivated cluster 19) mutant in *Streptomyces* sp. S 4.7
S.4.7_SARP1 and S.4.7_SARP2	Overexpressed SARP in *Streptomyces* sp. S 4.7 wild type-Mutant
Escherichia Coli XL Blue	
Escherichia Coli ET12567	Used for conjugation with PSOK vector and pDNA

5.1.2 Vectors and Plasmids

Table 2: Vectors and Plasmids used in this work

Name	**Type**	**Properties**	**Source/ Reference**
pSOK201	Vector	pSG5 and ColE1 replicons, oriT and Am^R	[1]
pSOK806	Vector	integrative plasmid encoding conjugation machinery; strong promotor (PermE*); AmR	[3]
pUWLoriT	Plasmid	*E. coli*/ *Streptomyces* shuttle vector with oriT, Amp^R, $Thio^R$	[2]
pUWLoriT_SARP	Plasmid	Modified pUWLoriT carrying SARP gene	This work

5.1.3 Primers

Table 3: Primers for mutants in Cluster 19 in *Streptomyces* sp. S 4.7

Name	Sequence	Size of expecting product	Annealling temp
S47_KN_*Hind*III	GCTAGAAGCTTGCAGCCGGT CGTACATGA	2197 bps	60 °C
S47_KN_EcoRI	GAGCGAATTCCACCACCGAC TGGCTGAAG	2197 bps	60 °C
S47_KN_DTF	CGAGATGCGCAACTCCTAC	2298 bps	58 °C
S47_KN_DTR	TGGGTGTCCAGGAGACTAC	2298 bps	58 °C
SARP_C19_HindIII	GCGCCAAGCTTTCTTCGCCT GCTTGATCGTC	9405 bps	72 °C
SARP_C19_SpeI	GAAACTAGTACACGGTGGTG TTCGGTGAG	9038 bps	68 °C

5.1.4 Test organisms for Bioassay with disc diffusion test

Table 4: Test organisms of various types with incubation temperature and media

Organisms for Bioassay	Temperature	Media
Gram-negative bacteria		
Escherichia coli DH5αF	37 °C	LA
Pseudomonas putida	28 °C	LA
Erwinia persicina	28 °C	YPD Agar
Gram-positive bacteria		
Staphylococcus carnosus	37 °C	LA
Enterococcus mundti	37 °C	LA
Kocuria rhizophila	28 °C	LA
Micrococcus luteus	28 °C	LA

Bacillus subtilis	28 °C	LA
Fungi		
Saccharomyces cerevisiae	28 °C	YPD Agar

5.1.5 Kits

Table 5: Kits for DNA isolation

Name of kit	Manufacturer	Aim
QIAGEN Dneasy Tissue Kit	QIAGEN	Genomic DNA isolation from *Streptomyces*
Wizard® Plus SV Minipreps DNA Purification System	PROMEGA	Plasmid DNA isolation
Zymoclean™ Gel DNA Recovery Kit	Zymo Research	DNA recovery from gel

5.2 Media

5.2.1 Media used for cultivation of *Escherichia coli*

L-Broth medium (LB)

Bacto™ tryptone	10 g
Bacto™ yeast extract	5 g
NaCl	5 g
dH_2O	up to 1000 mL

L-Agar (LA)

Bacto™ tryptone	10 g
Bacto™ yeast extract	5 g
NaCl	5 g
Agar	10g
dH_2O	up to 1000 mL

5.2.2 Media used for cultivation of *Streptomyces* strains

5.2.2.1 Pre-culture media

Tryptic Soy Broth medium (TSB)

Tryptone soy broth powder	30 g
dH_2O	up to 1000 mL

R5 medium

Sucrose	103 g
Glucose	10 g
Yeast Extract	5 g
Difico Casamino Acids	5 mg
$MgCl_2$ * 6 H_2O	10.12 mL
K_2SO_4	0.25 g
TES	5.73 g
dH_2O	up to 1000 mL

YEME media

Oxoid Yeast Extract	3 g
Difico Bacto TM Peptone	5 g
Malt extract	5 g
Glucose	10 g
Sucrose	340 g
ddH_2O	up to 1000 mL

5 mL 1 M $MgCl_2.6H_2O$ were added after autoclaving.

5.2.2.2 Fermentation media

Tryptic Soy Broth medium (TSB)

Tryptone soy broth powder	30 g
dH_2O	up to 1000 mL

CSM

Triptic soy broth	30 g
Yeast extract	3 g
$MgSO4*7H_20$	4.09 g
Glucose	5 g
Maltose	4 g
dH2O	up to 1000 mL

R5 medium

Sucrose	103 g
Glucose	10 g
Yeast Extract	5 g
Difico Casamino Acids	5 mg
$MgCl_2$ * 6 H2O	10.12 mL
K_2SO_4	0.25 g
TES	5.73 g
dH2O	up to 1000 mL
Autoclave and add:	
$CaCl_2$ x 2 H_2O (5 M)	0.4 mL
KH2PO4 (0.5 %)	1 mL
L-Proline (20 %)	1.5 mL
NaOH (1 N)	0.7 mL
Trace elements as for R2YE	2 mL

R3 medium

Glucose	10 g
Yeast Extract	5 g
Difico Casamino Acids	0.1 mg
MgCl2 * 6 H2O	10 mL
K2SO4	0.2 g

TES	5.6 g
dH2O	up to 1000 mL

Autoclave and add:

$CaCl_2$ x 2 H_2O (5 M)	0.4 mL
KH_2PO_4 (0.5 %)	1 mL
L-Proline (20 %)	1.5 mL
NaOH (1 N)	0.7 mL
Trace elements as for R2YE	2 mL

MYM medium

Maltose	4 g
Yeast Extract	4 g
Malt Extract	10 g
Tap Water	500 mL
dH2O	up to 500 mL
R2 Trace Element	2 mL

Adjust to pH 7.8, autoclave, and store at 4 °C.

YPD
medium

Difco Bacto Yeast Extract	10 g
Bacto ™ Peptone	20 g
Glucose	20 g
dH2O	up to 1000 mL

5.2.2.3 Solid Media

ISP medium 2 (ISP2)

Bacto™ yeast extract	4 g
Malt extract	10 g

Dextrose	4 g
Agar	20 g
dH2O	up to 1000 mL

ISP medium 4 (ISP4)

Soluble Starch	10 g
Dipotassium Phosphate	1 g
Magnesium sulphate	
USP	1 g
NaCl	1 g
Ammonium sulphate	2 g
Calcium carbonate	2 g
Ferrous sulphate	1 g
Manganese chloride	1 g
Zinc sulphate	1 g
Agar	20 g
dH2O	up to 1000 mL

Mannitol soya flour medium (SFM)

Soya flour	20 g
Mannitol	20 g
Agar	20 g
TMS-1 Solution	1 mL
Tap water	up to 1000 mL

YPD medium

Bacto™ peptone	20 g
Bacto™ yeast extract	10 g
D-Glucose	20 g
Agar	15 g
dH2O	up to 1000 mL

5.2.3 Media used for cultivation of *Saccharomyces cerevisiae*

YPD medium

Difco Bacto Yeast Extract	10 g
Bacto ™ Peptone	20 g
Glucose	20 g
dH_2O	up to 1000 mL

YPD agar

Difco Bacto Yeast Extract	10 g
Bacto ™ Peptone	20 g
Glucose	20 g
Agar	15 g
dH_2O	up to 1000 mL

5.2.4 Additives for media

Artificial Seawater

Reagents

NaCl	26.29 g
KCl	0.74 g
$CaCl_2$	0.99 g
$MgCl_2*6H_2O$	6.09 g
$MgSO_4*7H_20$	3.94 g
dH_2O	up to 1000 mL

Adjust to pH 7.8, sterile filtrate in laminar air flow bank and store at 4 °C.

5.3 Stock solutions

5.3.1 Antibiotic stock solutions

Stock solutions of antibiotics were prepared to select genetically modified strains of *E. coli* or *Streptomyces*. An overview of the antibiotic stock solutions can be found in table 6. After preparation, all stock solutions, except for chloramphenicol (Cml) and Thiostrepton (Thio), were filtered using a syringe filter, Rotilabo PTFE 0.2 micrometers by Roth. Antibiotic stock solutions were stored at -20 °C.

Table 6: Overview of antibiotic stock solutions used in this experiment

Antibiotic	**Stock concentration**	**Concentration (µg/mL) used for *E. coli***	**Concentration (µg/mL) used for *Streptomyces***	**Diluent**
Ampicillin (Amp)	100	100	-	H_2O
Ampramycin (Am)	100	100	50	H_2O
Chloramphenicol (Cml)	25	25	-	EtOH abs.
Kanamycin (Kan)	25	25	-	H_2O
Nalidixic acid (NaI)	30	-	30	0.1 M NaOH
Thiostrepton (Thio)	30	30	30	DMSO

5.3.2 Buffers and other solutions

A lot of additional stock solution were used to perform diverse experiments (see list below).

Trace elements same as for R2YE

$ZnCl_2$	40 mg/L
$FeCl_3$ x 6 H_2O	200 mg/L
$CuCl_2$ x 2 H_2O	10 mg/L
$MnCl_2$ x 4 H_2O	10 mg/L
$Na_2B_4O_7$ x 10 H_2O	10 mg/L
$(NH_4)_6Mo_7O_{24}$ x 4 H_2O	10 mg/L

Solutions for GenBank screen

Table 7: Solutions for GenBank Screenings

Name	Content
Sol I	50 mM TrisHCl, pH 8.0 + 10 mM EDTA, 50 mM Glucose
Sol II	0.2 N NaOH + 1 % (w/v) SDS
Sol III	3 M KOAc, pH 4.8

Solution for preparation of cell suspension

Glycerol 20 %

Glycerol	15 g
dH2O	75 mL

Solution for preparation of competent cells

$CaCl_2$ (50 mM)

$CaCl_2 * 2 H_2O$	14.7 g
dH_2O	up to 2000 mL

$CaCl_2$ (50 mM) + 10 % Glycerol

$CaCl_2 * 2 H_2O$	3.67 g
Glycerol	50 g
dH_2O	up to 500 mL

Solution for conjugation experiment

$MgCl_2$

(1M)

$MgCl_2 * 6$ H2O	47.6 g
dH_2O	up to 500 mL

$CaCl_2$ (0.6M)

$CaCl_2 * 2$ H2O	44.10 g
dH_2O	up to 500 mL

Solutions used in preparation of media

Trace elements for R3 medium and R5 medium

$ZnCl_2$	40 mg
$FeCl_3 * 6\ H_2O$	200 mg
$CuCl_2 * 2\ H_2O$	10 mg
$MnCl_2 * 4\ H_2O$	10 mg
$Na_2B_4O_7 * 10\ H_2O$	10 mg
$(NH_4)_6Mo_7O_{24} * 4$ H_20	10 mg
dH_2O	Up to 1000 mL

Buffers used for gel electrophoresis

TBE buffers (10x)

Tris base

Boric acid

EDTA

dH2O

Lysis buffer

TRIS HCl (200 mM, pH= 8)

EDTA (10 mM, pH= 8)

Triton $_X$100 (1.2 %)

dH2O

Lysozyme (was added fresh before use)

Polymerases for PCR reactions

Q5 ® High-Fidelity DNA Polymerase

5x Q5 High GC Enhancer

Taq DNA Polymerase

5.4 Agarose Gel

For this project agarose gel for routine analysis and extraction of DNA and RNA electrophoresis were used with 0.5 %, 0.8 % and 2 % concentration of agarose in 1x TBE buffer. To the melted solution, GelRed Nucleic Acid Stain (10.000x) was added. Agarose gels have a broad range of separation capabilities and is suitable for resolving DNA fragments ranging from a few hundred base pairs up to several kilobases.

6 Methods

6.1 Genetic Modification and Bioinformatic Analysis

6.1.1 Bioinformatic tools

In this project, two bioinformatic tools were utilized for genome analysis and the planning of strategies for gene targeting, antiSMASH 5.0 and Clone Manager 9.

6.1.1.1 Genome Analysis by antiSMASH 5.0

This study was based on the results of genome sequencing data analysis, which was performed using the open-source online program antiSMASH 5.0 (Antibiotics and Secondary Metabolite Analysis Shell). This bioinformatic tool is used for the automated identification and analysis of putative biosynthetic gene clusters (BGCs) in bacterial genomes. AntiSMASH 5.0 was used to analyze the genome of *Streptomyces* sp. S 4.7, providing detailed information about the size, type, and predicted functions of the biosynthetic gene clusters (BGCs). Based on this analysis, specific primers were designed to amplify regulatory genes, which were then used to construct an insertional knock-out mutant in *Streptomyces* sp. S 4.7 KN.

6.1.1.2 Primer Design by Clone Manager

Clone Manager was an essential tool in our experimental workflow for designing the primers needed to generate knock-out cassettes. The precise design of these primers was important for the success of our gene manipulation experiments. By using Clone Manager, we were able to carefully plan and construct the knock-out cassettes to target specific genes of interest within the organism.

6.1.2 Genomic DNA Isolation from *Streptomyces* sp. S 4.7

To isolate genomic DNA from *Streptomyces* sp. S 4.7 using the DNeasy Kit from QIAGEN, following steps from the manual were followed: Culture of *Streptomyces* sp. S 4.7 grew as described in 6.4.1. The cell-culture was centrifuged and supernatant was removed. Pellet was resuspended with 180 µl lysis buffer (20 mM Tris-HCl, 10 mM EDTA, 1.2 % Triton X100, 15 mg/ml lysozyme; pH 8). Then this sample was incubated for 15 minutes in 37 °C and flipped every 5 minutes. Ethanol was added to this mix and sample was put through a DNeasy Mini

spin column. The flow-through was centrifuged and discarded. Then the column was washed with buffers and the genomic DNA was eluted. Storage of isolated gDNA was in – 20 °C.

6.1.3 Plasmid DNA Isolation

For the isolation of plasmid DNA of various *E. coli* strains, that were used during this work with *Streptomyces* sp. S 4.7, steps were followed from the manual of Wizard® Plus SV Minipreps DNA Purification System from Promega. Isolated plasmid DNA was stored in – 20 °C.

6.1.4 Polymerase Chain Reaction (PCR)

To perform a Polymerase Chain Reaction a PCR cycler is used. Its function is to provide precise temperature control and cycling conditions required for denaturation, annealing and extension steps, to enable efficient DNA amplification. In order to start this reaction, a Master mix was created in an Eppendorf tube with following ingredients and a program with following conditions were chosen to perform in a PCR cycler:

Reaction setup to perform a PCR with Q5 High-Fidelity DNA Polymerase:

Component	**for 25 µl reaction**
5X Q5 Reaction Buffer	5 µl
10 mM dNTPs	0.5 µl
10 µM Forward Primer	1.25 µl
10 µM Reverse Primer	1.25 µl
Template DNA	2 µl
Q5 High-Fidelity DNA Polymerase	0.25 µl
5X Q5 High GC Enhancer	5 µl
Nuclease-Free Water	to 25 µl

Reaction setup to perform a PCR with Taq DNA Polymerase:

Component	**for 25 µl reaction**
10X Standart *Taq* Reaction Buffer	2.5 µl
10 mM dNTPs	0.5 µl
10 µM Forward Primer	1.25 µl
10 µM Reverse Primer	1.25 µl

Template DNA	2 µl
Taq DNA-Polymerase	0.25 µl
5X Q5 High GC Enhancer	5 µl
Nuclease-Free Water	to 25 µl

Thermocycling conditions for a routine PCR with Master mix mentioned above:

Step	**Temperature**	**Time**
Initial Denaturation	95 °C	3 min
30 Cycles	95 °C 60 °C (primers in MM: *Hind*III/*EcoR*I) or 58 °C (primers in MM: DTF/DTR) 68 °C	30 sec 45 sec 2 min 50 sec
Final Extension	72 °C	7 min
Hold	4-10 °C	-

Initially, various Master Mix Compositions were tested under a range of PCR conditions. This involved adjusting the timing for different steps (denaturation, annealing, and extension temperatures), as well as the proportions of ingredients in the master mix. During the mixing process, all components, excluding the polymerase and primers, were combined. Subsequently, the polymerase was added and gently mixed. In the final step, both the forward and reverse primers were introduced into the master mix. PCR reagents and enzymes were stored in accordance with the manufacturer's recommendations. Primers were diluted with freshly autoclaved water at a 1:10 ratio and stored at –20 °C. Genomic DNA was similarly diluted to 1:10 using nuclease-free water.

6.1.5 Gel Electrophoresis

To assess DNA quality and fragment size, agarose gel electrophoresis was performed. DNA samples were mixed with 6× purple gel loading dye (Thermo Scientific™) and loaded onto a 0.8% agarose gel prepared with GelRed® for nucleic acid staining. To estimate the size of DNA fragments, 1 µL of GeneRuler 1 kb DNA ladder (Thermo Scientific™) was included as a molecular weight marker. Electrophoresis was carried out at a voltage appropriate for the gel size—typically 80–100 V for 40 minutes for smaller gels, and up to 120 V for larger gels. DNA

bands were subsequently visualized under UV light using the ChemiDoc™ Touch Imaging System (Bio-Rad).

6.1.6 DNA Recovery from Gel

During the experiment, DNA isolation and purification from a gel were performed. DNA fragments of interest were excised from an agarose gel using a sterile scalpel. The gel slice containing the DNA was then placed in a microcentrifuge tube and subjected to gel extraction using the Zymoclean™ Gel DNA Recovery Kit from ZYMO RESEARCH, following the manufacturer's instructions. The purified DNA was eluted in an appropriate buffer and used for downstream applications. Additionally, the gel pieces not used for gel recovery after the cut-out were stored at -20°C for potential future analysis.

6.1.7 Ligation

In this experiment, the T4 DNA ligase enzyme obtained from New England Biolabs GmbH was used to modify the *cmdD*_PCR_cut sample, leading to the formation of the PSOK_*cmdD*_KN vector. This vector was specifically designed to deactivate a gene within cluster 19 in the *Streptomyces* sp. S 4.7 wild-type strain.

Table 8: Ligation protocol

Component	**Volume in µl**
Vector	1
Insert	7
T4 ligation buffer 10x	2
T4 DNA Ligase	1
ddH_2O	Up to 20

To initiate the experimental procedure, genomic DNA was extracted from *Streptomyces* sp. S 4.7. Subsequently, the *cmdD* gene was selectively amplified using primers produced for this purpose. The resulting amplified product was then subjected to restriction enzyme digestion, using *HindIII* and *EcoRI* enzymes. After the digestion process, the resultant fragment was labeled as *cmdD*_PCR_cut. These steps were performed to generate the necessary starting material for following analyses.

6.1.8 Preparation of Competent Cells

Competent cells were used to perform experiments like conjugations or transformations. There are many different methods to create competent cells of E. coli XL Blue and DH5alphaF`.

6.1.8.1 Preparation of Competent *E. coli* Cells by Calcium Chloride Method

Competent cells were created according to a protocol: a single colony was chosen and picked with a sterile toothpick from a LA plate with *E. coli* and placed into a bottle with 2 mL of LB to grow over night in an incubator at 37 °C with 200 rpm. 0.5 mL of this overnight culture was added to a bottle with 50 mL of LB and incubated. After 2 hours of incubating the OD_{600} of this culture was monitored by a UV/VIS spectrophotometer. If the OD_{600} reached 0.4-0.5, this culture was cooled on ice. After that it was transferred into a sterile Falcon tube and centrifugated at 6000 rpm for 8 min at 4 °C. Supernatant was discarded and the cells (remaining in a pellet) were resuspended in 20 mL of cooled 0.5 M $CaCl_2$. This culture was incubated for 20 min. Then the process of centrifugation was repeated. Supernatant was put into a waste and the cells in form of a pellet were resuspended with 2.5 mL 0.5 M $CaCl_2$ with 20 % Glycerol. Competent cell solution was divided into small tubes with each 100 µl, freezed in liquid nitrogen and stored in – 80 °C.

6.1.8.2 Preparation of Competent *E. coli* Cells for Electroporation

Big plasmids can be transformed into E. coli cells more successfully using electroporation than through chemical transformation. This protocol was followed to prepare competent *E. coli* cells: An *E. coli* overnight culture was grown in 10 mL of LB medium containing the appropriate antibiotic at 37 °C with shaking at 200 rpm. The next day, 1 mL of the overnight culture was inoculated into 100 mL of fresh LB medium with the same antibiotic and incubated at 30 °C for 3–4 hours at 200 rpm, until the optical density at 600 nm (OD_{600}) reached approximately 0.4–0.6. The culture was then split into two sterile 50 mL Falcon tubes and centrifuged at 4000 rpm for 5 minutes at 4 °C. The supernatant was carefully discarded, and the resulting pellet was resuspended in 40 mL of ice-cold sterile 10 % glycerol. This washing step was repeated twice: the cells were centrifuged again, resuspended in 30 mL ice-cold 10 % glycerol, centrifuged once more, and finally resuspended in approximately 500 µL to 1 mL of 10 % glycerol. The competent cell suspension was then aliquoted into 50 µL portions, snap-frozen in liquid nitrogen, and stored at –80 °C until further use.

6.1.9 Transformation of Exogenous DNA and Conjugation

In this work, genetically modified material was transferred through conjugation from a donor bacterium to a recipient bacterium. Conjugative plasmid DNA was transformed to *E. coli* ET12567/pUZ8002. For a successful conjugation, the best conditions had to be found. For this reason, many approaches with different methods were tested. 20 to 30 plates (SFM and ISP2 media) were produced for each conjugation experiment. Other variations included using different plasmid vectors, spreading the plates with antibiotics after 2 days instead of 1 day, or maintaining the plates at different temperatures. The conjugation of *Streptomyces* sp. S 4.7 had been carried out following a protocol. Initially, plasmid DNA (pDNA) was transformed into *E. coli* ET12567/pUZ8002 cells. Four colonies of E. coli ET12567/pUZ8002 cells transformants were selected and spread onto plates containing three selective antibiotic markers: Kanamycin, Chloramphenicol, and Apramycin. Plates were incubated at 37 °C for one day. The colonies of *E. coli* ET12567/pUZ8002 cells from the plate were carefully transferred and suspended in 500 µl of 2x YT media. Among them, the best-grown colony from the previous step was chosen for further experimentation. Next, 50 µl of *Streptomyces* spore suspension was mixed with 350 µl of 2x YT and incubated for 7 minutes at 50 °C. The reaction tube was then cooled for 5 minutes at room temperature. Following this, 400 µl of heat-shocked spores were mixed with 100 µl of *E. coli* ET12567/pUZ8002 cell suspension. The mixture was then spun down for 1 minute at 5000 rpm. Subsequently, 250-300 µl of supernatant were removed, and the rest was spread on ISP2 media without the selective marker but with 1 M MgCl2. The plates were left to dry and cool down to room temperature.

After incubating at 28 °C for 18-20 hours, plates were checked for any signs of growth. If there was growth, 1 mL of H2O was mixed with 30 µl of Nalidixic acid and Apramycin and put on the plates. The incubation plates were placed back at 28 °C for approximately 2 days.

After the incubation period, the plates were checked for growth. If colonies were present, one was picked with a sterile toothpick and then spread on new ISP2 plates with addition of Apramycin and Nalidixic acid. Incubation was for a few days at 28 °C. Finally, when sporulating colonies could be observed, the preparation of spore suspension could be completed and stored at – 80 °C.

6.2 Preparation of Media and Stock Solutions

6.2.1 Preparation of Media

During this work, a variety of different media were used. The standard procedure for media preparation involved weighing out solid chemicals according to a recipe, placing them into a bottle, and then adding the appropriate volume of water. This solution was thoroughly mixed using a magnetic stirrer. To prevent contamination and ensure result accuracy, all media were autoclaved at 121 °C for 20 minutes. After autoclaving, the media were stored at room temperature. For media containing agar, it was necessary to melt the agar by heating it in the microwave. Once melted, the media was poured into Petri dishes. Approximately 100 mL of media was sufficient to fill three Petri dishes. The melted media had to cool down to 40 °C before adding antibiotics. Agar plates were then stored in a cold room at 4 °C. The shelf life of agar media plates under these conditions is approximately 1.5 months.

6.2.2 Preparation of Media with Artificial Water

In the production of media with the addition of artificial seawater, the following procedure was followed: Reagents are dissolved in 50 % of total volume water and then autoclaved in 121 °C for 20 min. After autoclaving, remaining total volume is added as artificial water.

6.2.3 Antibiotic Stock Solutions

Antibiotics were weighed and solved with appropriate solvents (see more detailed information in table 6 under chapter 5.3.1. All antibiotic stock solution, except Thiostrepton and Chloramphenicol, were sterile filtrated with PTFE 0.22 µm. Antibiotic stocks were stored at – 20 °C.

6.3 Glycerol Stocks

6.3.1 Glycerol stocks from Spore-Forming Organisms

Spore forming bacteria were grown on respective solid media for preparation of glycerol stocks. For the *Streptomyces* sp. S 4.7 strains (wildtype and recombinant) this media was ISP2 with artificial seawater. All *Streptomyces* strains were incubated at 28°C. After sporulation was observed, 5 mL of 20 % Glycerol was added to the bacteria grown on the agar. With the help of a pipette, bacteria were scratched from the solid media and the suspension was filtrated through a cotton in a sterile syringe for separation of mycelium of the spores. Spore suspension was stored aliquoted in cryopreservation tubes at – 80 °C.

6.3.2 Glycerol stocks from Non-Spore-Forming Organisms

Bacterial test organisms as shown in table 9, which were used for the bioactivity testing, are not spore forming organisms.

Table 9: List of bioassay organisms including growing parameters (media and incubation temperatures)

Organisms	Temperature conditions	Media
Escherichia coli DH5αF	37 °C	LA
Staphylococcus carnosus	37 °C	LA
Enterococcus mundti	37 °C	LA
Pseudomonas putida	28 °C	LA
Kocuria rhizophila	28 °C	LA
Micrococcus luteus	28 °C	LA
Bacillus subtilis	28 °C	LA
Erwinia persicina	28 °C	LA
Saccharomyces cerevisiae	28 °C	YPD

6.4 Screening of Media for Secondary Metabolite Production by *Streptomyces* sp. S 4.7

6.4.1 Optimization of Fermentation Conditions

To optimize the production of secondary metabolites, various fermentation conditions and media combinations were tested using *Streptomyces* sp. S 4.7 wild-type and knock-out strains.

6.4.1.1 Pre-culture preparation

To initiate bacterial growth, 200 µL of spore suspension of *Streptomyces* sp. S 4.7 were inoculated into 10 mL of pre-culture medium. Tryptic Soy Broth (TSB) and R5 media, both with and without artificial seawater (ASW), were used as pre-culture media. Cultures were incubated at 28 °C with constant shaking at 200 rpm for 48 hours in 50 mL sterile flasks. The purpose of this step was to allow spore germination and adaptation to laboratory conditions prior to fermentation.

6.4.1.2 Fermentation cultivation

Following pre-incubation, 2 mL of each pre-culture were transferred into 50 mL of fermentation medium. Several nutrient-rich fermentation media were tested in parallel to stimulate the biosynthesis of secondary metabolites. Cultures were incubated in 250 mL baffled Erlenmeyer flasks for 7 days at 28 °C and 200 rpm. Daily visual inspections were carried out to monitor bacterial growth, pigment formation, and possible contaminations.

6.4.2 Extraction of Secondary Metabolites

After fermentation, the cultures were centrifuged to separate the **cell pellet** from the **supernatant**. Extraction of bioactive compounds was performed from both the cell pellet and supernatant using organic solvents. The crude extracts were dried and stored at –20 °C for further analysis.

Table 10: Overview of selected media and solvent of extracts of the *Streptomyces* sp. S 4.7 strains

Pre-media	Fermentation-media	Solvent of extract
R5 media	R5 media	MeOH
R5 media	CSM media with AW	MeOH
R5 media	CSM media	MeOH
R5 media	TSB media with AW	MeOH
TSB media with AW	R5 media	MeOH
TSB media with AW	CSM media with AW	MeOH
TSB media with AW	CSM media	MeOH
TSB media with AW	TSB media with AW	MeOH

To prepare samples for HPLC analysis, 50 mL of each fermentation broth were transferred into 200 mL round-bottom flasks and frozen. The frozen samples were then lyophilized (freeze-dried) and subsequently extracted with 50 mL of methanol under gentle agitation. The resulting mixture was filtered to separate the solid cell mass (pellet) from the liquid supernatant. The pellet was subjected to further extraction using 80% acetone to maximize compound recovery. The methanolic supernatant was concentrated using a rotary evaporator at 50 °C and 300 rpm. The dried residue was re-dissolved in 5 mL of methanol and stored at 5 °C. These extracts were later tested with antimicrobial bioassays to assess their biological activity against a selection of bacterial and fungal indicator strains.

6.5 Scale-up Fermentation and Peptide Extraction

For the large-scale isolation of the bioactive peptide produced by *Streptomyces* sp. S 4.7, 900 mL of fermentation broth were prepared under optimized conditions. These cultures were processed to extract and purify the antibiotic compound. There were optimizations of the method to purify the antibiotic peptide, which are described below.

6.6 Analytical Determination of Extracted Secondary Metabolites

6.6.1 Analytical HPLC

Screening device for the analytical HPLC was Shimadzu HPLC unit. Columns in use were Luna ® 5 µm C18 (2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex® and Luna ® 5 µm C18 (2) 100 Å, LC Column by Thermo Fisher Scientific® with the dimensions of 250 x 4.6 mm. Eluent A was ddH_2O and Eluent B was ACN. The method was performed with a flow of 1 mL/min with a specific gradient (see table 11).

Table 11: Gradient table for analytical Chromatography (Eluent A: ddH2O Eluent B: ACN)

Time (min)	Eluent A in %	Eluent B in %	Flow (mL/min)
0	95	5	1
45	5	95	1
55	95	5	1
65	95	5	1

6.6.2 Optimization of the HPLC method for purification of Antibiotic X

There are many approaches for optimization of HPLC methods: starting from column selection, mobile phase composition, selection of gradient and flow rate or sample loading. In this experiment, two 40 minutes isocratic methods with a flow rate of 1 mL/min and 0.5 mL/min were used with selected solvents (see table 12) for the Luna ® 5 µm C18 (2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex®:

Table 12: Concentration of solvents using Acetonitrile, Methanol, Tetrahydrofuran and ddH_2O in Isocratic method 1

ACN	MeOH	THF	ddH_2O
20 %	30 %	10 %	40 %

Table 13 Concentration of solvents using Acetonitrile, Methanol, Ammonium acetate and ddH_2O in Isocratic method 2

ACN	**MeOH**	**NH_4-Acetat**	**ddH_2O**
45 %	10 %	10 %	35 %

6.6.3 Bioactivity Assays by Disk Diffusion Method

A bioactivity test using the disc diffusion method was conducted to determine the presence of antibiotic compounds in the extracted secondary metabolites. Agar plates were prepared with 100 µl of selected test organisms (listed in table 9).

On sterile filter paper discs (OxoidTM Antimicrobial susceptibility test discs, 6 mm in diameter), the extract was added drop by drop and allowed to dry for 20 minutes. The dried filter paper discs with the extract were then placed onto the plates using sterile tweezers, gently pressed into specific positions, and marked accordingly.

As a negative control, a sample containing only methanol was used.

6.6.4 Mass Spectrometry

Mass spectrometry was done at the Institute for Analytical Chemistry, were the Mass Spectrometry Center of the University of Vienna is located. The implementation of the analysis and result was carried out by Dr. Martin Zehl.

7 Results

7.1 Characterization of Growth and Secondary Metabolite Production of *Streptomyces* sp. S 4.7

7.1.1 Sporulation conditions and morphology of *Streptomyces* sp. S 4.7

In order to determine optimal sporulation conditions, a range of solid agar media were tested: SFM, ISP2, ISP4 and SFM with 50 % commercial artificial seawater plates were inoculated with 50 µL spore suspension of *Streptomyces* sp. S 4.7 at room temperature and 28 °C. The best sporulation results were observed with ISP2 plates incubated at 28 °C. Better growth but no sporulation was observed on SFM plates.

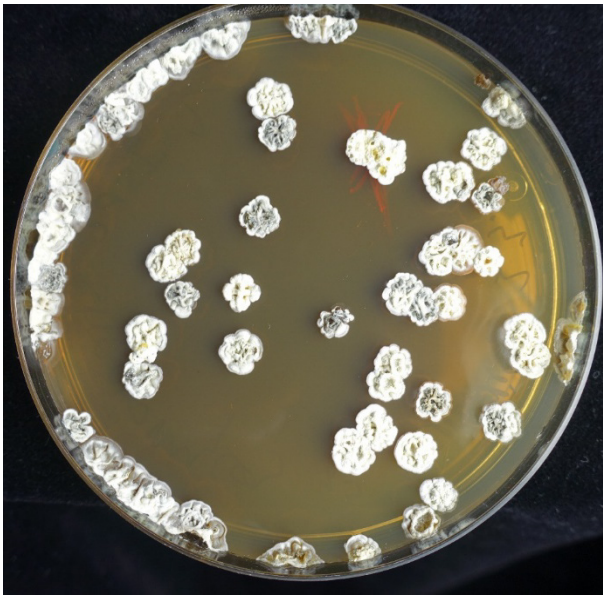

Figure 9: Morphology of *Streptomyces* sp. S 4.7 colonies on ISP2 plate

Streptomyces sp. S 4.7 colonies displayed a distinct morphology, characterised by aerial and substrate mycelium growth. Sporulation was directed upwards, forming aerial mycelium that extended above the surface of the colony, while vegetative mycelium penetrated the agar medium. The colonies were distinct, irregular, wave-shaped and the characteristic surface texture was wrinkled. Most of the colonies were dark grey in the middle and white on the outer surface of the colony. A detailed close-up (Figure 10) revealed an oily exudate on the surface of some colonies from *Streptomyces* sp. S 4.7. Some of the colonies were white or brown coloured.

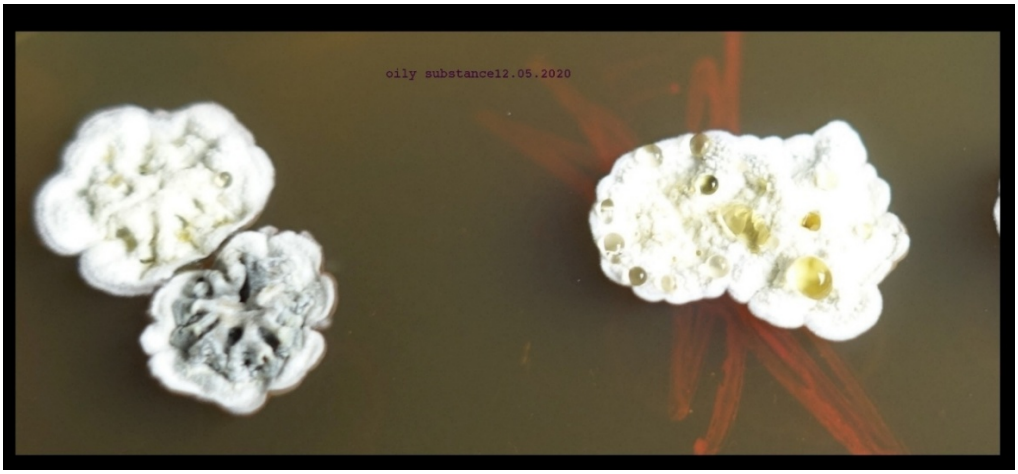

Figure 10: Detailed view of bacterial colonies displaying production of an oily exudate, possibly indicating secondary metabolites production.

7.1.2 Fermentation Conditions and Extract Preparation

To initiate the fermentation, *Streptomyces* sp. S 4.7 wild-type bacteria were cultivated in a pre-culture medium to ensure viability and adaptation to laboratory conditions. For this step, 200 µL of *Streptomyces* sp. S 4.7 spores were inoculated into 10 mL of a pre-culture medium (see 5.2.2.2 for details on R5 and TSB+AW). The pre-culture was incubated for 48 h in 28 °C and 200 rpm.

After confirming viability, 2 mL of this pre-culture were transferred into a number of fermentation media and incubated for 7 days under the same conditions. The cultures were visually checked for contamination and growth before further processing. The fermentation media were carefully selected to contain of various nutrients known to stimulate the production of secondary metabolites and therefore to yield bioactive extracts. To find the optimal conditions to stimulate secondary metabolites production of *Streptomyces* sp. S 4.7 wild-type bacteria, various combinations of pre-culture and fermentation media were tested. The resulting extracts were analysed through bioassays against different bacterial and fungal strains.

7.1.3 Proof of Principle: Bioassay-Based Detection of Bioactive Compounds in *Streptomyces* sp. S 4.7 Extracts

The crude extracts of *Streptomyces* sp. S 4.7 were tested in bioassays against selected bacterial and fungal strains. The results showed differences in bioactivity based on the pre-culture and fermentation media used. The inhibition zones (in mm) observed for each extract are presented in Tables 14/15) and represent bioactivity. *Streptomyces* sp. S 4.7 was grown with either R5 or

TSB+AW as pre-culture, then cultivated in R5, CSM+AW, CSM, TSB+AW as the fermentation-culture. These extracts demonstrated selective antimicrobial activity against Gram-positive bacteria, such as *Staphylococcus carnosus*, *Micrococcus luteus*, and *Bacillus subtilis*, while no inhibition was observed for *Pseudomonas putida* or *Saccharomyces cerevisiae*. Notably, fermentation in CSM+AW or R5 medium tended to result in stronger antimicrobial effects. As noted in Table 15, the highest bioactivities were observed when CSM+AW or R5, with S4.7 growing in TSB+AW as the pre-culture, with large inhibition zones against *Micrococcus luteus* and *Staphylococcus carnosus*.

Table 14 Bioassay results of *Streptomyces* sp. S 4.7 extracts using R5 as pre-culture medium and only medium (CSM and CSM+AW). Zones of inhibition (in mm) against bacterial and fungal test organisms are shown for each fermentation condition. "-" indicates no inhibition.

Name of Extract (Ferment. Media)	**R5**	**CSM+AW**	**CSM**	**TSB+AW**	**Only medium CSM**	**Only medium CSM+AW**
Test Organism	**Inhibition zone in mm**					
E. coli DH5α	–	13	–	–	–	–
Staphylococcus carnosus	12	15	1	11	–	–
Enterococcus mundtii	1	13	1	1	–	–
Pseudomonas putida	–	–	–	–	–	–
Kocuria rhizophila	–	11	–	–	–	–
Micrococcus luteus	12	20	12	19	–	–
Bacillus subtilis	1	20	1	–	–	–
Saccharomyces cerevisiae	–	–	–	–	–	–

Table 15 Bioassay results of *Streptomyces* sp. S 4.7 extracts using TSB + Artificial Seawater as pre-culture medium and only medium (CSM and CSM+AW). Zones of inhibition (in mm) against bacterial and fungal test organisms are shown for each fermentation condition. "-" indicates no inhibition.

Name of Extract (Ferment. Media)	**R5**	**CSM+AW**	**CSM**	**TSB+AW**	**Only medium CSM**	**Only medium CSM+AW**
Test Organism	**Inhibition zone in mm**					
E. coli DH5α	14	13	–	–	–	–
Staphylococcus carnosus	17	18	1	14	–	–

Enterococcus mundtii	13	11	1	14	–	–
Pseudomonas putida	–	–	–	–	–	–
Kocuria rhizophila	–	1	–	–	–	–
Micrococcus luteus	20	20	11	15	–	–
Bacillus subtilis	13	17	1	10	–	–
Saccharomyces cerevisiae	–	–	–	–	–	–

7.1.4 Semipreparative HPLC-assisted Purification of Bioactive Fractions

In order to identify the compound(s) responsible for the antibacterial activity, the extract was separated using semi-preparative HPLC. For this purpose, *Streptomyces* sp. S 4.7 was fermented for seven days in R5 medium, as this medium provided more homogeneous and enhanced growth of the strain. After lyophilization of the culture, the resulting pellet was extracted with methanol and concentrated using a rotary evaporator. The extract was then fractionated by semi-preparative HPLC, yielding 13 fractions that were collected according to their retention times.

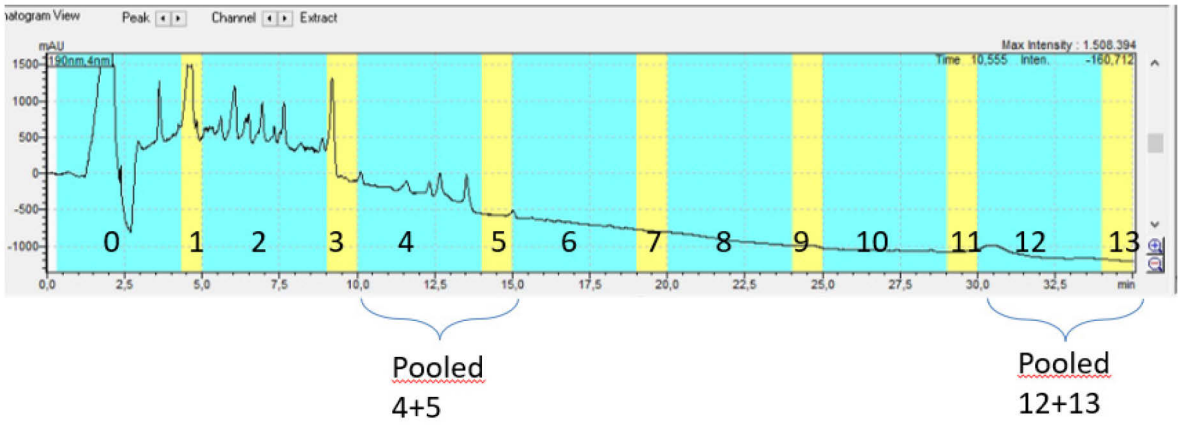

Figure 11 Semi-preparative HPLC chromatogram of the crude extract obtained from *Streptomyces* sp. S 4.7. Each fraction was subsequently evaluated for antimicrobial activity by bioassay. The retention times of the bioactive fractions are listed in Table 16.

The antimicrobial activity of the collected fractions was tested with *E. coli* and *M. luteus*. Fraction 0 exhibited the strongest activity, with inhibition zones of 12 mm against *E. coli* and 21 mm against *M. luteus*. Fraction 2 also showed a strong bioactivity: 11 mm and 18 mm. Moderate inhibition of *E. coli* was detected in fraction 4-5 (10 mm), while weak inhibition against *M. luteus* was also noted. Fraction 12-13 produced a weak inhibitory effect against *E. coli*. No activity was observed in the remaining fractions. Bioassays were conducted to identify

fractions with antibacterial activity and localize bioactive compounds based on their retention times.

e 16 Bioassay results of active fractions

Active fractions	Retention time (in min)	Bioassay result with *E. coli*	Bioassay result with *M. luteus*
0	0 – 4.4	Moderate inhibition	High inhibition
2	5 – 9	Moderate inhibition	High inhibition
4-5	10 – 15	Moderate inhibition	Slightly inhibition
12-13	30 – 35	Slightly inhibition	No inhibition

7.2 Genetic and Functional Characterization of *Streptomyces* sp. S 4.7

7.2.1 Cluster 19 analysis of *Streptomyces* sp. S 4.7 wild-type

This thesis focuses on BGC 19 from the *Streptomyces* sp. S 4.7 strain. An antiSMASH 5.0 analysis showed that the gene cluster encodes for a NRPs (nonribosomal peptide synthase). Based on previous work by Prof. S. B. Zotchev and Arthur Kaplan, and the bioassay results from this work, it was proposed that BGC 19 may be responsible for producing an antibacterial compound. Table 17 represents the list of genes, potentially involved in the biosynthesis of secondary metabolite specified by BGC 19. Among these genes, the NRPS-encoding gene S4.7_corr_29380 and a SARP-encoding gene S4.7_corr_29455 were identified and analysed further in this work.

Table 17 AntiSMASH 5.0 listed biosynthetic gene cluster of *Streptomyces* sp. S 4.7

Gene number	**Predicted Domain Architecture/Protein**
S4.7_corr_29335	hypothetical protein
S4.7_corr_29340	hypothetical protein
S4.7_corr_29345	Long-chain-fatty-acid--CoA ligase
S4.7_corr_29350	AB hydrolase superfamily protein YdjP
S4.7_corr_29355	D-alanyl-alanine synthetase A
S4.7_corr_29360	AMP-binding enzyme
S4.7_corr_29365	Multidrug resistance protein
S4.7_corr_29370	LOG family protein ORF6 in fasciation locus
S4.7_corr_29375	NRPS
S4.7_corr_29380	**NRPS**
S4.7_corr_29385	NRPS

S4.7_corr_29390	NRPS
S4.7_corr_29395	NRPS
S4.7_corr_29400	NRPS
S4.7_corr_29405	MbtH-like protein
S4.7_corr_29410	bifunctional 3-demethylubiquinone-9 3-methyltransferase/ 2-octaprenyl-6-hydroxy phenol methylase
S4.7_corr_29415	Helix-turn-helix protein
S4.7_corr_29420	Putative exporter
S4.7_corr_29425	N-carbamoyl-L-amino acid hydrolase
S4.7_corr_29430	Transposase DDE domain protein
S4.7_corr_29435	Transposase DDE domain protein
S4.7_corr_29440	Transposase DDE domain protein
S4.7_corr_29445	Flavin-dependent tryptophan halogenase RebH
S4.7_corr_29450	Flavin reductase (NADPH)
S4.7_corr_29455	**Regulatory protein SARP**
S4.7_corr_29460	hypothetical protein
S4.7_corr_29465	Putative peptidoglycan binding domain protein
S4.7_corr_29470	hypothetical protein
S4.7_corr_29475	hypothetical protein
S4.7_corr_29480	hypothetical protein
S4.7_corr_29485	Integrase core domain protein

In order to confirm that BGC19 is involved in the biosynthesis of an antibiotic compound, two genetic approaches were applied: inactivation of the S4.7_corr_29380 gene and overexpression of the SARP regulator within the cluster.

7.2.2 Inactivation of S4.7_corr_29380 in BGC 19

The knock-out mutant pSOK201_29380_KN was constructed from the wild-type *Streptomyces* sp. S 4.7 to find out if this gene cluster is responsible for the biosynthesis of antibiotically active compound in *Streptomyces* sp. S 4.7 wild-type. The inactivation of the gene cluster and thus terminating the biosynthesis of the product of this gene is possible via insertion of the vector into the gene, which is called insertional disruption. The extracts from the knock-out mutant should produce one less non-ribosomal peptide or fewer peptides compared to extracts from the

wild-type strain. Ideally, it would be the peptide that is responsible for the antibiotic effect and in the bioassays the extracts from the knock-out mutants would not have any antibiotic effect.

7.3. Construction and Verification of the S4.7_corr_29380 Knock-Out Mutant

Primers were designed to amplify a fragment in cluster 19 of *Streptomyces* sp. S 4.7 (wild type). To amplify the internal fragment of the S4.7_corr_29380 gene from the BGC 19 in a PCR reaction, the following primers were used: S47_KN_EcoR and S47_KN_Hind (see Materials and Methods).

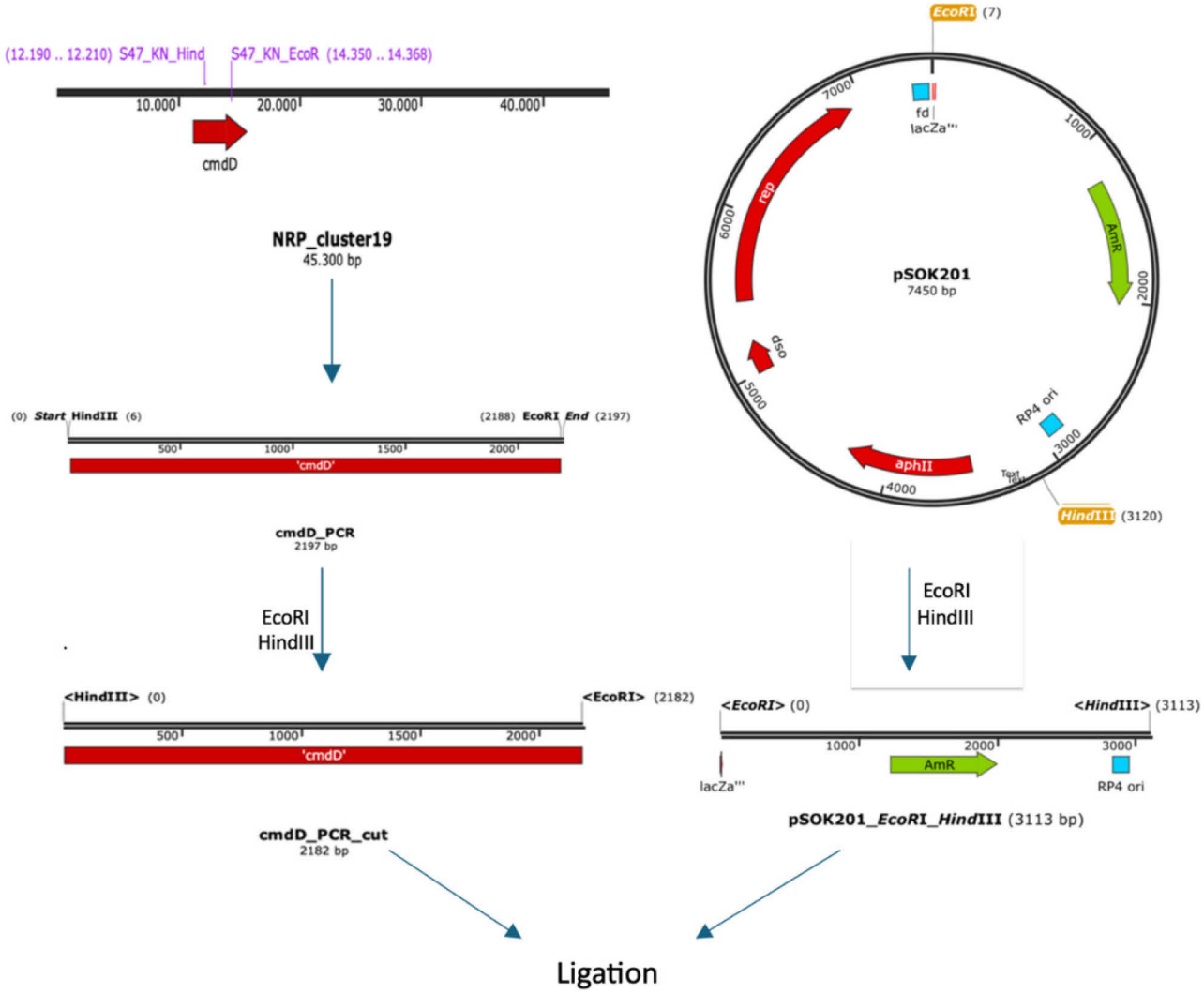

Figure 12 This is an overview of the PCR product and plasmid pSOK201 before ligation to form pSOK201_cmdD_KN construct. The replication site is indicated by 'rep', the apramycin resistance gene by 'AmR', and the origin of plasmid transfer via conjugation by 'RP4 ori'.

After amplification, the PCR product strain was analysed by gel electrophoresis to confirm the presence by size. The DNA was observed in a band of the size of ~ 2.5 kB, corresponding to the expected fragment size (Figure 13).

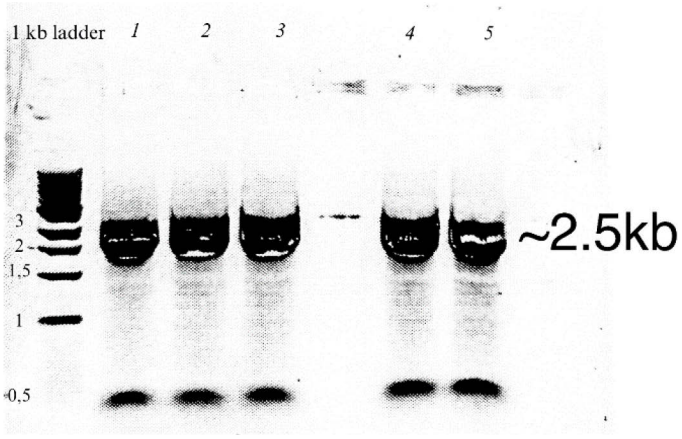

Figure 13: 1 kb ladder and PCR products (samples 1–5) separated by gel electrophoresis.

The PCR product was cut from the agarose gel band and extracted using the Zymoclean Gel DNA Recovery Kit. The purified PCR product and the pSOK201 plasmid were digested using the same restriction enzymes, *EcoRI* and *Hind*III to ensure compatible overhangs for the ligation. Following digestion, the PCR product was ligated with the 3.1 kb fragment pSOK201 vector to generate the recombinant construct pSOK201_29380 _KN. This construct carries a selective marker gene for apramycin, enabling efficient selection of recombinant strains. The 3.1 kb pSOK201_*EcoR*I_*Hind*III cassette also contains the RP4 origin, which allows conjugative transfer of the plasmid into *Streptomyces* sp. S 4.7. However, it lacks the origin of replication for the S 4.7 strain, meaning that the final construct, once inserted into the S4.7_corr_29380 gene, it cannot be further amplified in the host. This strategy helps to avoid false-positive clones and simplifies the identification of correct recombinants.

The purified plasmids were digested with the restriction enzymes *KpnI*-HF and *Nhel*-HF, and analysed by agarose gel electrophoresis to verify the presence of the correct inserts. Based on the expected restriction pattern, the recombinant construct pSOK201_29380_KN was predicted to yield bands of around 861 bp and 4434 bp. The empty plasmid pSOK201 was expected to yield bands of 3331 bp and 4119 bp. As shown in Figure 14, the test plasmid and ligated vectors (clone 4 and 5) displayed the expected bands, and were therefore selected for further experiments.

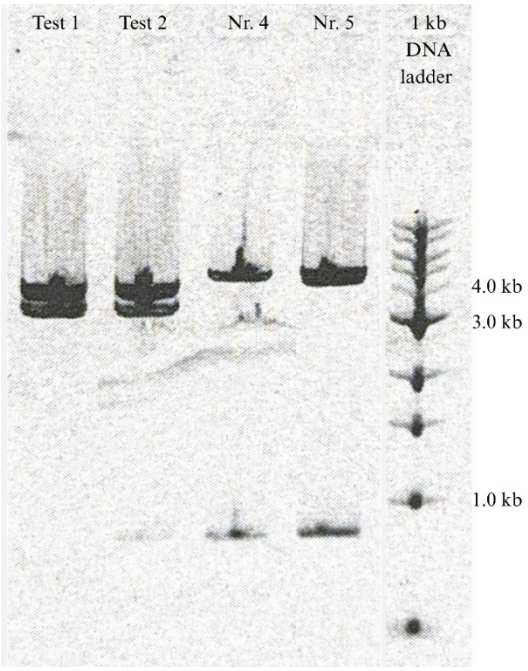

Figure 14 Restriction analysis of control plasmids and recombinant clones via gel electrophoresis. Control plasmid pSOK201 (test 1 and test 2) and recombinant plasmids (Nr. 4= clone 4, Nr. 5= clone 5) were analyzed. A 1 kb DNA ladder was used for reference. The Test 1 and Test 2 produced bands of ~ 3.5 kb and 4 kb. Nr. 4 and Nr. 5 showed bands of ~ 861 bp and 4.4 kb. This matches the recombinant construct pSOK201_29380_KN.

Constructed plasmid vector pSOK201_29380_KN was first introduced into *E. coli* ET12567/pUZ8002 (see in 6.1.9). The plasmid was transferred by conjugation (see details in 6.1.9) into *Streptomyces* sp. S 4.7. After incubation under selective conditions, several transconjugant colonies were obtained. From these, a colony (transconjugant Nr. 2) was picked for further verification, and genomic DNA was isolated for PCR analysis.

After conjugation, the insertion of the pSOK201_29380_KN into *Streptomyces* sp. S 4.7 was determined. Genomic DNA from transconjugants and wild-type strain was isolated and purified for PCR using a Qiagen kit. For this verification, primers were designed which bind outside of the DNA region targeted for insertion in *Streptomyces* sp. S 4.7: S47_KN_DTF (forward primer) and S47_KN_DTR (reverse primer). As a result, it was expected that the PCR product using the recombinant gDNA template would have a strain of ~ 9.0 kb, whereas the PCR product of wild-type gDNA was expected to be around 2.0 kb in size.

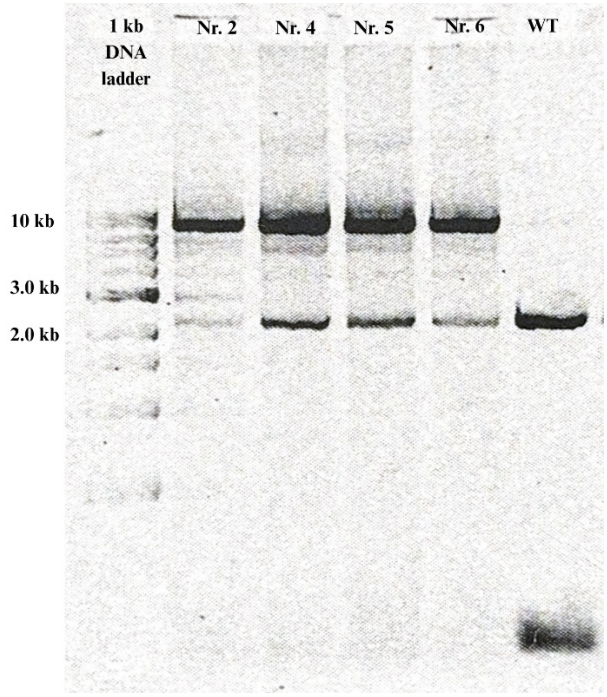

Figure 15 Verification of *Streptomyces* sp. S 4.7/pSOK201_29380_KN by PCR products via gel electrophoresis. Nr. 2 = PCR product of transconjugant Nr. 2; Nr. 4 = PCR product of transconjugant Nr. 4; Nr. 5 = PCR product of transconjugant Nr. 5; Nr. 6 = PCR product of transconjugant Nr. 6; WT = PCR product using gDNA of the *Streptomyces* sp. S 4.7 wild-type

As shown in Figure 15, the fragment amplified from the gDNA of the knockout mutant *Streptomyces* sp. S 4.7/pSOK201_29380_KN is larger (~ 9.0 kb) than fragment amplified from *Streptomyces* sp. S 4.7 wild-type gDNA (~ 2.0 kb). Several transconjugants, including transconjugant Nr. 2 (KN2), Nr. 4 (KN4), Nr. 5 (KN5), and Nr. 6 (KN6), displayed the expected band size, indicating successful plasmid integration. Among verified mutants, KN2 was selected for detailed downstream analysis due to its comparatively strongest bioactivity in first bioassays (see Supplementary S4).

7.4 Construction and Verification of the SARP Overexpression Mutant of *Streptomyces* sp. S 4.7

A detailed analysis of the genomic biosynthetic gene cluster 19 (BGC19) revealed the presence of a gene homologous to SARP. Since SARPs are known to act as positive regulators that activate the expression of secondary metabolite biosynthetic clusters, a SARP-overexpressing recombinant strain was constructed. For this purpose, the SARP gene from BGC 19 of *Streptomyces* sp. S4.7 was amplified and cloned into the multi-copy replicative vector pUWLoriT. Amplification of the SARP gene was performed using the primer pair SARP_C19_*Hind*III/SARP_C19_*Spe*I. The resulting PCR product (SARP_C19_PCR) and the

pUWLoriT vector were digested with *Hind*III-HF and *Spe*I-HF, yielding SARP_cut (~ 2.5 kb) and pUWLoriT_cut (~ 7.3 kb) cassettes, respectively. The digested fragments were subsequently ligated to generate the recombinant construct. Figure 16 presents a schematic overview of the cloning steps and the final construct, designated pUWLoriT-SARP.

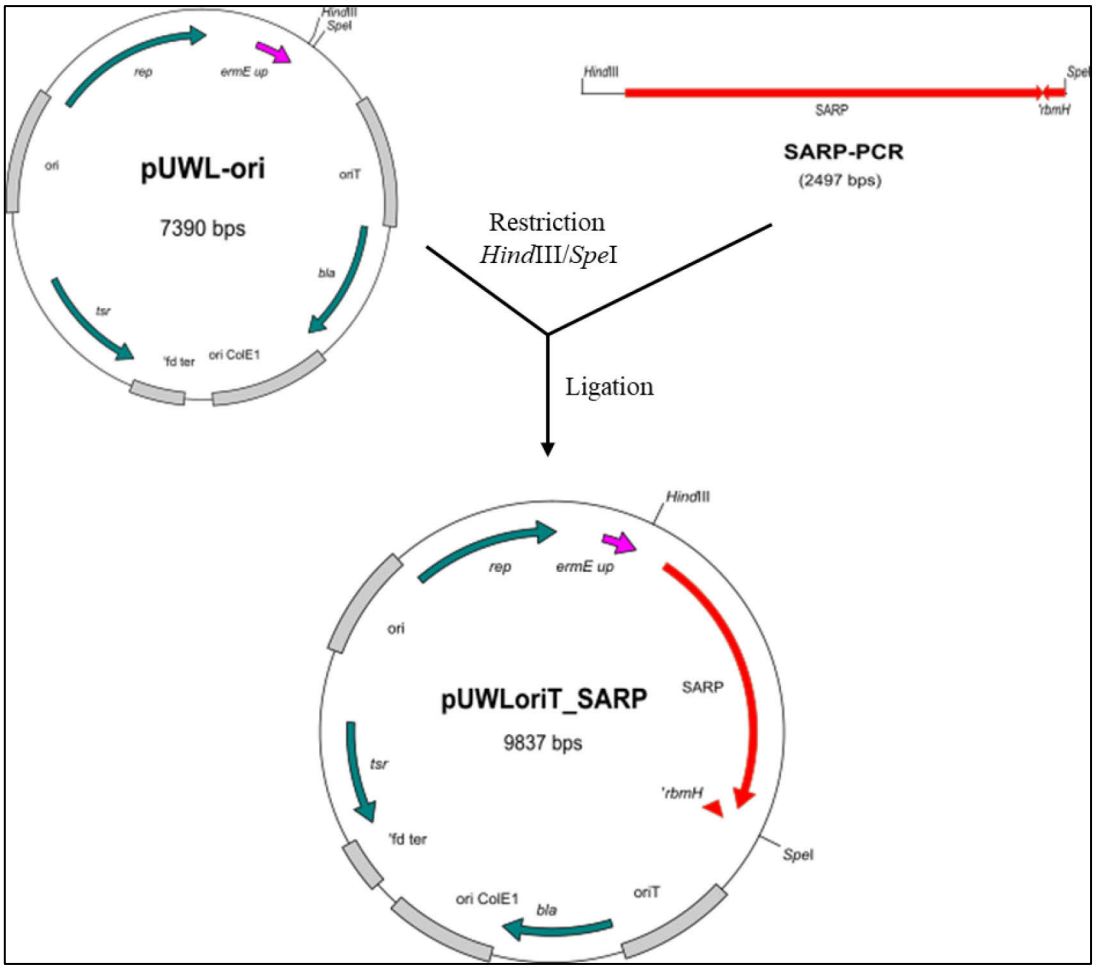

Figure 16 Schematic overview of the cloning strategy used to generate the recombinant plasmid pUWLoriT-SARP. The pUWLoriT backbone contains the following functional elements: rep – replication gene enabling plasmid maintenance in *Streptomyces*; oriT – origin of transfer for conjugation; ori and oriColE1 – origins of replication for *Streptomyces* and *E. coli*, respectively; *bla* – ampicillin resistance gene for selection in *E. coli*; tsr for selection in Streptomyces; ermE* – constitutive promoter driving expression of the inserted gene; SARP – regulatory gene from BGC19 of *Streptomyces* sp. S 4.7 cloned under the control of the ermE* promoter.

The ligation product is the SARP_pUWLoriT construct. The recombinant plasmid contains an ampicillin resistance gene, thiostrepton resistance gene, an origin for conjugative transfer, and constitutively expressed promoter for overexpression of the inserted gene. The ampicillin resistance (bla) was used for selection in *E. coli*, while thiostrepton resistance (tsr) enabled selection of recombinant strains in *Streptomyces*. The recombinant plasmids from *E. coli* strain were purified and confirmed by restriction digestion. Based on the expected restriction pattern, clone 1 and clone 3 were predicted to yield two bands of around 8.1 kb and 1.7 kb. As shown

in Figure 17, the digestion of two selected clones resulted in the expected band sizes and therefore confirming the correct ligation of the SARP insert into the pUWLoriT vector.

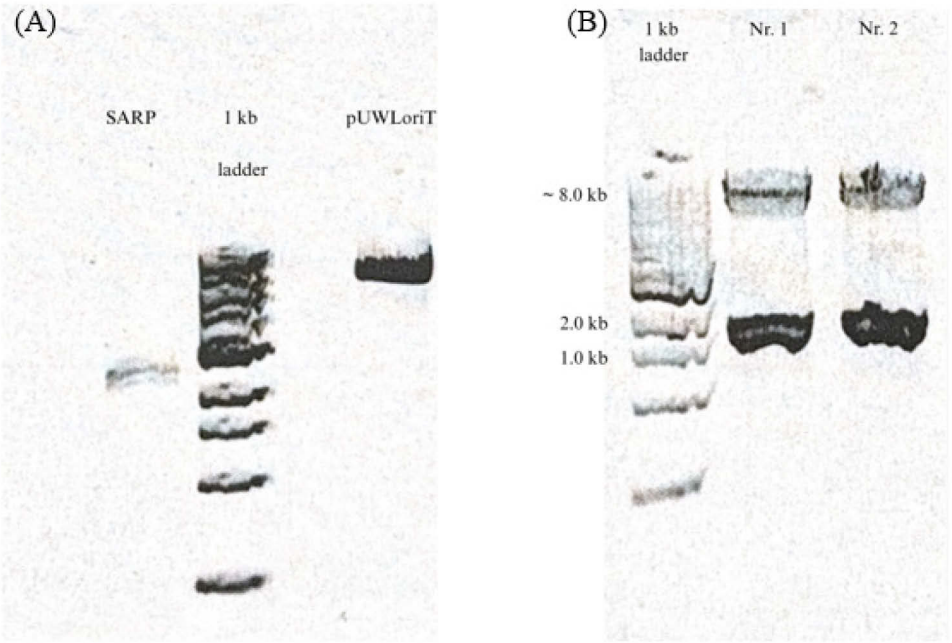

Figure 17 Agarose gel electrophoresis (A) Restriction analysis with *Hind*III/*Spe*I of SARP gene (2.5 kb), pUWLoriT (ca 7.4 kb), 1 kb DNA ladder (B) Analytical restriction of finale product pUWLoriT-SARP with *Xba*I and *EcoR*I (8.1 kb and 1.7 kb): Nr. 1 (= clone 1) and Nr. 2 (= clone 2).

Following verification of the correct construct, clone 3 (SARP-pUWLoriT construct) was transformed into *E. coli* ET12567. As a control, the empty vector pUWLoriT (without insert) was also transformed. Both strains were grown on LA plates with ampicillin, confirming plasmid presence in *E. coli* ET12567. Conjugation was then performed according to the protocol (see in 6.1.9), resulting in the appearance of transconjugant colonies that were further analysed.

7.5 Fermentation and Analytical Characterization of Metabolites

After the conjugative transfer of the knockout constructs and their confirmation via PCR, growth conditions were optimized to achieve dense cultures suitable for subsequent fermentation experiments and extract preparation. In this work, various media were tested to determine the optimal conditions for growth. Interestingly, the R5 medium, which is used in most cases as secondary metabolite-production medium, proved to be the most suitable for pre-culture development and was therefore selected as the standard pre-culture medium.

Pre-cultures of the S4.7 wild-type strain and several knockout mutants (KN2, KN4, KN5, KN6, KN7, and KN8) were prepared in R5 medium supplemented with apramycin. After four days of incubation, 2.5 mL of each pre-culture were transferred into 50 mL of CSM+AW fermentation medium without antibiotic for further cultivation. Although several knockout mutants were obtained, KN2 was chosen as the representative strain for detailed downstream metabolite analysis.

Similarly, S4.7 strains harbouring overexpressed SARP genes (S4.7_SARP1 and S4.7_SARP2) and their respective controls were also grown in R5 medium under thiostrepton selection. From these pre-cultures, 2.5 mL were inoculated into 50 mL of CSM+AW medium supplemented with thiostrepton.

After seven days of fermentation, the cultures were lyophilized, and extracts were prepared for subsequent analysis as described in Materials and Mehtods.

These methanolic extracts were analyzed by high-performance liquid chromatography (HPLC) to assess metabolite profiles and were also subjected to bioassays to evaluate antimicrobial activity.

Analytical HPLC of the culture supernatant from the strain with an inactivated S4.7_corr_29380 gene, *Streptomyces* sp. S 4.7 KN6, grown in CSM+AW medium, revealed an additional peak at 19.2 minutes (Figure 18) that was absent in the control strain *Streptomyces* sp. S 4.7 WT. This result was consistently observed for all tested KN mutants; however, the mutant KN2 showed a more abundant production of the compound corresponding to this peak and was therefore considered the main candidate for further testing, including disc diffusion assays and scale-up experiments aimed at isolating the potential new metabolite.

Apart from the additional signal, the chromatographic profiles of all strains remained comparable (Figure 18).

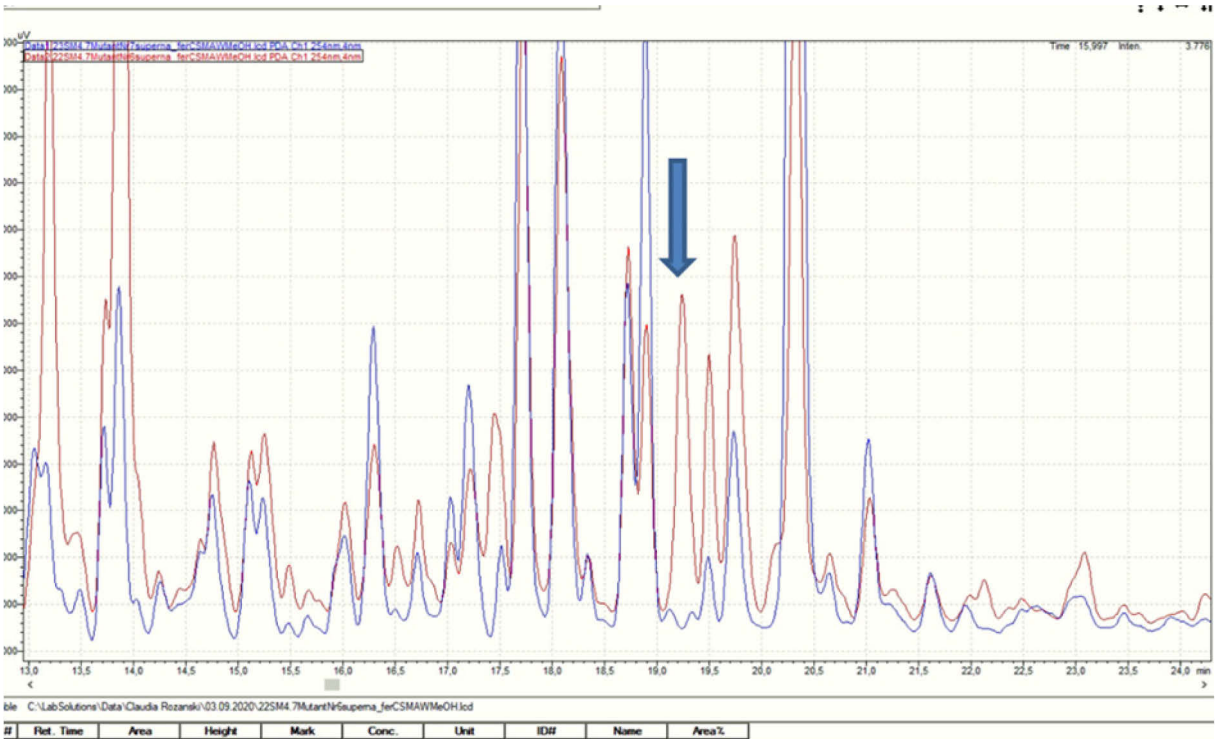

Figure 18 Analytical HPLC chromatogram of *Streptomyces* sp. S 4.7 KN2 with a inactivated *S4.7_corr_29380* gene (red) compared to the control strain (blue). The chromatogram of KN2 shows the appearance of a distinct peak at a retention time of 19.2 minutes (indicated by an arrow), which is absent in the control.

Parallel to the bioassays, all KN-extracts were analysed by LC- MS. The results showed that all strains, including the wild type, consistently produced tetronasin. Tetronasin, a polyether ionophore naturally synthesized by *Streptomyces* species, is well known for its antibacterial activity against Gram-positive bacteria. The identity of tetronasin in the extracts was confirmed based on its characteristic mass and retention time, corresponding to previously published data (Newbold et al., 1988).

AntiSMASH 5.0 analysis performed in the context of this thesis did not highlight a biosynthetic gene cluster clearly annotated as tetronasin. However, previously published genome analyses of *Streptomyces* sp. S.4.7 reported the presence of a PKS type I gene cluster putatively associated with tetronasin biosynthesis [26]. The cluster investigated in this work (BGC 19) is dominated by non-ribosomal peptide synthetase (NRPS) genes and lacks the type I polyketide synthase modules that are characteristic of polyether ionophore antibiotics such as tetronasin.

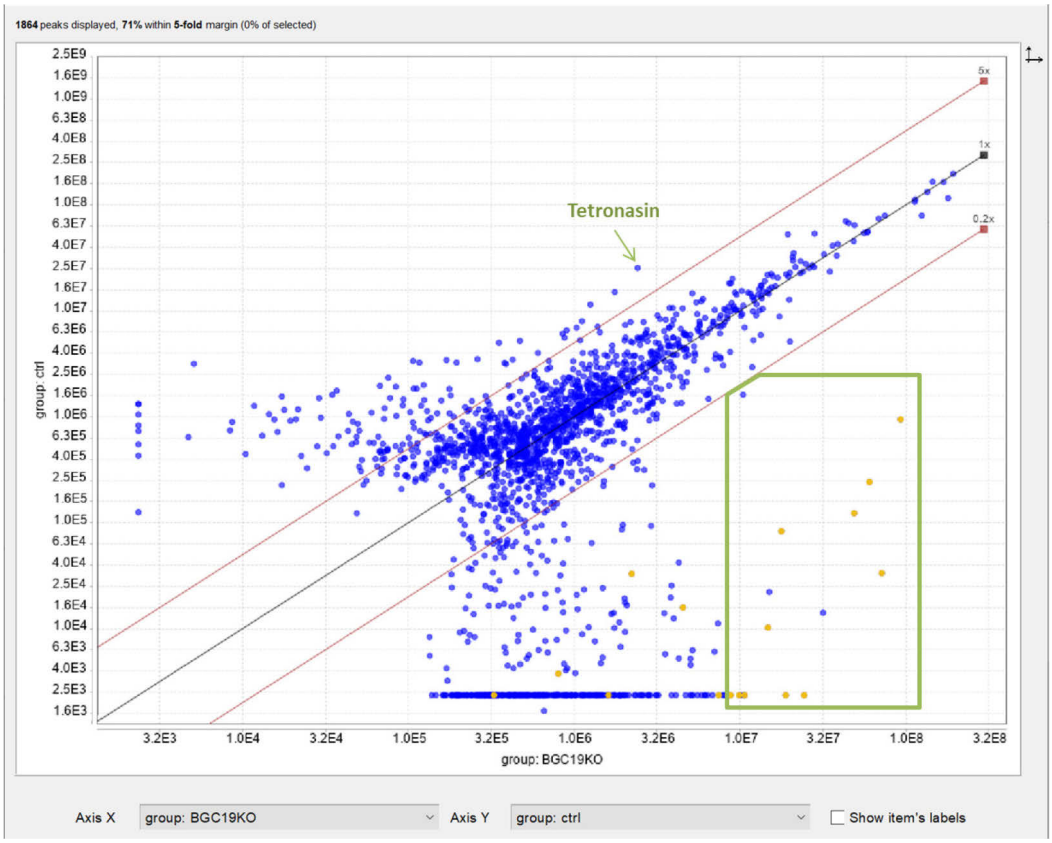

Figure 19 Scatter plot of the peak areas in the KN mutants (2, 4, 5, 6) vs. control (7, 8)

Figure 19 summarizes the LC- MS data as a scatter plot of peak areas across the knockout mutants. Notably, the analysis revealed several newly detected, yet unidentified compounds appearing only in the BGC19 knockout strains. These features, highlighted in the green box, suggest that the inactivation of BGC19 alters the metabolic profile and may activate previously silent metabolic pathways.

7.6 Preparative Scale-Up and Purification of Bioactive Compounds

Based on the HPLC chromatogram, *Streptomyces* sp. S 4.7_KN2 exhibited a dominant peak at approximately 19 minutes, suggesting a high production level of a specific metabolite. To obtain sufficient material for compound isolation and characterization, a larger-scale cultivation was carried out. In total, 18 parallel fermentations were performed, each containing 50 mL of CSM+AW medium, resulting in a combined culture volume of 900 mL. Pre-cultures were grown in R5 medium to ensure homogeneous growth prior to inoculation.

A comparative HPLC analysis was performed to evaluate metabolite production in the large-scale *Streptomyces* sp. S 4.7_KN2-FLII culture (Sample II) and, for comparison, the extract obtained from a 50 mL fermentation of the *Streptomyces* sp. KN4 strain. The chromatographic profiles of both extracts were largely comparable, except for a distinct peak observed at a retention time of approximately 11 minutes.

In this region, the KN4 extract showed a markedly higher peak intensity than KN2-FLII, indicating that the production level of the corresponding metabolite(s) was higher in KN4 (Figure 20). Nevertheless, the KN2-FLII extract was selected for further purification by flash chromatography.

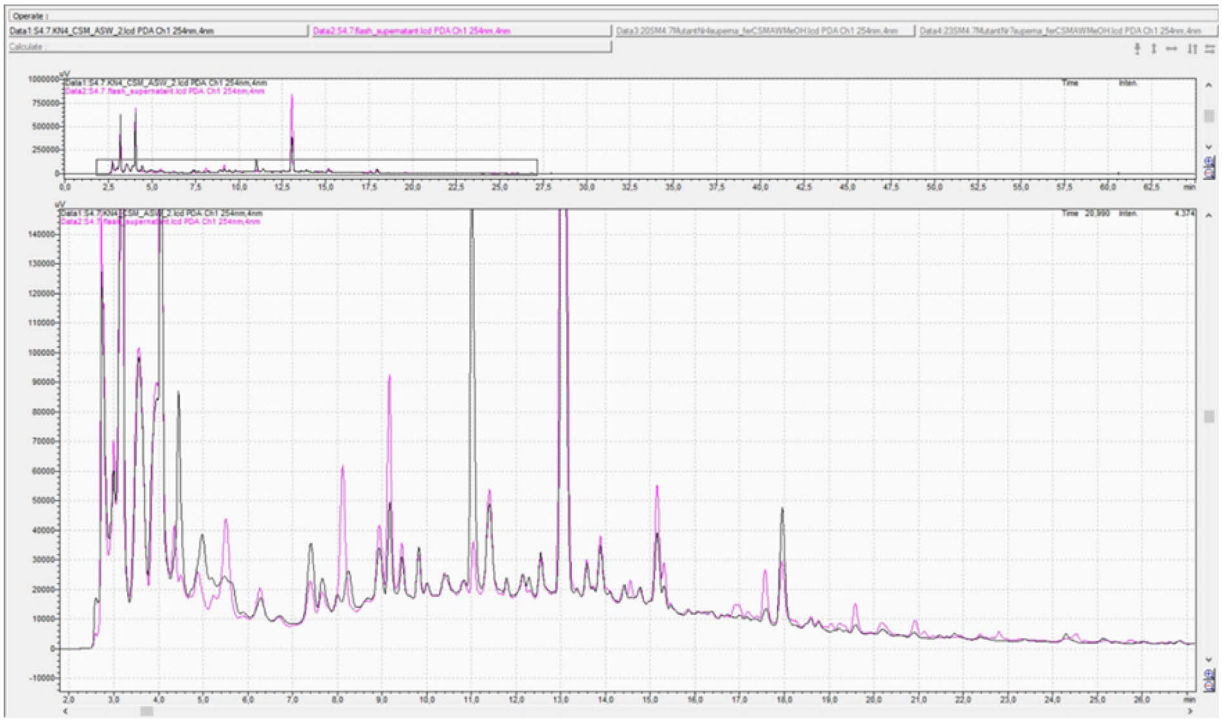

Figure 20 Comparative HPLC chromatograms of KN2 (pink) and KN4 (black) extracts.

Large-scale extraction of *Streptomyces* sp. S 4.7_KN2 resulted in approximately 9.3 g of crude extract from each portion (KN2-FLI and KN2-FLII). The obtained crude extracts were resuspended in 45 mL of methanol and subsequently subjected to antimicrobial bioassays.

As shown in Figure 21, no antibacterial activity was detected against *Escherichia coli*, while only weak inhibitory activity was observed against *Micrococcus luteus*. These results suggest that the compounds responsible for the previously observed bioactivity may be present in low

concentrations or require further purification to exhibit detectable antimicrobial effects. KN2-FLII was selected for further separation via flash chromatography.

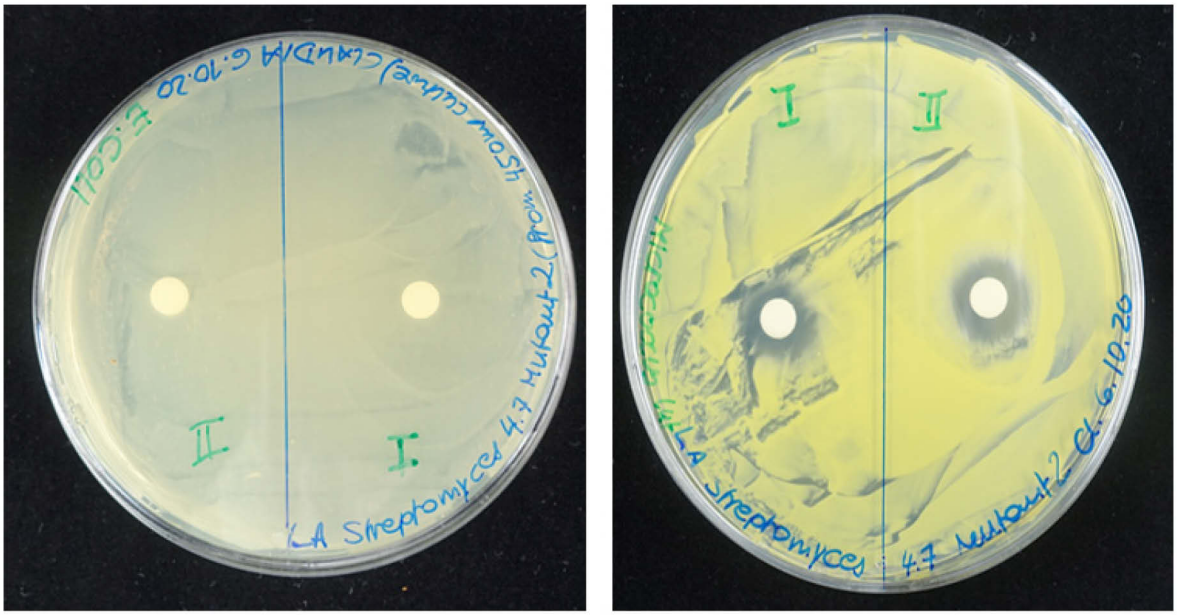

Figure 21 Bioassay of KN2 crude extracts yielded from large-scale cultivation. Bioactivity tests were performed with the two crude extract samples I (KN2-FLI) and sample II (KN2-FLII). The left plate shows the assay against *E. coli,* where no inhibition zones were observed. The right plate shows the assay against *M. luteus*, where both samples exhibited only weak antibacterial activity.

KN2-FLII was put through preparative flash chromatography and the fractions were collected according to the column volumes (CVs) (see figure 22). Chromatographic monitoring was performed using multiple detection channels, including UV scans from 200 to 600 nm and a UV signal at 220 nm. The strongest and most distinct peaks were observed in these two channels, especially during the first 10 CV of elution. A total of 19 fractions were obtained. These fractions were then subjected to bioassays to test their antibacterial activity.

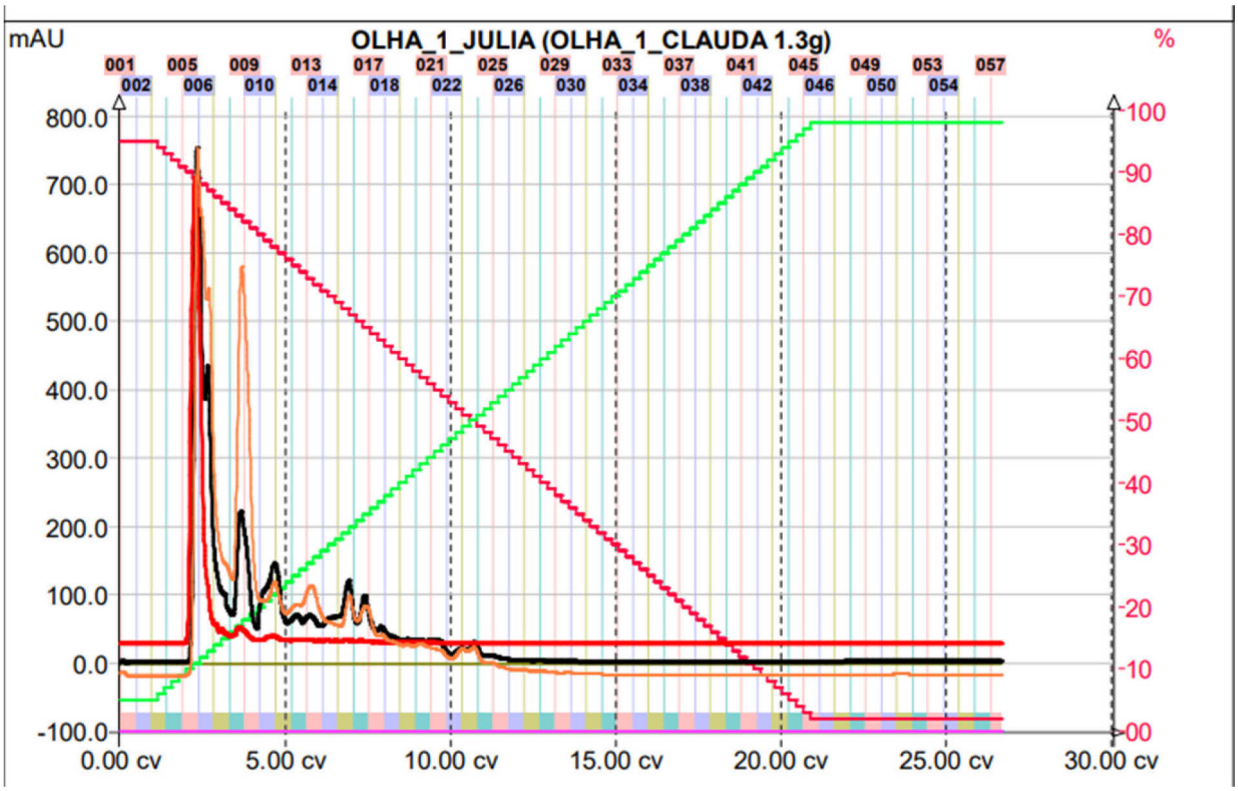

Figure 22 Flash chromatography of KN2 (Sample II) using a C18 reverse-phase column: The chromatogram shows the UV absorbance (black trace) and evaporative light scattering detector (ELSD, orange trace). Elution was performed with a gradient from 95% water (solvent A, red line) to 98% methanol (solvent B, green line). The red and green lines indicate the changing proportions of the aqueous and organic phases throughout the run.

The antibacterial activity of the flash chromatography fractions was tested against *E. coli* and *M. luteus*. Surprisingly, fractions 19 and 20 exhibited weak inhibition zones measuring 10 mm and 9 mm. By contrast, *M. luteus* was more sensitive to the fractions, with several showing clear inhibition. Fraction 19 displayed the strongest activity, with an inhibition zone of 25 mm. This was followed by fractions 20 (20 mm) and 28 (23 mm). Moderate activity was detected in fractions 14-18 and 21-22, while weak activity, indicated by growth disturbance rather than clear inhibition, was observed in fractions 11-13, 22-23 and 41.

Table 18 Antibacterial activity of flash chromatography fractions against *E. coli* and *M. luteus*. The inhibition zones are given in millimeters (mm). "-" indicates no inhibition; "*" indicates weak inhibition without a defined inhibition zone.

Fraction	*E. coli*	*M. luteus*
11	-	*
12	-	*
13	-	*
14	-	9
15	-	9
16	-	10
17	-	10
18	-	15
19	10	25
20	9	20

21	-	15
22	-	*
23	-	*
26	-	10
27	-	13
28	-	23
41	-	*
42	-	15

Fractions 19 and 20 were further analysed by LC–MS and compared with the crude supernatant prior to flash chromatography in order to assess the degree of purification and to support the identification of potential bioactive compounds. The chromatographic profiles of both fractions displayed a markedly reduced level of complexity relative to the original extract, indicating that the flash chromatography step successfully enriched specific components.

The LC–MS traces revealed that fractions 19 and 20 contained almost identical peak patterns, suggesting that they represent closely related elution segments of the same compound mixture. Several peaks that dominated the crude supernatant were either significantly reduced or completely absent in these fractions, demonstrating a substantial removal of non-target matrix components. This corresponds well with their relatively strong antibacterial activity against *E. coli*.

Despite this improvement in purity, the LC–MS data also showed the presence of multiple co-eluting peaks within each fraction, indicating that the compounds responsible for the biological activity are not yet fully purified. It was expected that the flash chromatography would yield more highly purified fractions; however, the overlapping profiles of fractions 19 and 20 suggest that the targeted constituents eluted over a broader retention range than anticipated. This implies that additional purification steps, such as further chromatographic

separation or more selective fractionation, may be required to achieve compound-level purity for definitive structural identification.

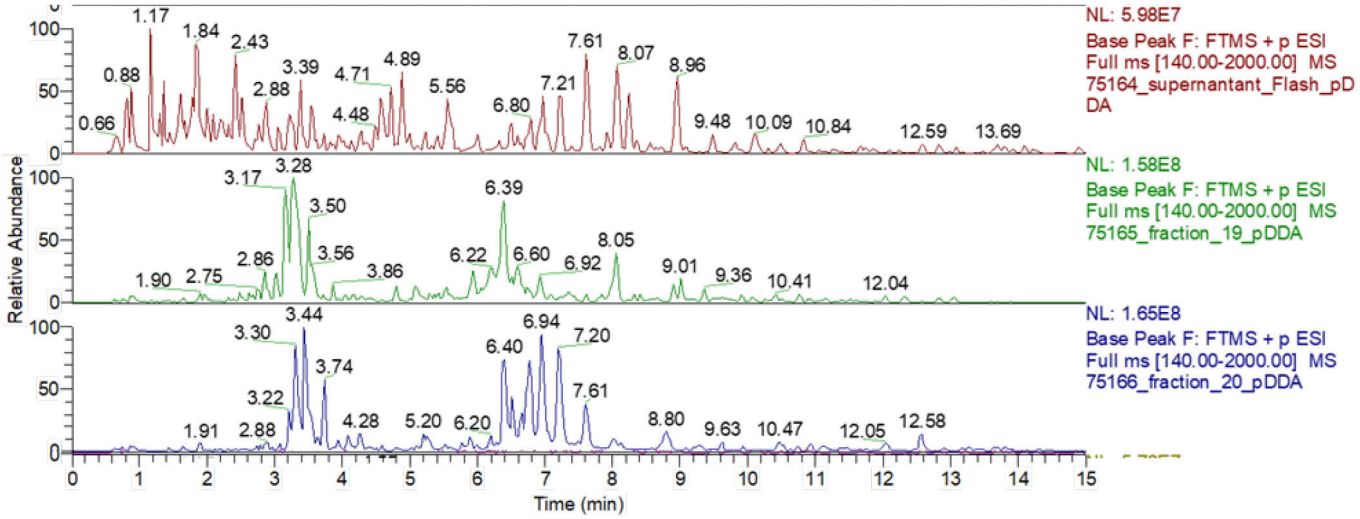

Figure 23 **LCMS analysis of the samples from 27.11.2020 – S.M4.7 BGC19KO**: Base peak chromatograms (m/z 140-2000) comparing the KN2 original sample large scale (red), fraction 19 (green), fraction 20 (blue). Data provided by Dr. Martin Zehl.

LC–MS analysis of the most active fractions revealed several distinct molecular features, indicating the presence of multiple small compounds. Several masses correspond to putative cyclic dipeptides such as Cyclo(Arg,Leu), Cyclo(Arg,Phe), and Cyclo(His,Phe), which are known to occur in microbial extracts and can exhibit moderate antibacterial properties (Rhee, 2004).

Overall, the fractions contain a mixture of identifiable and non-identifiable compounds, suggesting that the antibacterial activity may arise from one or several of these cyclic peptides or from yet-unidentified molecules. Further purification or structural analysis would be required to confirm the exact active component.

7.7 Bioactivity assessment of KN and SARP Mutants

Table 19 Antibacterial activity of the KN mutants extract against *E. coli* and *M. luteus*. The inhibition zones are given in millimeters (mm). "-" indicates no inhibition.

Extracts	**Bioassay result with *E. coli***	**Bioassay result with *M. luteus***
KN4	17	25
KN5	15	24
KN6	15	24
KN7 control	12	25

KN8 control	11	24
WT	13	24
Medium control	-	-

For all KN mutants and the wildtype strain, pre-cultures were grown in R5 medium supplemented with apramycin, and then cultivated in CSM+AW (with no supplementation of apramycin) for fermentation. The medium control was processed in the same way as the KN mutants and the wild type. 2.5 ml of R5 pre-culture medium containing apramycin was added to the fermentation medium. Therefore, the control also contained a small amount of residual apramycin.

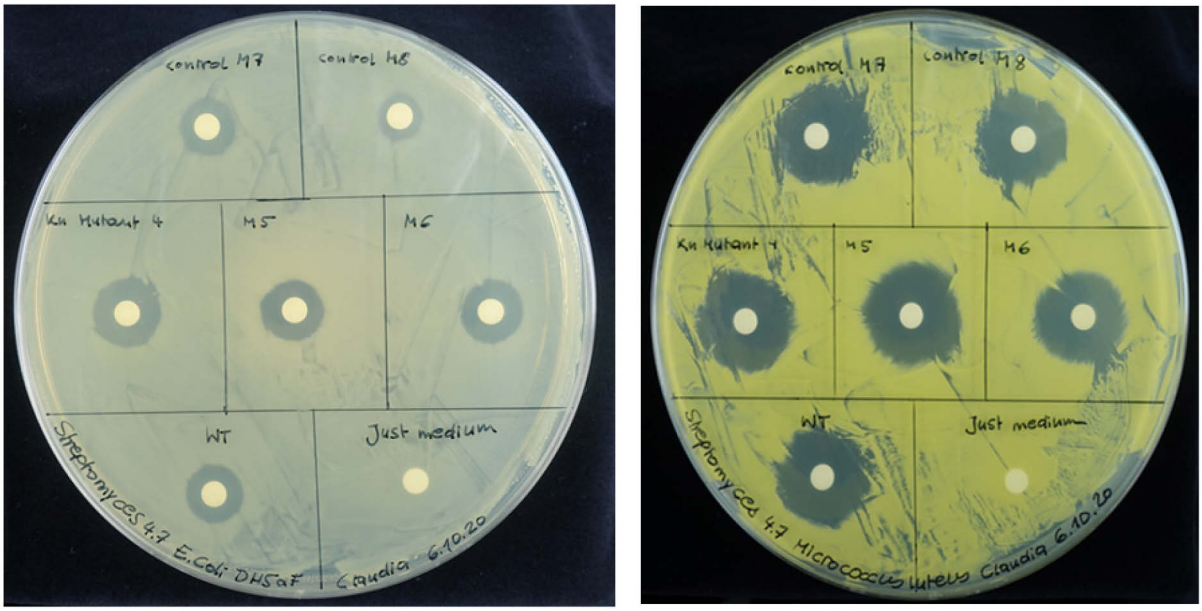

Figure 24 Bioassay plates showing antibacterial activity of the tested extracts (KN Mutant 4= KN4, M5= KN5, M6= KN6, control M7= KN7 control, control M8= KN8, WT, Just medium) against *E. coli* (showed on the left side) and *M. luteus* (right*).*

The antibacterial activity of the extracts was tested using agar diffusion assays against *E. coli* and *M. luteus* (see Table 19 and Figure 24). All the KN mutants (KN4, KN5 and KN6) exhibited a slightly higher bioactivity than the control strains (KN7 and KN8) or wildtype strain. The strongest activity was observed for KN4 (17 mm), followed by KN5 and KN6 (both 15 mm). The wildtype strain displayed moderate inhibition (13 mm), while KN7 and KN8 showed comparatively weaker activity with inhibition zones of 12 mm and 11 mm. The medium control showed no inhibitory effect. The bioassays against *M. luteus* demonstrated stronger inhibition. The KN4 and KN7 control, both exhibited an inhibition zone of 25 mm, while the other samples displayed inhibition zones of 24 mm. The medium showed no inhibition. Taken together, these results indicate that the bioactive compound is unlikely to originate from BGC 19, as inactivation of this cluster did not reduce antibacterial activity in the mutant strains.

To assess antibacterial activity, bioassays were performed using both pellet and supernatant extracts obtained from KN strains cultivated in R5 and CSM+AW media after setting pre-cultures with R5 and apramycin. The inhibition zones were tested against *E. coli DH5α* and *M. luteus*. The results are summarized in Supplementary Details S4 and S5.

For the extracts obtained from the pellet cultivated in R5 medium, inhibition of *E. coli* was only observed from KN4 (1 mm); no inhibition was detected for the other samples. Against *M. luteus*, inhibition zones of 9-18 mm were observed, with the highest values recorded for KN2 and KN4 (18 mm). When the strains were cultivated in CSM+AW medium, inhibition against *E. coli* was recorded for KN2, KN4 and KN5 at 15; 9 and 9 mm. No inhibition was detected for KN6, KN7 or KN8. Against *M. luteus*, the inhibition zones ranged from 1 to 21 mm. The highest values were observed for KN2 (21 mm), KN4 and KN5 (20 mm each), KN7 (9 mm) and KN8 (1 mm).

In R5 medium supernatant extracts, inhibition against *E. coli* was observed for KN2, KN4, and KN5 (1 mm each). There was no inhibition detected for KN6, KN7 and KN8. Inhibition against *M. luteus* ranged from 1 to 25 mm, with the largest zone observed for KN2 (25 mm).

The antibacterial activity of the extracts was tested on day 1 and again after 7 days of storage in – 20 °C. Activity remained stable for all samples except KN4, which showed a slight decrease in inhibition from 19 mm to 17 mm. The S 4.7 wildtype extract even tested slightly more bioactive after 7 days. No inhibition was observed in the medium control. A tabulated overview of all measurements is provided in Supplementary Details S6.

To evaluated the potential role of the SARP gene in antibiotic production, the antibacterial activity of extracts obtained from SARP overexpressed mutants (S 4.7_SARP1 and S 4.7_SARP2) and their respective control strains (S 4.7_SARP1 (control) and S 4.7_SARP2 (control)), were tested against *E. coli DH5α* and *M. luteus*. The strains were cultivated in different fermentation media (R5, CSM, and CSM+AW) supplemented with thiostrepton as a selective antibiotic. The results are summarized in Supplementary Details S7.

S 4.7_SARP1 exhibited moderate inhibitory activity in CSM+AW and R5 (11 mm and 9 mm), with no inhibition observed in CSM. S 4.7_SARP2 and all the control strains exhibited

no inhibition against *E. coli DH5α* and were less bioactive against *M. luteus* than S 4.7_SARP1. In the presence of thiostrepton, just medium had no inhibitory effect on *E. coli DH5α*; therefore against *M. luteus*, the inhibition zones were between 10 and 13 mm.

Although S 4.7_SARP1 and S 4.7_SARP2 were constructed using the same overexpression strategy and were expected to exhibit similar levels of antibacterial activity, the results summarised in Supplementary Details S7 revealed significant differences between the two mutants. While S 4.7_SARP1 exhibited moderate inhibitory activity in CSM+AW and R5, S 4.7_SARP2 displayed significantly lower activity under the same conditions. As both mutants were cultivated and processed in parallel and should theoretically display similar bioactivity profiles, these inconsistent results suggest that the outcome may have been influenced by experimental variability or a potential contamination event. Therefore, a repetition of the experiment would be required to verify the reproducibility of the observed activity patterns.

To confirm the reproducibility of antibacterial activity, extracts of the KN mutants and S.4.7_SARP2 were tested against *E. coli DH5α* and *M. luteus.* The results of the bioassays are listed in Supplementary Details S8.

No inhibition was observed for the KN2 extract against *E. coli*, while a moderate inhibition zone of 18 mm was detected against *M. luteus.* KN4 (I) exhibited stronger inhibition against *E. coli* (9 mm) and *M. luteus* (22 mm), whereas KN4 (II) showed stronger inhibition against both organisms (17 mm and 26 mm). These differences are likely related to variations in preculture growth (see Figure 25). No visual differences were observed in the growth of fermentation culture. S.4.7 SARP2 extract from CSM medium showed slight inhibition against *E. coli* and a 24 mm inhibition zone against *M. luteus*, whereas the extract from CSM+AW medium displayed 11 mm inhibition against *E. coli* and 20 mm against *M. luteus* (S8). The bioassay plates with the results described above are shown in Figure 26.

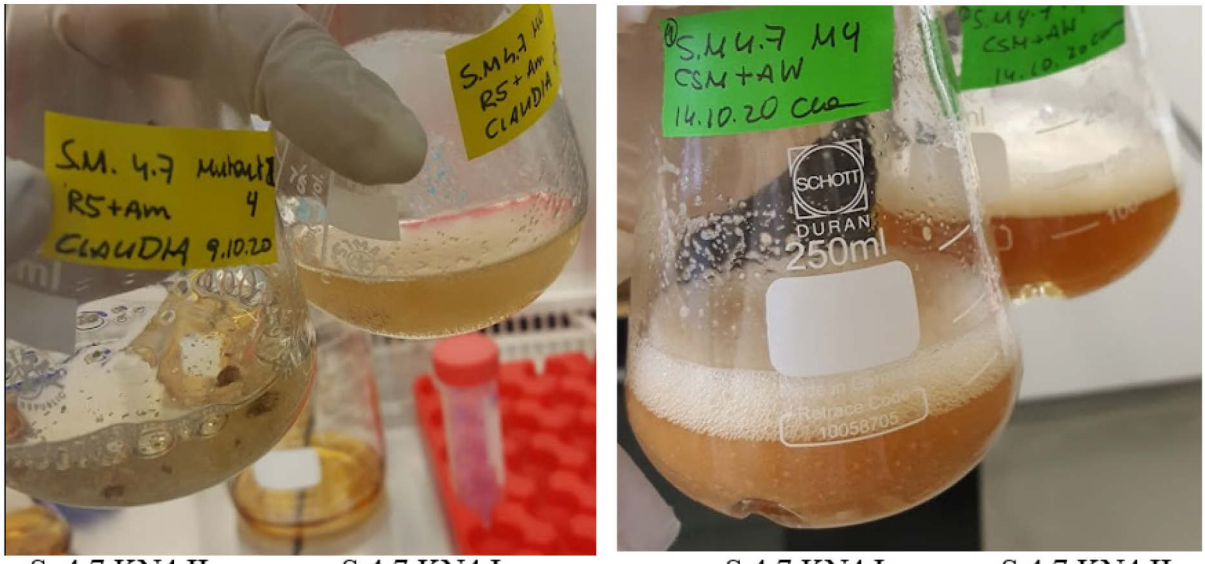

Figure 25 Comparison of KN4 (I) and KN4 (II): pre-cultures (left) and fermentation culture (right).

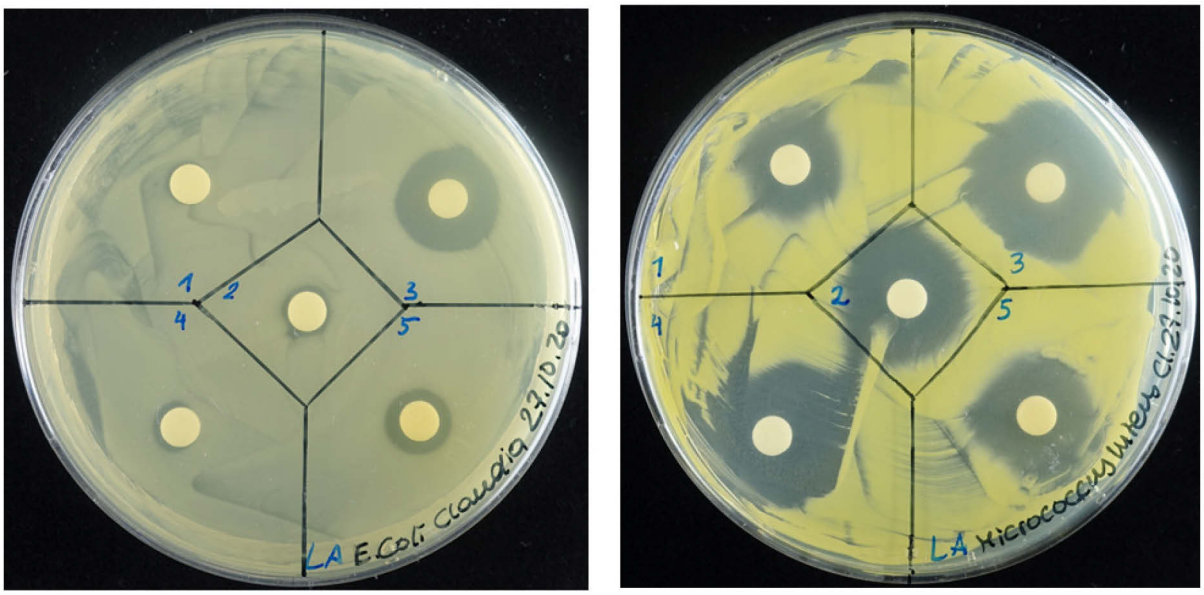

Figure 26 Bioassay plates showing antibacterial activity of the tested extracts (1 = KN2, 2 = KN4 (I), 3 = KN4 (II), 4 = S. 4.7_SARP2 grown in CSM, 5 = S. 4.7_SARP2 grown in CSM+AW) against E. coli (showed on the left side) and M. luteus (right).

8 Discussion

This work investigated the secondary metabolite production of *Streptomyces* sp. S 4.7 by combining bioassay-guided screening with targeted genetic modifications of biosynthetic gene cluster 19 (BGC 19). The aim was to evaluate whether this NRPS-encoding gene cluster is responsible for the observed antibacterial activity. In contrast to the initial hypothesis, the inactivation of the NRPS-encoding gene S4.7_corr_29380 within BGC19 did not lead to a reduction of bioactivity. Instead, knockout mutants exhibited comparable or even increased antibacterial effects compared to the wild-type strain.

Analytical HPLC data supported these findings. The knockout strains showed an additional peak at a retention time of approximately 19.2 minutes, which was not detected in the wild type. This suggests that the inactivation of BGC19 changes the metabolic profile of the strain. One possible explanation is that the inactivation of this cluster affects the balance between different biosynthetic pathways, which then leads to overall changes in secondary metabolite production. In addition, several new molecular features were detected in the LC-MS profiles of the knockout strains. These compounds were not present in the wild-type extract and may indicate that other biosynthetic pathways was activated after disruption of BGC19. However, further experiments would be required to confirm the origin of these metabolites and to determine whether they are produced by previously silent gene clusters.

The SARP overexpression mutants showed inconsistent results between independently constructed strains. Although both mutants were generated using the same strategy, they did not show the same level of antibacterial activity. This could be caused by differences in gene expression, biological variation between cultures, or possible experimental issues such as contamination during cultivation. Because of these differences, the results should be interpreted with caution, and repetition of the experiments would be necessary to confirm the observed effects.

During the fermentations, small but visible differences in culture appearance were also observed, including differences in pigmentation and growth. These variations suggest that secondary metabolite production in *Streptomyces* sp. S 4.7 is sensitive to small changes in growth conditions.

In particular, media such as R5 and CSM+AW showed higher levels of bioactivity, indicating that secondary metabolite production strongly depends on environmental conditions. This is consistent with the OSMAC approach, where different cultivation conditions can activate different biosynthetic pathways. The results suggest that *Streptomyces* sp. S 4.7 has a high potential for condition-dependent secondary metabolite production.

Bioassay-guided fractionation showed that crude extracts generally had higher antibacterial activity than the fractions obtained after flash chromatography. This suggests that the activity may not be caused by a single compound, but rather by a mixture of compounds that may act synergistically. The loss of activity after fractionation also suggests that some compounds may be unstable or require other components to show full activity.

Overall, the results indicate that BGC19 is not directly responsible for the production of a single antibacterial compound. Instead, it appears to be part of a broader metabolic network that influences secondary metabolite production in *Streptomyces* sp. S 4.7. Its inactivation seems to change the metabolic balance of the strain and may lead to the activation or increased production of other metabolites.

9 Outlook

Although this study provided important insights into the secondary metabolite production of *Streptomyces* sp. S 4.7, several aspects remain unresolved and require additional investigation. In particular, the exact role of BGC19 is still unclear. While knockout experiments did not reduce bioactivity, they led to clear changes in the metabolic profile, including additional peaks in HPLC and LC-MS analyses. These findings suggest that disruption of BGC19 may alter the regulatory balance of secondary metabolism and potentially trigger the activation of silent biosynthetic gene clusters. LC-MS data further supported this hypothesis by revealing several previously undetected compounds exclusively in knockout strains, while tetronasin was consistently produced in all strains.

Future work should therefore focus on the isolation and structural elucidation of newly detected metabolites, as well as genetic approaches to confirm involvement of silent gene clusters. The variability observed in SARP overexpression mutants further suggests the need for repeated experiments to ensure reproducibility and to better understand regulatory effects.

In addition, artificial intelligence-assisted approaches could support future studies by enabling more efficient analysis of complex metabolomic datasets, prioritization of bioactive features, and improved prediction of biosynthetic gene cluster functions. In combination with human expertise, such augmented strategies may help to better exploit the biosynthetic potential of *Streptomyces* sp. S 4.7.

Overall, *Streptomyces* sp. S 4.7 remains a promising strain for natural product discovery due to its complex and condition-dependent secondary metabolism.

10 Supplementary Details

S4

Table 4 Bioassay results of pellet extracts obtained from KN strains cultivated in R5 and CSM+AW media. The table summarizes the inhibition zones (in mm) determined in agar diffusion assay against *E. coli* and *M. luteus*. "-" indicates no inhibition.

Pellet extracts	**Fermentation media**	***E. coli DH5α***	***Micrococcus luteus***
KN 2	R5	-	18
KN 4	R5	1	18
KN 5	R5	-	9
KN 6	R5	-	11
KN 7 (control)	R5	-	11
KN 8 (control)	R5	-	-
KN 2	CSM+AW	15	21
KN 4	CSM+AW	9	20
KN 5	CSM+AW	9	20
KN7 (control)	CSM+AW	-	9
KN8 (control)	CSM+AW	-	1

S5

Table 5 Bioassay results of pellet extracts obtained from KN strains cultivated in R5 and CSM+AW media. The table summarizes the inhibition zones (in mm) determined in agar diffusion assay against *E. coli* and *M. luteus*. "-" indicates no inhibition.

Supernatant extracts	**Fermentation media**	***E.Coli DH5α***	***Micrococcus luteus***
KN 2	R5	1	16
KN 4	R5	1	18
KN 5	R5	1	13
KN 6	R5	-	1
KN 7 (control)	R5	-	20
KN 8 (control)	R5	-	-
KN 2	CSM+AW	18	25
KN 4	CSM+AW	12	18
KN 5	CSM+AW	13	21
KN 6	CSM+AW	16	20
KN 7 (control)	CSM+AW	-	1
KN 8 (control)	CSM+AW	-	-

S6

Table 6 Stability of antibacterial activity of the extracts after 7 days. The table summarizes the inhibition zones (in mm) determined in agar diffusion assay against *E. coli*. "-" indicates no inhibition.

Supernatant extract	***E. coli DH5α* (day 1)**	***E. coli DH5α* (day 7)**
KN 4	19	17
KN 5	15	15
KN 6	15	15
KN 7 (control)	12	12
KN 8 (control)	11	11
S 4.7 WT	12	13
Just medium	-	-

S7

Table 7 Bioassay results of SARP overexpressed mutants. The table summarizes the inhibition zones (in mm) determined in agar diffusion assay against *E. coli* and *M. luteus*. "-" indicates no inhibition and "*" indicated for slight inhibition of bacterial growth.

Samples	**Fermentation media**	***E. coli DH5α***	***M. luteus***
S 4.7_SARP1	CSM	-	15
S 4.7_SARP1	CSM+AW	11	21
S 4.7_SARP1	R5	9	19
S 4.7_SARP2	CSM	-	11
S 4.7_SARP2	CSM+AW	-	10
S 4.7_SARP2	R5	*	17
S 4.7_SARP1 (control)	CSM	-	8
S 4.7_SARP1 (control)	CSM+AW	-	8
S 4.7_SARP1 (control)	R5	-	-
S 4.7_SARP2 (control)	CSM	9	19
S 4.7_SARP2 (control)	CSM+AW	-	11
S 4.7_SARP2 (control)	R5	*	20
Just medium	CSM	-	13
Just medium	CSM+AW	-	12
Just medium	R5	-	10

S8

Table 8 Bioassay results of KN mutants (KN2 and two different extracts of KN4) and S.4.7_SARP2 (in CSM and CSM+*AW).* The table summarizes the inhibition zones (in mm) determined in agar diffusion assay against *E. coli* and *M. luteus*. "-" indicates no inhibition and "*" indicated for slight inhibition of bacterial growth.

Samples	**Fermentation media**	***E. coli DH5α***	***M. luteus***
KN2	CSM+AW	-	18
KN4 (I)	CSM+AW	9	22
KN4 (II)	CSM+AW	17	26
S. 4.7_SARP2	CSM	*	24
S. 4.7_SARP2	CSM+AW	11	20

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