The University of South Bohemia in České Budějovice

Faculty of Science

Searching for the polyenes in *Streptomyces* associated with bark beetles

Master thesis

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Annotation

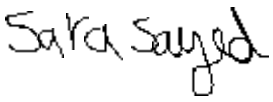
This thesis was focused on investigating unexplored sources, such as bark beetles, to find new potential producers of polyene antifungal compounds with low cytotoxicity. *Streptomyces* strains were isolated, identified, and their bioactivity and spectrum of fungal inhibition were evaluated. Additionally, data mining from polyene databases was conducted to search for specific gene markers involved in production of polyenes.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

Linz, 13.12. 2023

Sara Khaled Youssef Sayed



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Abbreviations

ACP	Acyl Carrier Protein
AMR	Antimicrobial resistance
Asp	Aspartic acid
AT	Acyltransferase
BCCO	Biology Centre Collection Organisms
BGC	Biosynthetic Gene Cluster
BLAST	Basic Local Alignment Search Tool
CAAL	*Candida albicans*
CYP 450	Cytochrome P450
DH	Dehydratase
DOBISCUIT	Database of Biosynthesis clusters Curated and Integrated
DSM	Difco Sporulation Medium
ER	Enol Reductase
GC Content	Guanine-Cytosine content
His	Histidine
KR	Ketoreductase
KS	Ketosynthase
MBS	McBeth Scale medium
MiBIG	Minimum Information about a Biosynthetic Gene cluster
NCBI	National Center for Biotechnology Information
PBS	Phosphate Buffered Saline
PKS	Polyketide synthase
PSAE	*Pseudomonas aeruginosa*
R2A	Reasoner's 2A agar
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
Ser	Serine
STAU	*Staphylococcus aureus*
TE I	Thioesterase type I

Abstract

Actinomycetes are filamentous gram-positive bacteria that are characterized by having a high GC content in their genomes. They form symbiotic relationships with various organisms and produce secondary metabolites crucial for interactions with hosts. Moreover, they serve as an important source for development of new clinical drugs as antifungal agents. Polyenes are the 2nd most frequently used antifungals. Most polyenes that have been identified so far are produced by streptomycetes. However, they have limitations, such as drug resistance, low bioavailability after oral administration, and high cytotoxicity.

The main objectives of the thesis were to explore new niches, particularly bark beetles to search for novel putative producers of polyenes or other antifungals with low cytotoxicity and to search for specific gene markers involved in the production of polyenes.

First, isolation, purification, characterization, and identification of the *Streptomyces* strains were done. Then the inhibitory activity against different indicator strains were evaluated. Promising strains were tested for inhibitory activity against fungi obtained from different environments. Second, the amino acid and nucleotide sequences of the polyene biosynthetic gene clusters of *Streptomyces* sp. strains were downloaded from databases. Phylogenetic analyses of CYP 450 and TE type I were performed to determine conservative regions and to identify the possibility of the usage of these enzymes and their coding regions as gene markers for polyene biosynthesis. The amino acid sequences of the conserved region of TE type I for polyenes were translated back to nucleotide sequences and used for designing the primers and tested them against *Streptomyces* strains using *in-silico* PCR.

The results of morphological characterization showed that some isolates were *Streptomyces*-like strains, while other isolates did not resemble actinomycetes. These results were confirmed by 16S rRNA identification. Moreover, the bioassay results indicated that most *Streptomyces* strains exhibited inhibitory activity against *Candida albicans* and inhibited β-hemolysis of *Staphylococcus aureus*. The four most promising isolates showed a weak inhibitory effect against fungal strains obtained from different areas. The analysis of cytochrome P450 of the polyene BGCs revealed the presence of two distinct groups of CYP 450 enzymes, each group demonstrates regiospecific oxidation. However, some polyene BGCs possess no cytochrome P450. Therefore, CYP 450 is not specific to polyenes and cannot be used as a gene marker. Moreover, the results of thioesterase type I gene analysis showed the presence of some domains, which are unique and specific to polyenes, but lack a high level of conservation to design specific PCR primers.

1. Introduction

Actinomycetes are aerobic filamentous gram-positive bacteria that are characterized by having high GC content in their genomes [1]. The most common and important genera are *Actinomyces, Corynebacterium, Frankia, Gardnerella, Mycobacterium, Nocardia, Propionibacterium and Streptomyces* [2] . Actinomycetes are found free in soil, freshwater, and marine environments. Moreover, they are living in symbiosis with fungi, plants, insects as well as in the gastrointestinal tract of animals and humans [3]. Natural products produced by actinomycetes play a key role in the interaction with their hosts [4].

1.1.Actinomycetes associated with arthropods.

The majority of the interactions between actinomycetes and their hosts are beneficial as actinomycetes are not only producing natural products that enable their hosts to defend themselves, their offspring and their food source against pathogens or pests, but also, they produce enzymes that break down complex natural polymers like lignocellulose into simple compounds that can serve as source of nutrients and energy for the host [4].

One of the most well-studied examples of a highly integrated symbiosis is the association between leaf-cutting ants, their mutualistic fungal cultivar, and actinomycetes [5]. Leaf-cutting genera of ants as *Atta* and *Acromyrmex* provide the mutualistic fungus *Leucoagaricus gongylophorus* with fresh leaf materials needed for their growth, producing swollen tips of fungal hyphae called gonglydia that serve as a nutrient source for the ants. Actinomycete strain of *Pseudonocardia* is hosted on specific regions of ant's cuticle for the protection of the fungal garden from potential invasion by harmful pathogens by producing antibiotics as dentigerumycin, nystatin P1 and selvamicin [4].

Similar to ants, fungi and actinomyces tripartite symbiosis, fungus-growing termites are associated with *Streptomyces*. They feed on plant material and provide their plant-containing faecal matter to their fungal cultures known as *Termitomyces* producing gonglydia that collected by termites for nourishment [5]. Although the host-associated *Streptomyces* species are producing natural products as microtermolides A and B that show inhibitory activity against the competitor fungus *Pseudoxylaria*, it is unclear if they protect the termites or their fungus against infection [4].

Moreover, different genera of female digger wasps as *Philanthus*, *Trachypus* and *Philanthinus* are cultivating *Candidatus Streptomyces philanthi* in their antennal glands to protect their offspring against fungal infections [4]. In addition to that endophytic strain of *Streptomyce*s

bacteria protects strawberry plants from gray mold pathogens and protects pollinating bees against insect pathogens [6].

Furthermore, bark beetles (subfamily of beetles Scolytinae) cultivate ambrosia fungi in a specific storage compartment known as mycangium to cover the nutritional needs for themselves and their developing larvae [4], [7]. They are also surrounded by *Streptomyces* in their mycangia and subcortical galleries, which produce antifungal antibiotics with the potential to protect themselves against competitor fungi [8].

1.2. *Streptomyces*

Streptomyces is the largest genus of *Actinobacteria* that belong to the family Streptomycetaceae and order Actinomycetales [9]. It contains 1,184 species and 73 subspecies that are identified (http://www.bacterio.net/streptomyces.html, accessed on 13th October 2023). *Streptomyces* has received the attention of researchers due to its significant role in soil ecology, its diverse phylogenetic distribution, and its production of valuable secondary metabolites [10]. It produces a wide range of bioactive compounds, including antibiotics, enzymes, anti-hypertensives, immunosuppressants and antitumor agents, making them significant in both medical and industrial applications [11]. Two-thirds of all known antibiotics are produced by *Actinobacteria*, of which the majority are produced by *Streptomyces* (Figure 1) [10].

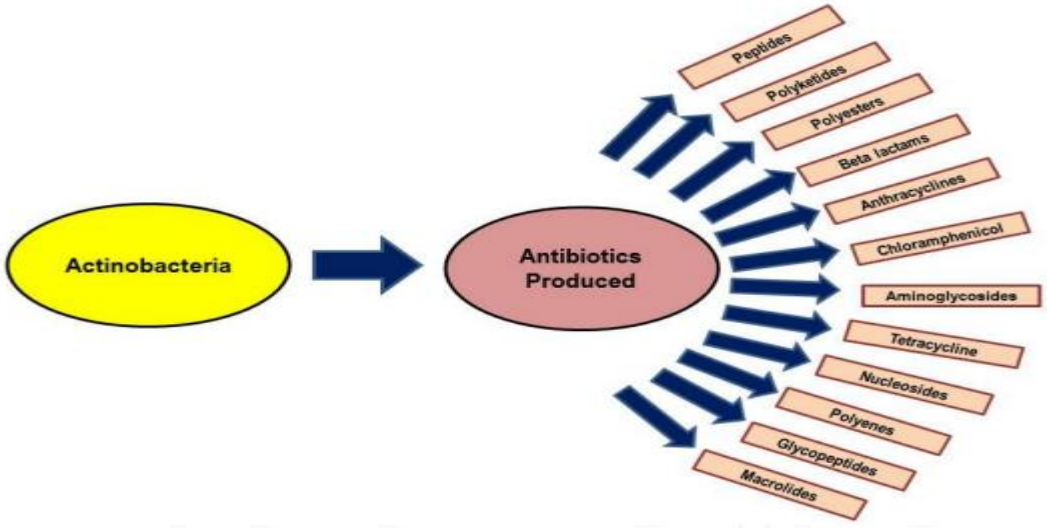

Figure 1: Antibiotics produced by *Actinobacteria* [3].

1.2.1. Genomic features and morphology of *Streptomyces*

They are aerobic filamentous gram-positive bacteria. Their genomes typically exhibit a GC content ranging from 68 to 78 mol% [12]. Streptomycetes have some of the largest genomes

compared to other bacterial species. Their genome sizes typically range from 8.7 Mbp to 11.9 Mbp [13]. The number of protein-coding sequences (CDSs) ranges between 5,361 and 11,170 [14]. The number of total biosynthetic gene clusters (BGCs) in streptomycetes genomes varies from 18 (e.g., *Streptomyces* sp. CLI2905 and *Streptomyces tendae* 139) to 53 (e.g., *Streptomyces hygroscopicus* XM201 and *Streptomyces sp*. YIM 121038), with an average of 32 [14]. Biosynthetic gene clusters (BGCs) are groups of organized genes that work together to produce secondary metabolites. Each BGC is responsible for synthesizing one or more similar compounds, which may differ mainly in terms of their strength and/or specificity of biological activities [15]. One *Streptomyces* strain can produce several secondary metabolites as it can possess several biosynthetic gene clusters (BGCs), so called one strain many compounds (OSMAC) approach [16], for example *Streptomyces* sp. SD85 (Figure 2) [17].

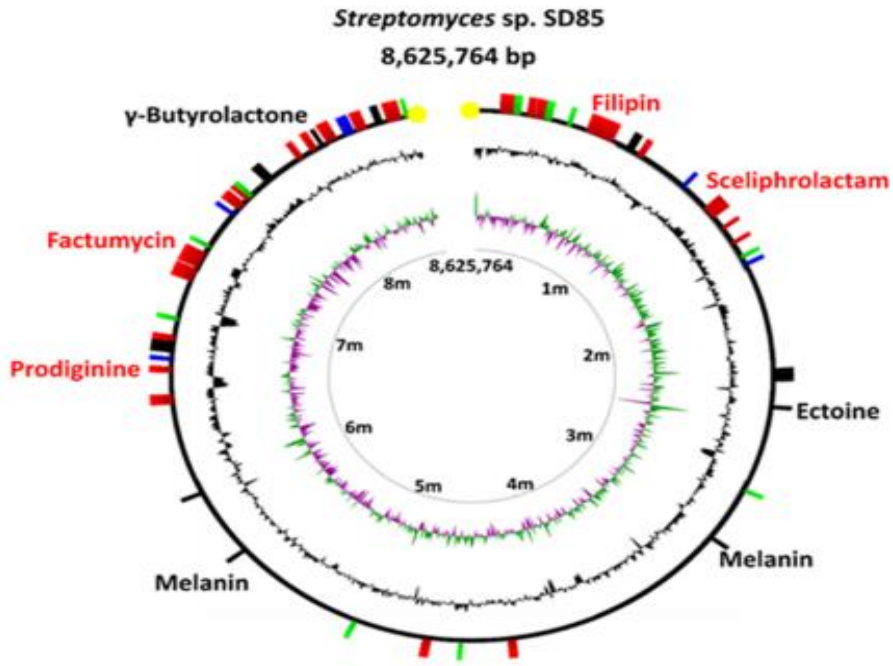

Figure 2: Circular representation of Streptomyces sp. SD85 chromosome [17].

The life cycle of the streptomyces is a complex process that composed of many stages, particularly when it is growing on solid surfaces such as agar or soil particles (Figure 3). Firstly, free spores are released into the environment. Then germination of the spores takes place, forming germ tubes that grow by branching into substrate mycelium. *Streptomyces* forms a long chain of spores after chromosome segregation and septation of the aerial mycelium [1], [12]. Substrate mycelium is responsible for absorption of nutrients [1], while aerial mycelium is responsible for production of reproductive spores [10].

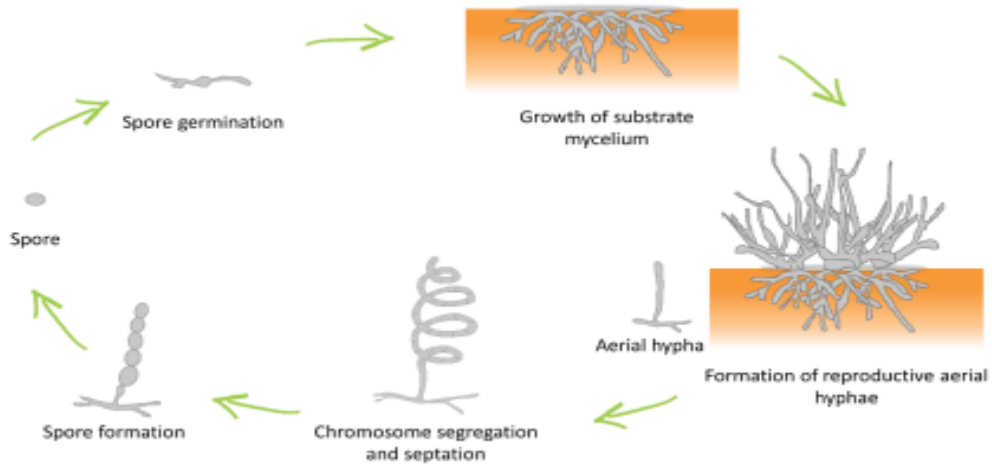

Figure 3: Schematic diagram of the Life cycle of *Streptomyces* [12].

1.2.2. Secondary metabolites produced by *Streptomyces* and their ecological importance

Secondary metabolites are small organic molecules that are mainly classified into volatile and soluble compounds. Geosmin is one of the volatile compounds, which is produced by *Streptomyces* and responsible for the earthy smell of the soil after rain [18].

Although secondary metabolites are not essential for bacterial growth, they have important ecological roles (Figure 4). Soil bacteria including *Streptomyces* produce antimicrobial compounds that have different bioactivities based on their concentration. At inhibitory concentrations, they can serve as microbial warfare to inhibit the growth of the other microbial competitors that are growing in the same niche. However, if they are produced at subinhibitory concentrations, they act as signaling molecules that have different cellular functions as cell development, biofilm formation, inducing cell motility, and nutrient utilization. Secondary metabolites with antimicrobial properties have the potential to stimulate sporulation and provide protection against predators by knocking their genes that are involved in antibiotic production, thus decreasing the growth rate on prey cells. As mentioned above, secondary metabolites as antibiotics also play a significant role in protecting hosts against infections [18].

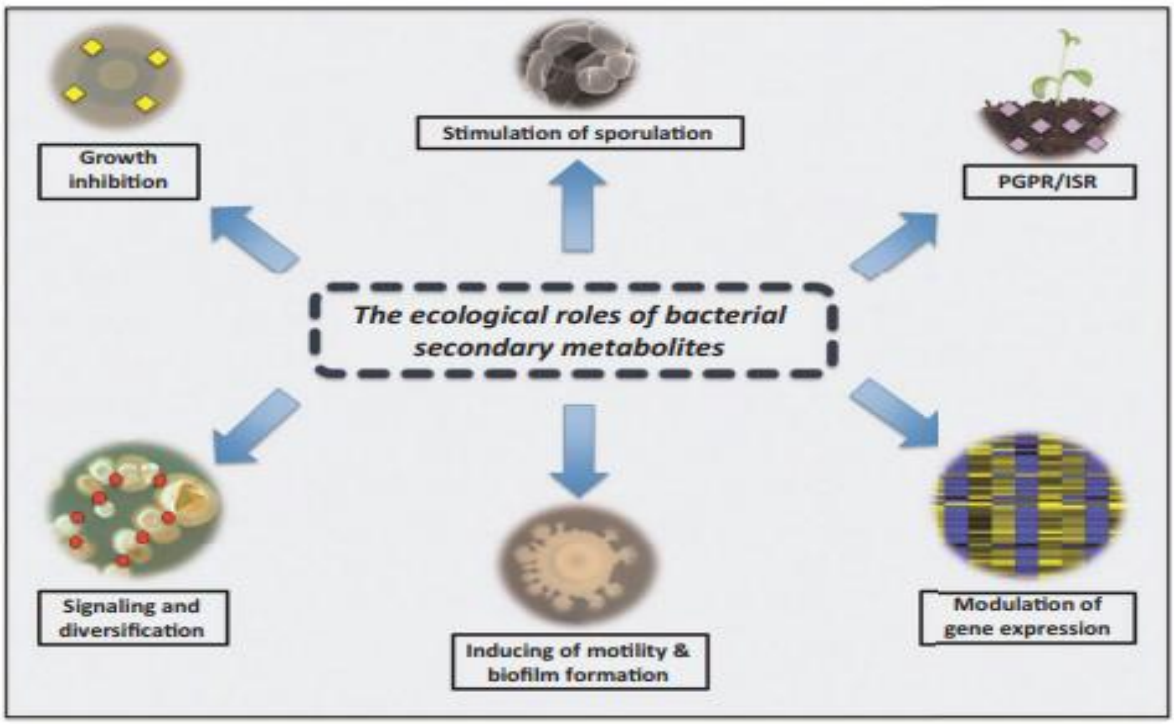

Figure 4: Schematic diagram for the ecological importance of bacterial secondary metabolites [15].

Polyenes antibiotics are one of the secondary metabolites that have antifungal activity [1]. The majority of polyenes identified so far are produced by soil filamentous actinomycetes of the *Streptomyces* genus [19].

1.3. Polyene antibiotics

1.3.1. History of polyenes as antifungal Drugs

Nystatin is the first polyene macrolide antifungal antibiotic. It was discovered in 1950 by Hazen and Brown and named fungicidin. Since then, over 90 different members of this group have been identified and more are being discovered each year. Moreover, Amphotericin B was discovered in the 1950s and was available on the market in 1957. It is considered the standard treatment for severe invasive fungal infections [20].

1.3.2. Polyenes structures

Polyenes are compounds that have polyketide chains with 3-8 conjugated double bonds and can have either linear or cyclic structures (macrolactam and macrolactone) [19]. They can be classified based on the number of conjugated double bonds into trienes, tetraenes, pentaenes, hexaenes, and heptaenes [20]. The main structural characteristics of polyenes include the presence of a hydrophobic polyene region combined with a hydrophilic polyol portion, which is often glycosylated [19].

Linear polyenes polyketides (LPPs) are one of the crucial members of the polyene family [21]. Typical LPPs such as mediomycin A, mediomycin B and clethramycin show potent antifungal activity. These three polyenes are produced by streptomycetes, and they have similar structures: the same polyene scaffold with the same numbers of polyols in the same positions. However, they differ by the moieties linked to the central structure: clethramycin possesses guanidine moiety instead of amino group and mediomycin B has no sulfate moiety (Figure 5) [22].

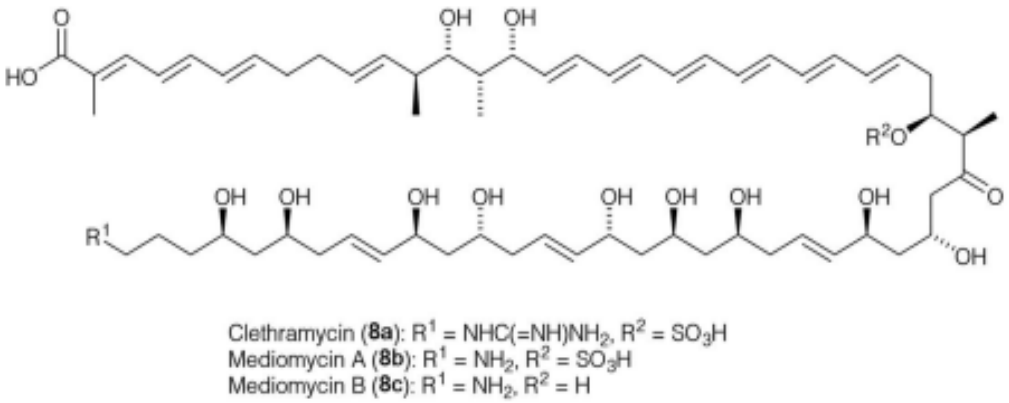

Figure 5:Structures of clethramycin and mediomycins [19].

Macrolactam polyenes are a group of macrolactam family that contain a conjugated carbon skeleton and a nitrogen-containing moiety that forms a ring via lactamization. Macrolactam polyenes can be further classified into two subgroups based on the different β-amino acid starter units (Figure 6). Both β-amino acid starter units are synthesized from L-glutamate. The first subgroup uses 3-methylaspartate (3-meAsp) as the starter unit, while the second subgroup utilizes 3-aminobutyrate [17].

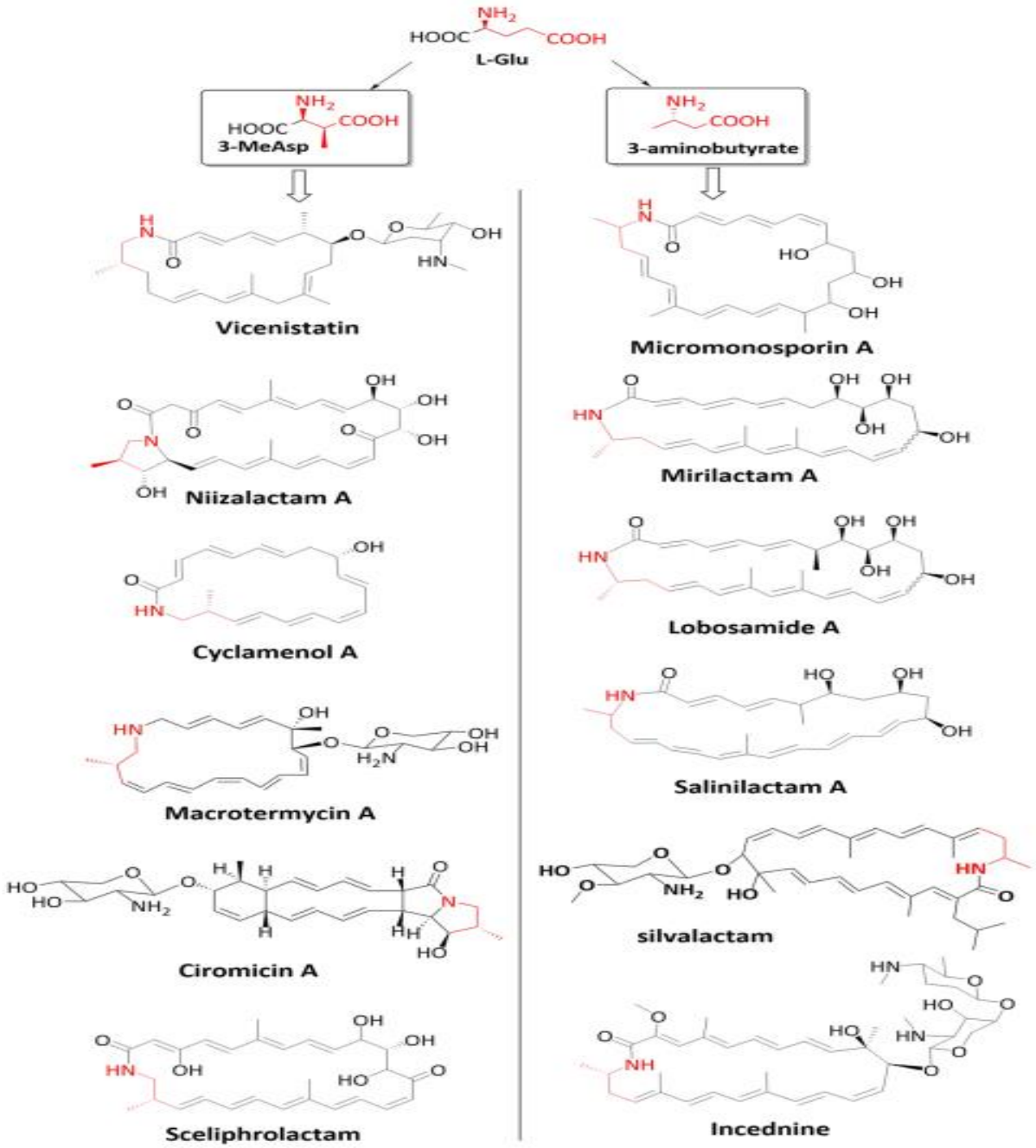

Figure 6: Structures of polyene macrolactams that are classified based on the β-amino acid starter unit.

Most of these polyene macrolactams are produced by *Streptomyces* genus, except for micromonolactam and macrotermycin A, which are produced by different *Actinobacteria Micromonospora* and *Amycolatopsis* strains, respectively [17].

Polyene macrolide antibiotics are strong antifungal secondary metabolites that are commonly used in human therapy nowadays [23]. They typically consist of a large ring made up of 20-40 carbon atoms, linked to a deoxysugar called mycosamine via a β-glycosidic bond and exocyclic

carboxy group (Figure 7) [23], [20]. They have four to eight conjugated carbon double bonds that constitute a part of their macrolide ring structure and a series of hydroxyl groups in positions opposite to double bonds [20] that form a ring via lactonization [19].

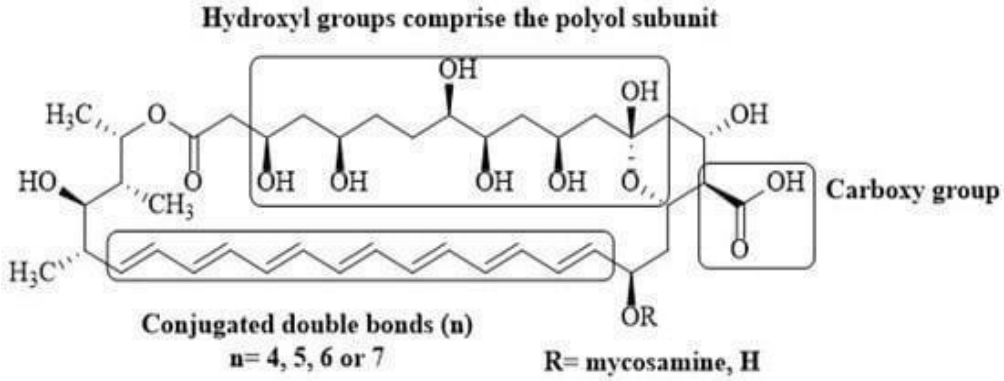

Figure 7: Common structure of polyene macrolide antibiotics [17].

1.3.3. Mechanism of action

The activity of the polyene antibiotics is closely linked to their structure, as their amphipathic structure enables them to penetrate the cytoplasmic membranes of different organisms [19]. The polyene chain is oriented towards the lipid environment, while the polyol chain faces the interior of the pores (Figure 8) [24]. This interaction can result in either irreversible destruction of the membrane or transient and reversible channel formation [19].

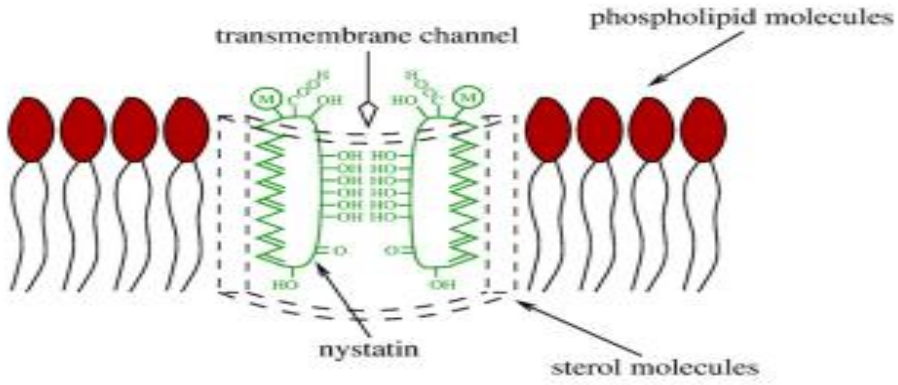

Figure 8: Schematic diagram for the orientation of nystatin in the cytoplasmic membrane forming transmembrane channel [22].

1.3.4. Biosynthesis of polyenes

Polyenes biosynthesis is a complex process involving multifunctional enzymes known as type I polyketide synthases (PKSs). Type I PKS are organized into modules, which consist of several domains (Figure 9). Polyenes are synthesized through three main steps: loading of the starting molecule, chain extension and termination of the final product. Many enzymatic reactions with different enzymes are involved in the process such as acyltransferase (AT), which catalyzes the attachment of the substrate (e.g., acetyl or malonyl) to the acyl carrier protein (ACP), and

ketosynthase (KS), which catalyzes the condensation of substrates attached in ACP followed by reducing of the keto ester catalyzed by ketoreductase (KR). Moreover, dehydratase (DH) catalyzes the dehydration of the compound resulting in formation of carbon – carbon double bond and releasing of water molecule and enol reductase (ER) catalyzes the reduction of the double bond in the molecule [25] .

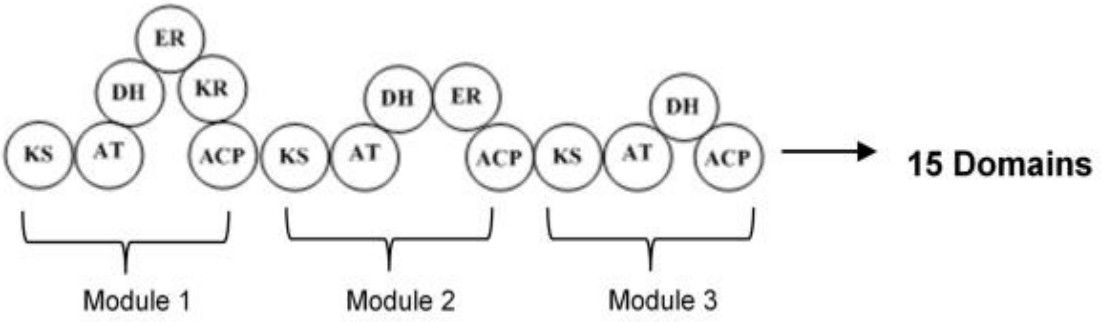

Figure 9: Type I PKS structure with 3 modules and 15 domains [25].

Thioesterase Type I (TE) is associated with ACP at the C-terminus of the last module of PKS. It catalyzes polyketide chain release by intramolecular cyclization or direct hydrolysis that results in the release of a linear product [26]. The catalytic mechanism of TE type I involves the utilization of a catalytic triad consisting of Aspartic acid (Asp), Histidine (His), and Serine (Ser). The mechanism for macrocyclization, which involves an intramolecular nucleophile, proceeds as follows: First, a linear intermediate from Acyl Carrier Protein (ACP) undergoes transesterification to bind to the catalytic serine of TE. Then, the product is offloaded through an intramolecular nucleophilic attack (Figure 10) [27].

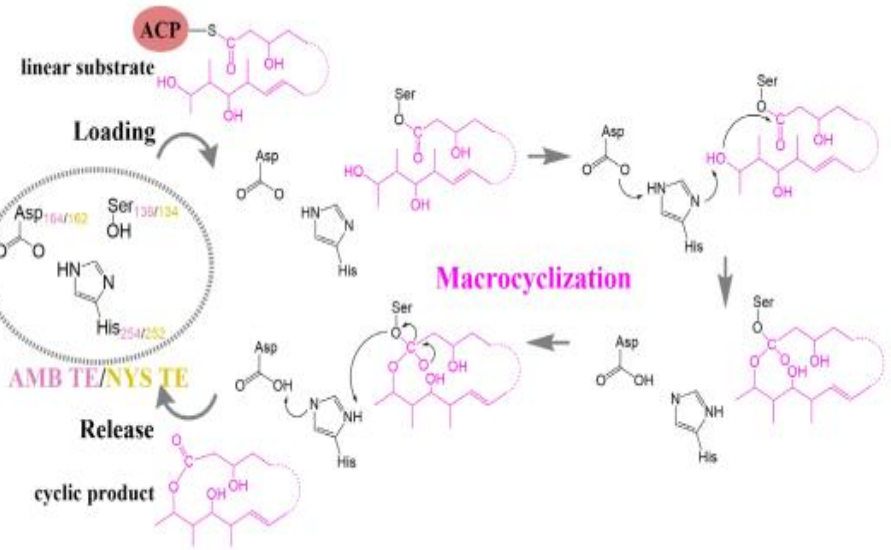

Figure 10: The mechanism of macrocyclization catalyzed by amphotericin B-thioesterases (AMB TE) and nystatin (NYS) TE [27].

Moreover, the mechanism for producing linear polyene, which involves an intermolecular nucleophile, follows a similar process: The linear intermediate from ACP undergoes transesterification to bind to the catalytic serine of TE. Then, the linear polyene is directly hydrolyzed and released via intermolecular nucleophilic attack (Figure 11) [28].

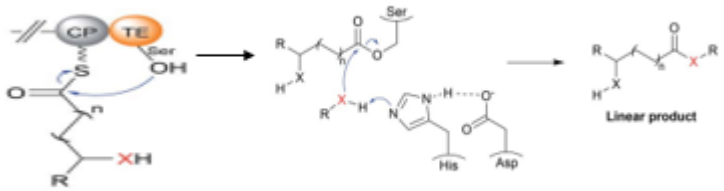

Figure 11: The catalytic mechanism for releasing linear polyenes.

The resulting polyketide product undergoes further modifications by one or two steps of oxidation and glycosylation via cytochrome P450 and glycosyltransferase respectively (Figure 12) [29]. Those enzymes are known as tailoring enzymes.

Oxidation is a common modification at which hydroxyl groups are introduced into the polyene backbone. This step is typically catalyzed by cytochrome P450 enzymes (CYP), which exhibit regiospecificity in hydroxylation reactions [30]. For CYP450s to carry out their enzymatic reaction, two electrons are required. Most of the electrons they receive come from ferredoxins that are soluble iron-sulfur (Fe-S) cluster proteins [31]. In a previous study, CYP 450 was used as a gene marker to isolate potential polyene-producing actinomycetes. This involved conducting a PCR-based genome screening, utilizing the specificity of CYP 450 for polyenes [30].

Glycosylation is a process at which amino sugar moieties are attached to specific sites on the polyene structure. Most polyene antibiotics are glycosylated with D-mycosamine (3,6-dideoxy-3-aminomannose). The process of N-glycosylation of D-mycosamine in polyenes results in the production of antibiotics that have reduced toxicity and exhibit similar activity to the original compound. However, these glycosylated antibiotics possess increased water solubility, leading to improved bioavailability [32].

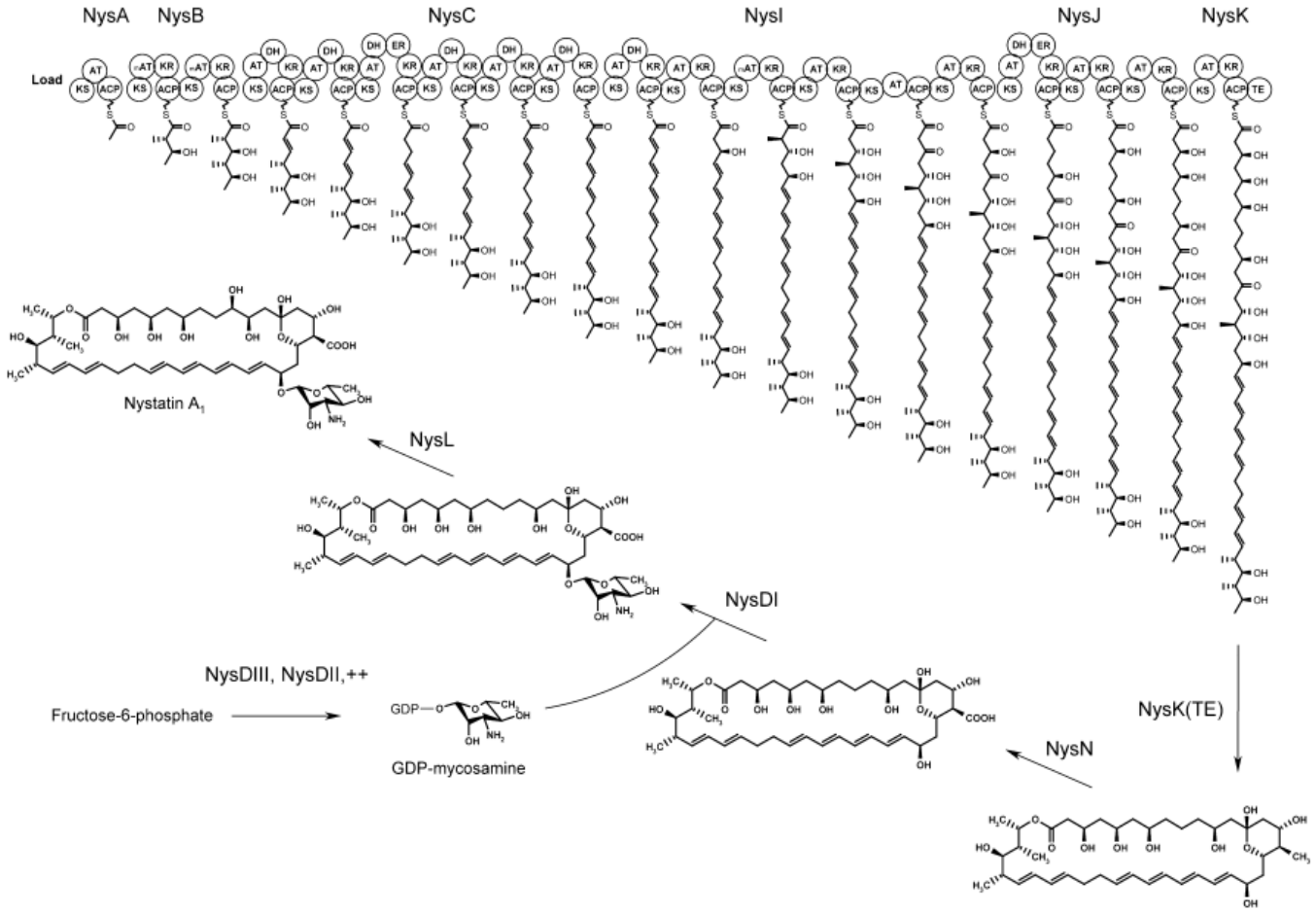

Figure 12: Schematic diagram for nystatin biosynthesis in *Streptomyces noursei* ATCC 11455, NysN and NysL (Cytochromes P450) are involved in oxidation, while NysDI is involved in glycosylation of nystatin [21].

1.3.5. Drawbacks of the polyenes in antibiotic therapy

Although polyene antifungals are effective in fungal infection treatment, they have several drawbacks that limit their use. These antibiotics are light-sensitive and some of them have high cytotoxicity as they exhibit high affinity to human cholesterol due to the similarity between human and fungal cell membranes. The ergosterol of the fungal cell membrane has an extra methyl group and 2 double bonds. (Figure 13) [33].

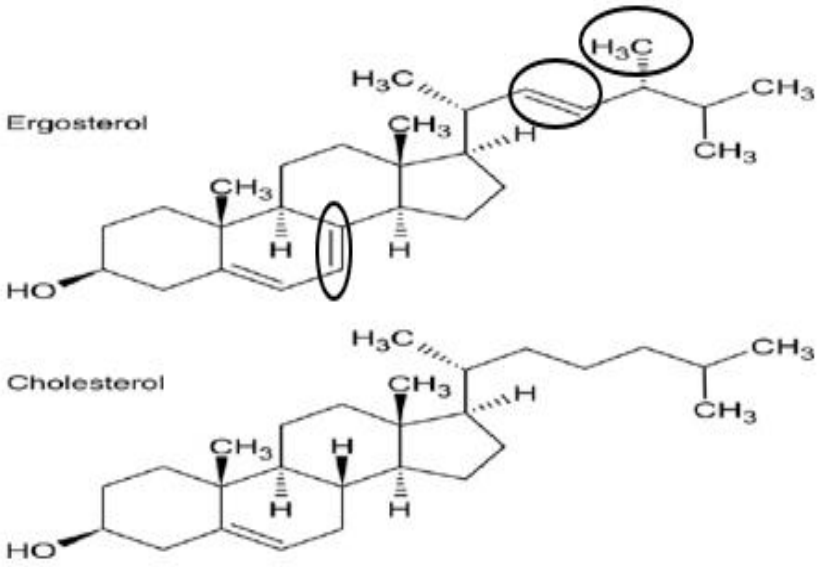

Figure 13: The structure of ergosterol and cholesterol with highlighted structural differences.

Moreover, polyene antibiotics have poor absorption when taken orally. They are also susceptible to the development of drug resistance. Antimicrobial resistance (AMR) threatens effective prevention and treatment of not only bacterial infections, but also fungal infections as they develop resistance against antimicrobial drugs that were previously effective in eliminating or controlling them [34].

Immunocompromised individuals, such as those undergoing chemotherapy or organ transplantation, are particularly vulnerable to fungal infections as candidiasis caused by *Candida* species and pulmonary aspergillosis caused by *Aspergillus* fungi. *Candida auris* is an example of an emerging multidrug-resistant pathogen that can affect various parts of the body, including the bloodstream, leading to systemic infections that can be fatal if not promptly treated. Furthermore, *Aspergillus fumigatus* is resistant to azole antifungal drugs and can lead to severe respiratory complications and even death if left untreated [35].

These examples highlighted the critical importance of effective prevention and treatment strategies for fungal infections, thereby the discovery of novel antifungals becomes a top priority for researchers.

2. Research objective

The primary objective of this thesis focused on investigating unexplored niches, particularly bark beetles, to search for novel putative producers of polyenes or other antifungals with low cytotoxicity. This involved isolating and identifying *Streptomyces* sp. strains, known for their potential in producing secondary metabolites with antimicrobial properties. These strains were then further characterized to evaluate their bioactivity and spectrum of fungal inhibition.

Another aspect of the research was to test the hypothesis whether streptomycetes inhibitory activities are specific to fungi present in the same niche or if they are random.

Moreover, data mining from polyene databases was carried out to search for a specific gene marker that is essential for the synthesis of polyenes. This was accomplished by a deep understanding of the biosynthetic gene clusters (BGCs) of the polyenes produced by *Streptomyces* sp. and evolutionary related actinomycetes. The first hypothesis was that Cytochrome P450 could be used as a specific gene marker for polyene production. The main aim of this work was to evaluate the matching of the primer pair that was designed in a previous study with CYP 450 sequences of a more robust dataset of polyenes. Furthermore, an alternative hypothesis was formulated after conducting an extensive data mining to search for a specific gene marker. The hypothesis was that thioesterase type I is a good gene marker for the identification of polyene-producing actinomycetes, specifically streptomycetes.

3. Materials and Methods

3.1. Field sampling

Branches and twigs of infested trees of different species were collected from Kunratický les forest, in Prague, Czech Republic. 19 samples of adults, larvae, and detritus (taken separately) were extracted from tree material and stored in Eppendorf tubes at 4°C until processing. The tubes contained either adults or larva and detritus. Each sample was labeled with the specific tree ID from where it was collected and the material type. Samples that contained adult beetles are labeled with tree ID+A, while the samples that contain larvae and detritus are labeled with tree ID+D. The number and the code of the samples are listed in Table 1.

Table 1: The number and the code of the collected samples.

Sample number	**Sample code**	**Tree ID**	**Bark beetle ID**	**Material type**
S1	PA1Da	*Picea abies*	*Cryphalus abietis pityographus*	Galleries with larvae and detritus
S2	QR3Da	*Quercus robur*	*Scolytus intricatus*	Galleries with larvae and detritus.
S3	PA2D	*Picea abies*	*Cryphalus abietis*	Galleries with larvae and detritus.
S4	QR1D	*Quercus robur*	*Scolytus intricatus*	Galleries with larvae and detritus
S5	QR3Db	*Quercus robur*	*Scolytus intricatus*	Galleries with larvae and detritus
S6	PA1Db	*Picea abies*	*Cryphalus abietis pityographus*	Galleries with larvae and detritus
S7	TC1D	*Tilia cordata*	*Enrnoporus tiliae*	Galleries with larvae and detritus.
S8	PA1A	*Picea abies*	*Cryphalus abietis pityographus*	Adults.
S9	AA2A	*Abies alba*	*Cryphalus piceae*	Adults
S10	AA2D	*Abies alba*	*Cryphalus piceae*	Galleries with larvae and detritus.
S11	PS1D	*Pinus silvestris*	*Pityophthorus pityographus*	Galleries with larvae and detritus.
S12	PS1A	*Pinus silvestris*	*Pityophthorus pityographus*	Adults.
S13	PS2D	*Pinus silvestris*	*Pityophthorus pityographus*	Galleries with larvae and detritus.
S14	AA1D	*Abies alba*	*Cryphalus piceae*	Galleries with larvae and detritus.
S15	FE1D	*Fraxinus excelsior*	*Hylesinus varius (=Leperisinus varius, L. fraxini)*	Galleries with larvae and detritus.

S16	QR2D	*Quercus robur*	*Scolytus intricatus*	Galleries with larvae and detritus
S17	PA1Dc	*Picea abies*	*Cryphalus abietis pityographus*	Galleries with larvae and detritus
S18	TC1D	*Tilia cordata*	*Enrnoporus tiliae*	Galleries with larvae and detritus.
S19	QR3Dc	*Quercus robur*	*Scolytus intricatus*	Galleries with larvae and detritus

3.2. Isolation buffer and media preparation

3.2.1. Isolation buffer preparation

100 mL of 1% Phosphate Buffered Saline (PBS) solution with Tween 80 (Carl Roth Gmbh & Co. KG, Germany) was prepared as follow:

Table 2: Composition of 1% PBS solution with Tween 80 [36].

Component	**Volume**
Distilled water	80 mL
Sodium chloride	0.8 g
Potassium chloride	0.02
Sodium phosphate	0.144 g
Potassium phosphate	0.0245
Tween 80	10 µL

Tween 80 was added after adjusting the pH to 7.4. Then the total volume was adjusted to 100 mL using a measuring cylinder. The buffer was sterilized by autoclaving for 20 minutes at 121 °C.

3.2.2. Isolation media preparation

Sterile McBeth Scale (MBS), Reasoner's 2A agar (R2A), Krainsky's asparagine (ATCC 236), Humic acid vitamin (HV) and Colloidal Chitin solid media were used for the isolation of bacterial strains. The composition of each medium is described as follow:

Table 3: Composition of MBS solid medium [37].

Component	**Amount (g/L)**
Starch	10.0
$CaCO_3$	3.0
K_2HPO_4	1.0

$(NH_4)_2SO_4$	2.0
$MgSO_4.7H_2O$	1.0
NaCl	1.0
Agar	25.0
Reverse osmosis water	Up to 1.0 L

The R2A solid agar medium contained 18.12 g L^{-1} of R2A agar (HiMedia Laboratories, Germany).

Table 4: Composition of R2A solid agar medium [38].

Component	**Amount (g/L)**
Casein acid hydrolysate	0.500
Yeast extract	0.500
Proteose peptone	0.500
Dextrose	0.500
Starch	0.500
Dipotassium phosphate	0.300
Magnesium sulphate	0.024
Sodium pyruvate	0.300
Agar	15.00

Table 5: Composition of ATCC 236 solid medium [39].

Component	**Amount (g/L)**
Glucose	11.0
K_2HPO_4	0.5
L-Asparagine	0.5
Agar	20.0
Reverse osmosis water	Up to 1.0 L

Table 6: Composition of Humic vitamin agar medium [40].

Component	**Amount (g/L)**
Humic acid	1.0 *
Na_2HPO_4	0.5
KCl	1.71
$MgSO_4.7H_2O$	0.05
$FeSO_4 \cdot 7H_2O$	0.01
$CaCO_3$	0.02
B-vitamins	**
Cycloheximide	0.05
Agar	18.0
Reverse osmosis water	Up to 1.0 L

* Humic acid was dissolved in 10 ml of 0.2 N NaOH.

** 0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxin-HC1, inositol, Ca-pantothenate, p-aminobenzoic acid, and 0.25 mg of biotin.

Table 7: Composition of Colloidal chitin solid medium [8].

Component	**Amount (g/L)**
Colloidal chitin	4.0
K_2HPO_4	0.77
$MgSO_4$	0.244
$KHPO_4$	0.37
$FeSO_4 \cdot 7H_2O$	0.001
$MnCl_2 \cdot 4H_2O$	0.001
$ZnSO_4 \cdot 7H_2O$	0.001
Agar	20.0
Reverse osmosis water	Up to 1.0 L

10 ml of stock solution containing 0.1 g of trace elements ($FeSO_4 \cdot 7H_2O$, g $MnCl_2 \cdot 4H_2O$ and $ZnSO_4 \cdot 7H_2O$) was prepared. Then 1 mL of the stock solution was added to 1 L of media resulting in a final concentration of 0.001 g/L of trace elements.

The pH of the media was adjusted to 7.0 ± 0.2 using HCl or NaOH. The media were subsequently sterilized through autoclaving for 20 minutes at 121 °C and approximately 20 mL were poured

into each sterile Petri dish in a laminar flow box (TelStar, Biostar). Then cycloheximide was added to inhibit fungal contamination (100 µg/mL) and nalidixic acid was added to inhibit fast growing bacteria (50 µg/ mL) as follow:

Table 8: Summary table for the antibiotics added to the isolation media.

	Isolation of samples 1 and 2.	**Isolation of samples 3 and 4.**	**Isolation of samples 5-19.**
Media used for isolation	MBS, ATCC, R2A and HV agar.	MBS, ATCC, R2A and HV agar	ATCC and Colloidal Chitin media.
Antibiotics added to the media	Cycloheximide to all isolation media.	Cycloheximide to all isolation media. Nalidixic acid to ATCC and R2A.	Cycloheximide to both media. Nalidixic acid to ATCC.

3.2.3. Media used for purification and cultivation of streptomycetes.

Moreover, M2 solid medium was used for purification of *Streptomyces*-like isolates and refreshing of the bacterial strains from glycerol stocks.

Table 9: Composition of M2 solid medium [37].

Component	**Amount (g/L)**
Malt extract	10.0
Yeast extract	4.0
Glucose	4.0
Agar	20.0
Reverse osmosis water	Up to 1.0 L

Oatmeal Agar medium was used for cultivation of streptomycetes before testing their antifungal activity.

Table 10: Composition of Oatmeal Agar medium [37].

Component	**Amount (g/L)**
Oatmeal	20.0
Agar	18.0
$FeSO_4$. $7H_2O$	0.001
$MnCl_2$ .4 H_2O	0.001
ZnSO4·7H2O	0.001

Reverse osmosis water	Up to 1.0 L

10 ml of stock solution containing 0.1 g of trace elements ($FeSO_4 \cdot 7H_2O$, g $MnCl_2 \cdot 4H_2O$ and $ZnSO_4 \cdot 7H_2O$) was prepared. Then 1 mL of the stock solution was added to 1 L of media resulting in a final concentration of 0.001 g/L of trace elements.

Nutrient agar was used for testing the *Sterptomyces*-like isolates against *Bacillus subtilis*. It contained 31 g/L of nutrient agar (HiMedia Laboratories, Germany) to which an additional 5 g agar was added.

The pH of the media was adjusted to 7.0 ± 0.2 using HCl or NaOH. The media were subsequently sterilized through autoclaving for 20 minutes at 121 °C and approximately 20 mL was poured into each sterile Petri dish in a laminar flow box (TelStar, Biostar).

3.2.4. Media for the cultivation of bioindicator microorganisms

Muller-Hinton agar with sheep blood (5%) (KHM agars) were purchased from Dulab, s.r.o. (Suché Vrbné, Czech Republic). It was used for the cultivation of bioindicator microorganisms such as *Pseudomonas aeruginosa* (PSAE), *Staphylococcus aureus* (STAU) and *Candida albicans* (CAAL).

3.2.5. Media used for cultivation of fungi (micromycetes) and the antifungal test.

Potato dextrose agar (PDA) was prepared for cultivation of filamentous fungi. It contained 24 g/L of the potato dextrose broth (HiMedia Laboratories, Germany) to which 13 g of agar were added. Moreover, malt extract agar was used for the antifungal test.

Table 11: Composition of malt extract solid medium [37].

Component	**Amount (g/L)**
Malt extract	20.0
Peptone	4.0
Agar	18.0
Reserve osmosis water	Up to 1.0 L

The pH of Potato dextrose agar was adjusted to 5.6 ±0.2, while the pH of the malt extract solid medium was adjusted to 7±0.2. The media were subsequently sterilized through autoclaving for 20 minutes at 121 °C and poured into sterile Petri dishes in a laminar flow box (TelStar, Biostar).

3.3. Bacterial isolation and purification of the promising strains

The volume of 1% PBS solution with Tween-80 added to the samples and the number of ten folds of serial dilutions that prepared for each sample were adjusted based on the amount of the material collected.

Firstly, 500 µL or 1000 µL of 1% PBS solution with tween 80 was added to the samples. Then the samples containing larvae and detritus were homogenized using manual glass homogenizer and mixed well by vortex while the samples containing adult bark beetles were homogenized using ultrasonic for 2 minutes and mixed by vortex. Two to three tenfold serial dilutions of the samples were prepared using 1% PBS solution with Tween-80 as depicted in figure 14.

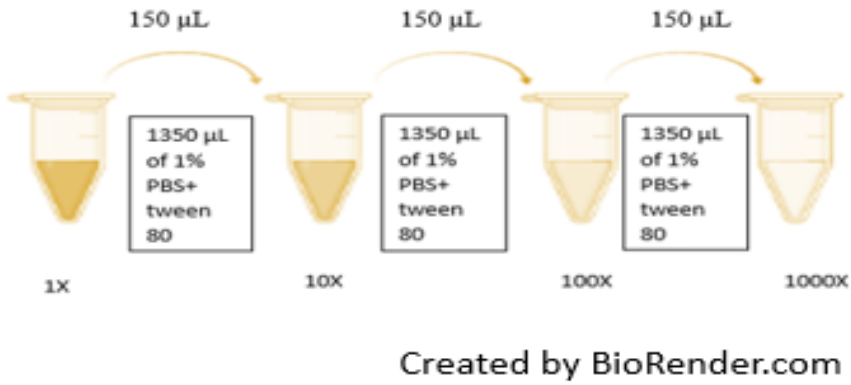

Figure 14: Schematic diagram for the serial dilutions of the samples.

The first two samples, PA1Da (S1) and QR3Da (S2) were used for pilot testing of the isolation procedure and dilutions. Dilutions were prepared for sample S1 at 10x, 100x, and 1000x, while dilutions for sample S2 were prepared at 100x and 1000x. Then 100 µl of the diluted were inoculated onto MBS, ATCC, R2A and HV agar media that contain cycloheximide (3 plates for each medium and concentration).

Then the conditions were slightly modified for the isolation of samples PA2D (S3) and QR1D (S4) to improve the growth of actinomycetes. Nalidixic acid was added to R2A and ATCC media and the samples were preheated (dry heating) at 70 °C for 15 minutes before adding the buffer to destroy the fast-growing bacteria, while streptomyces withstand high temperature.

100 µl of the diluted samples were inoculated onto the same four media (3 plates for each medium and concentration).

The conditions were further modified for the isolation of the other 15 samples (S5-S19) to optimize the growth of actinomycetes, specifically *Streptomyces* sp. Colloidal chitin medium was

used to enhance the growth of *Streptomyces* sp., as it grows at a slower rate compared to other bacteria. Nalidixic acid was added to ATCC 236 medium, and the 15 samples were preheated (dry heating) at 70 °C for 20 minutes before adding the buffer. 100 µl of the diluted samples were inoculated onto ATCC 236 and colloidal chitin media (3 plates for each medium and concentration).

Then the samples were spread evenly over the surface using sterile L-shaped hockey sticks. The samples were incubated at 28 °C and the growth of the colonies were checked regularly for 6 weeks.

Furthermore, all *Streptomyces*-like colonies from each plate were isolated on M2 medium. Then they were transferred again to M2 medium for further purification (Figure 15). Glycerol stocks of the promising isolates were prepared by adding a full loop of biomass to 300 µL of 50% glycerol and 700 µL of Luria-Bertani (LB) broth. Glycerol serves as a cryopreservant, so the glycerol stocks of bacteria can be stored stably at -20°C for short-term and at -80°C for long-term.

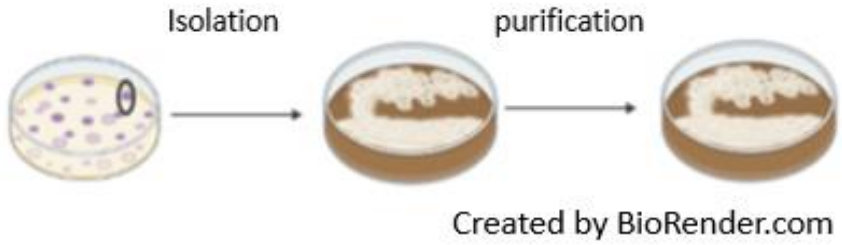

Figure 15: Schematic diagram for the isolation and purification of *Streptomyces*-like colonies.

3.4. Morphological characterization of the isolates

Morphology has been an important characteristic to identify *Streptomyces* like isolates [36]. The isolates were inoculated onto M2 agar. Various morphological observations, including color of aerial and substrate mycelium, and pigment production, have been used to identify streptomycetes.

Light microscopy was used for the observation of the filamentous type of growth to check the purity of the isolate and compare between the structure of filamentous spore chain of *Streptomyces* versus the non-filamentous spore of non-actinomycetes strains. One drop of LB submerse culture of each strain was added to the microscopic slide, covered with the coverslip, and observed under the microscope using objective lens (40x magnification).

3.5. Genotyping of the promising strains

3.5.1. DNA extraction

The DNA of 12 *Streptomyces*-like strains were extracted for identification of the strains. Firstly, Luria-Bertani (LB) broth was prepared as follow:

Table 12: Composition of LB broth [41].

Component	**Amount (g/L)**
Tryptone	10.0
Yeast extract	5.0
NaCl	5.0
Reverse osmosis water	Up to 1.0 L

20 mL of the medium was poured into small Erlenmeyer flasks using a measuring cylinder. The flasks were covered with stoppers and foil. Then the medium was sterilized using autoclave for 45 minutes. Submerse cultivation of the samples was done by adding a full loop of biomass into LB broth. The samples were incubated for three days at 28 °C on a shaker. After that, 100 µL of each sample was inoculated on M2 medium to ensure that there is no contamination. 40 mg of the biomass of each sample was harvested from the culture by centrifugation. Then the DNA of the samples were extracted using the protocol provided by NucleoSpin Microbial DNA kits (Macherey-Nagel GmbH, Germany). The concentration and purity of the DNA of each isolate was measured by Nanodrop One spectrophotometer (Thermo Scientific). The most frequently used method to determine the purity of DNA is by comparing the absorbance of DNA at 260 nm (A_{260}) to that of proteins at 280 nm (A_{280}). Additionally, the A_{260}/A_{230} ratio was measured to identify the presence of impurities coming from extraction solutions like Trizol, phenol, guanidine hydrochloride, and guanidine thiocyanate.

3.5.2. Polymerase chain reactions (PCR) and gel electrophoresis

- **BOX-PCR Fingerprinting**

BOX-PCR is a fast and inexpensive technique that is used for fingerprinting analysis of morphologically similar strains (S6, S10 and S18) by amplifying repetitive element regions (boxA) in the bacterial genome [37].

Firstly, the master mix for 12 reactions was prepared as follow:

Table 13: Master mix components for BOX-PCR [42].

Master mix components	**Concentration**	**Final concentration**	**1x (**µL)	**Total** (µL)
MilliQ water			6.8	81.6
2x LA Hot Start Master Mix (Top-Bio)	2x	1x	12.5	150
DMSO	100%	1%	2.5	30
BSA	5 mg/ml	0,04 mg/ml	0.2	2.4
BoxA1-primer 5′-CTACGGCAAGGCGACGCTGA CG-3′	(0,3 ug/ul)	0,012 ug/ul	1.0	12.0

Then 23 µL of the master mix and 2 µL of the purified DNA of the samples (200 ng/ µL) and positive control were added to each Eppendorf microtubes. For the DNA samples with a concentration higher than 200 ng/ µL, only 1 µL of the purified DNA and 1 µL of MilliQ water were added. Negative control was prepared by adding 2 µL of MilliQ water instead of DNA to ensure that no contaminated nucleic acid has been added to the master mix or samples during sample processing and positive control was used to test the efficiency of the polymerase chain reaction. The thermal cycling was 95°C for 7 min, followed by 30 cycles of 90 °C for 30 s, 53°C for 1 min, and 65°C for 8 min, with a final extension step of 65°C for 15 min.

Moreover, PCR products were separated by gel electrophoresis using 1% of agarose gel. It was prepared by mixing 1.2 g of agarose powder and 120 mL of 1X Tris-acetate-EDTA (TAE) buffer in an Erlenmeyer flask. The mixture was swirled and heated using the microwave till boiling. The solution was then slowly poured into the gel mold after being slightly cooled with tap water. Then the comb was added, and the gel was allowed to cool for 20 minutes. The gel was placed into the gel rig and enough 1X TAE was added to cover the top of the gel. 5 µL of Gene ladder (GeneRuler 1 kb, Thermo Fisher scientific), the samples, positive and negative controls were loaded on the gel. Then the gel was set to run at 100V and 400 mA for 50 minutes. Then the gel was stained by immersing it in a 1X TAE bath supplemented with ethidium bromide for 20 minutes and observed under UV302.

- **16S rDNA PCR**

The prokaryotic 16S rRNA gene is 1550 base pairs (bp) long. It is used for amplification of 16S rDNA of the promising strains to send them for sequencing.

Firstly, the master mix for 17 reactions was prepared as follow:

Table 14: Master mix components for 16S rDNA PCR [43].

Master mix components	**Concentration**	**Final concentration**	**1x (**µL)	**Total** (µL)
MilliQ water			10.0	170.0
pAf (5`-AGAGTTTGATCCTGGCTCAG-3`)	10 uM		0.75	12.75
pHr (5`-AAGGAGGTGATCCAGCCGCA-3`)	10 uM		0.75	12.75
2X Fast Start Master (Roche)	5000 U/ml	30 U/ml	12.5	212.5

Then 24 µL of the master mix and 1 µL of the purified DNA of the 12 samples and positive control. For the negative control 1 µL of MilliQ water was added. The thermocycler protocol that used for amplifying of 11 *Streptomyces*-like strains was 95°C for 3 min, followed by 34 cycles of 94 °C for 1 min, 66°C for 30 s, and 72°C for 1 min 30 s, with a final extension step of 72°C for 5 min. The same protocol was used for amplifying one strain (non-*Streptomyces*), except the annealing temperature was 61°C rather than 66°C.

Furthermore, gel electrophoresis was carried out to ensure the presence of the PCR products. Firstly, 1 µL loading dye (6X) was added to 5µL of the samples. Then 6µL of this mixture and 5µL of the gene ladder were loaded on 1% agarose gel. The running conditions, staining and observation steps were kept the same. Amplified DNA fragment was bi-directionally sequenced using Sanger technology by SEQme, s. r. o. (Dobříš, Czech Republic). Then the closest relatives of the strains were determined, along with their accession and strain numbers.

3.5.3. Identification of the strains using 16S rRNA

The sequences of the 12 samples were analyzed and manually edited by evaluation of the chromatograms. Then the forward and reverse reads of the sequences were assembled using Geneious software version R8.1.9. and a FASTA file of the consensus sequence was created. Then FASTA files were uploaded onto the National Center for Biotechnology Information (NCBI) platform and analyzed by nucleotide BLAST (blastn) against rRNA/ITS databases of 16S rRNA sequences (Bacteria and Archaea) to search for closely related species.

3.6. Screening of isolates for antimicrobial activity

3.6.1. Bioassay against indicator microorganisms

6 different *Streptomyces* sp. that were previously obtained from bark beetles from Papua New Guinea and Morocco (BCCO strains) [14] and 14 *Streptomyces*-like isolates were tested against different pathogens as *Pseudomonas aeruginosa* (Gram-negative bacteria), *Staphylococcus aureus* (Gram-positive bacteria) and *Candida albicans* (yeast) to determine the inhibitory effect of the secondary metabolites produced by the strains. The strains were refreshed from glycerol using M2 media and incubated for 2 weeks at 28 °C. The streptomycetes. were inoculated in lines on the blood agar and cultivated for 48 hours. Then lines of the indicator strains were inoculated perpendicularly to the samples. The size of the inhibitory zone of the pathogens and the hemolytic zone of the samples as well as the pathogens were measured after three days. Moreover, the sporulation of streptomycetes was evaluated, with a scoring that is ranging from 0 to 3. A score of 3 indicated full sporulation, while a score of 0 indicated that the strains were not sporulating.

Then the bioassay was repeated at different incubation periods of the promising isolates (S6/6, S10/3, S10/25, and S18/15), in order to determine the optimum duration required for the isolates to produce secondary metabolites. In this bioassay, the inhibitory activity of the promising isolates was tested against *Staphylococcus aureus* and *Candida albicans*, as the previous bioassay had shown their susceptibility to inhibition. The *Streptomyces* strains were inoculated in vertical lines and incubated for different time intervals (2 days to 6 days). Then lines of *Staphylococcus aureus* and *Candida albicans* were inoculated on the same plate perpendicularly to the samples and the results were evaluated after three days.

Moreover, to test if the inhibitory effect is due to the secondary metabolite produced by the sample only or it is a synergistic effect, the bioassay was repeated at which *Staphylococcus aureus* and *Candida albicans* were inoculated separately after 3-6 days of incubation of the *Streptomyces* strains.

3.6.2. Bioassay against *Bacillus subtilis*

Bacillus subtilis is a gram-positive bacterium which is not pathogenic to humans. Nine *Streptomyces* like isolates were tested against bacillus subtilis. Firstly, the isolates have grown for 2 weeks at 28 °C. Then 100 µL of bacillus subtilis Difco Sporulation Medium (DSM) was inoculated onto nutrient agar and spread well over the surface using sterile L-shaped hockey sticks. The plates were kept for 20 minutes to dry. Then, two 11 mm-diameter agar discs containing the cultivated *Streptomyces* like isolates (S) were transferred to the nutrient agar

containing *Bacillus subtilis* with a distance 3 cm between them as depicted in Figure 16. The plates were kept overnight at 37 °C.

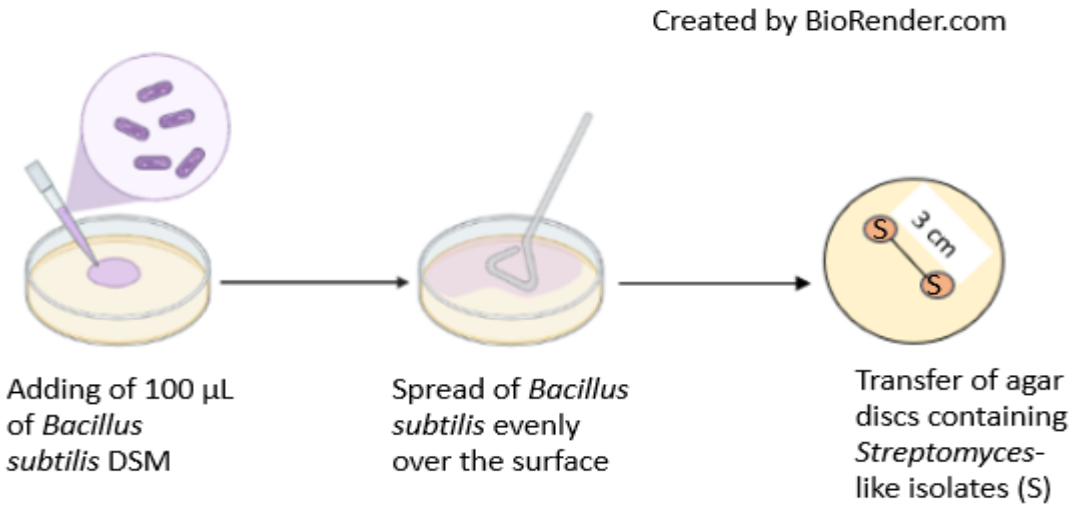

Figure 16: Schematic diagram for the Cultivation method of the Isolates with *Bacillus subtilis*.

3.6.3. Antifungal activity test

To determine whether the inhibitory activities by *Streptomyces* strains are specific to fungi present in the same niche or if they are random, four promising isolates (S6/6, S10/3, S10/25, and S18/15) and three previously obtained streptomycetes (BCCO 10_1099, 10_1104 and 10_1106) isolated from different environment (Morocco and Papua New Guinea) were tested against four fungi collected from various locations.

Table 15: Bacterial and fungal strains used for the antifungal activity test.

Strain code	**Strain number**	**Strain Identification**	**Countries**
CCF 3535	-	*Graphium asporum*	Czech Republic
CCF 3546	-	*Ophiostoma piceae*	Czech Republic
CCF 4450	-	*Ophiostoma minus*	Poland
CCF 6041	-	*Akanthomyces muscarium*	Hungary
BCCO 10_1099	B1	*Streptomyces violascens* (closest relative)	Papua New Guinea
BCCO 10_1104	B2	*Streptomyces albolongus* (closest relative)	Papua New Guinea
BCCO 10_1106	B3	*Streptomyces fumigatiscleroticus, spiralis* (closest relative)	Morrocco
S6/6	B4	*Streptomyces*-like strain	Czech Republic
S10/3	B5	*Streptomyces*-like strain	Czech Republic

S10/25	B6	*Streptomyces*-like strain	Czech Republic
S18/15	B7	*Streptomyces*-like strain	Czech Republic

Firstly, the selected Bacteria (B) and Fungi (F) were cultivated on oatmeal agar and potato dextrose agar (PDA), respectively. They were incubated at 28 °C for 10 days. Then 11 mm-diameter agar discs with grown Fungus/Bacterium were transferred to the malt extract agar in duplicates for each strain combination. The distance between the discs was approximately 3 cm. Moreover, an agar disc with each fungus was transferred to a separate malt extract plate as a control (Figure 17). The plates were incubated at 28°C for one month. The results - size of the inhibition zone - were recorded after 3 weeks for fast growing fungal strains and 4 weeks for slow growing ones.

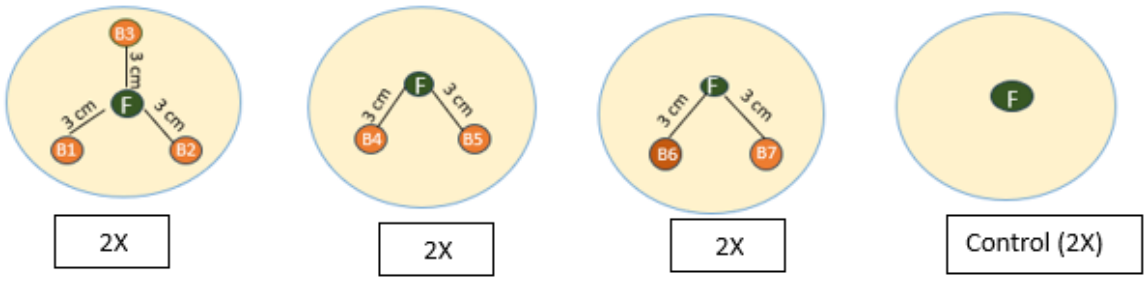

Figure 17: Transferring of discs of the selected bacteria and fungi to malt extract agar.

3.7. Genome Screening of antifungal polyenic secondary metabolites.

3.7.1. Data mining from polyene databases

Since polyenes have a common structure essential for their functions, they have common genes which may act as gene markers (Figure 18). Data mining from polyene databases such as the National Center for Biotechnology Information (NCBI), Minimum Information about a Biosynthetic Gene cluster (MIBiG) and Database of Biosynthesis clusters Curated and Integrated (DOBISCUIT), was carried out to identify a specific gene marker required for the production of polyenes.

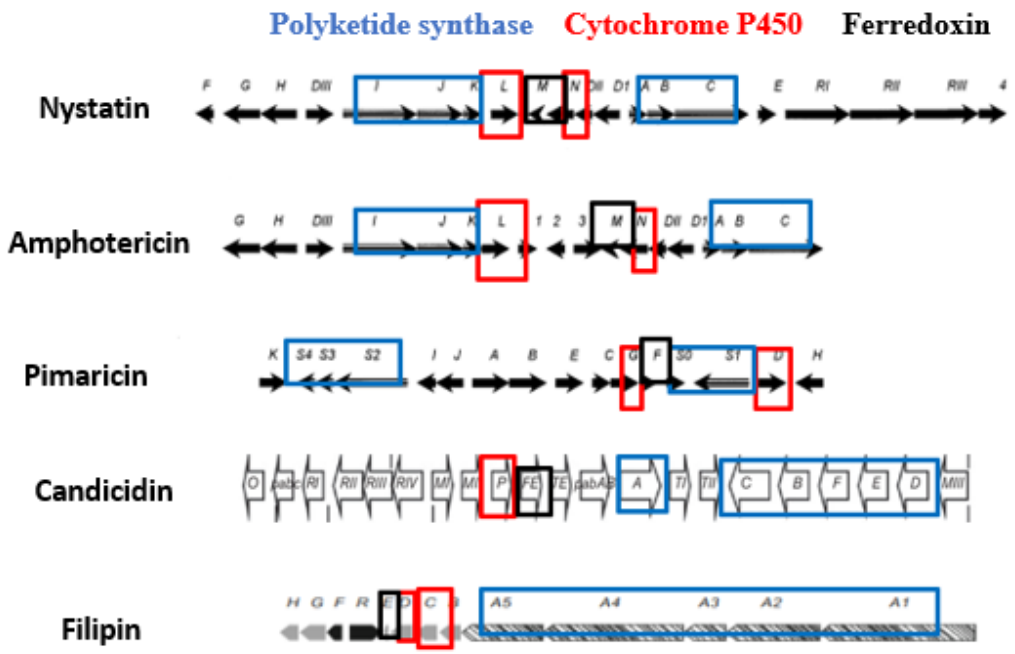

Figure 18: Illustration for the biosynthetic gene cluster of the polyenes with common genes highlighted.

- **Cytochrome P450 as a gene marker**

Based on a previous study, where a primer pair was specifically designed for cytochrome P450 and utilized for PCR-based genome screening to isolate potential polyene-producing actinomycetes strains [30].

Firstly, nucleotide and amino acid sequences of polyene BGCs were downloaded from publicly available databases such as DOBISCUIT (Accessed in October 2022) [38] and MIBiG (Accessed in October 2022) [39]. Then a FASTA file was created for the amino acid sequence of CYP 450 of 6 polyenes. Multiple sequence alignment of CYP 450 using the graphical interface of Geneious version 2022.0.2 (Biomatters Ltd., Auckland, New Zealand) was done using Muscle (Version 3.5). The aligned sequences were edited manually by removing the gaps and used for phylogenetic tree construction using Molecular Evolutionary Genetics Analysis (MEGA) software version 11.0.13 [40]. Maximum likelihood statistical method and 100 bootstrap replications were selected.

- **Thioesterase type I as a gene marker**

After conducting an extensive data mining process to search for another gene marker, the alternative hypothesis was that thioesterase type I could serve as an effective gene marker for identifying polyene-producing actinomycetes, particularly streptomycetes. It was based on a previous literature that highlighted the presence of a distinct group of PKS-TE domains responsible for producing polyene macrolides. These domains exhibited relatively low sequence

similarity compared to TEs of other non-polyene macrolides such as Erythromycin and Pikromycin [29].

Firstly, nucleotide sequences of polyene biosynthetic gene clusters were downloaded from JDB, NCBI and MIBiG. Then FASTA file was created for nucleotide sequences of the last module of type I polyketide synthase that associated with thioesterase type I, which included 26 polyenes and 2 non-polyenes (Pikromycin and Erythromycin). The sequences were checked that they are starting with start codon and have stop codon at the end. Then the sequences of the last module were uploaded on AliView software (Alignment Viewer and Editor). The sequences were translated into amino acid sequences using bacterial genetic code and translation frame 1. Then multiple sequence alignment of the last module of polyketide synthase using AliView was performed using Muscle and selecting the option "Realign everything as Translated Amino Acids". The aligned amino acid sequences were checked, edited manually and the gaps were removed. Then the amino acid sequences of ACP-TE were annotated using antiSMASH bacterial version [41] and extracted from the whole module. The results of the alignment of amino acid sequences of the thioesterase (TE) gene were compared to the results provided by Zhou, Yucong, et al. [29]. The Conserved region of TE for the polyenes and non-polyenes was extracted and the large gaps that occupied only by non-polyenes were removed. Then the Amino acid sequences were translated back to nucleotide sequences and used for designing the forward and reverse primers.

3.7.2. Designing of primer pairs

Ten primer pairs were designed on the consensus sequences using Geneious software version R8.1.9 (Biomatters Ltd., Auckland, New Zealand) by Clicking the Primers button and selecting Design New Primers. The parameters were adjusted as shown in Figure 19 for optimization of the primer design. The optimum product size was adjusted to 500 bp. Then the best option for the primer pairs was selected based on the % GC content, primer melting temperature (Tm), Hairpin Tm and Self-Dimer Tm and used for designing degenerate primer pairs. Moreover, it is important that the sequence of the primers have guanine (G) or cytosine (C) within the last 5 nucleotides at the 3’ end as they form 3 hydrogen bonds thus better stability. This is known as GC clamp [42].

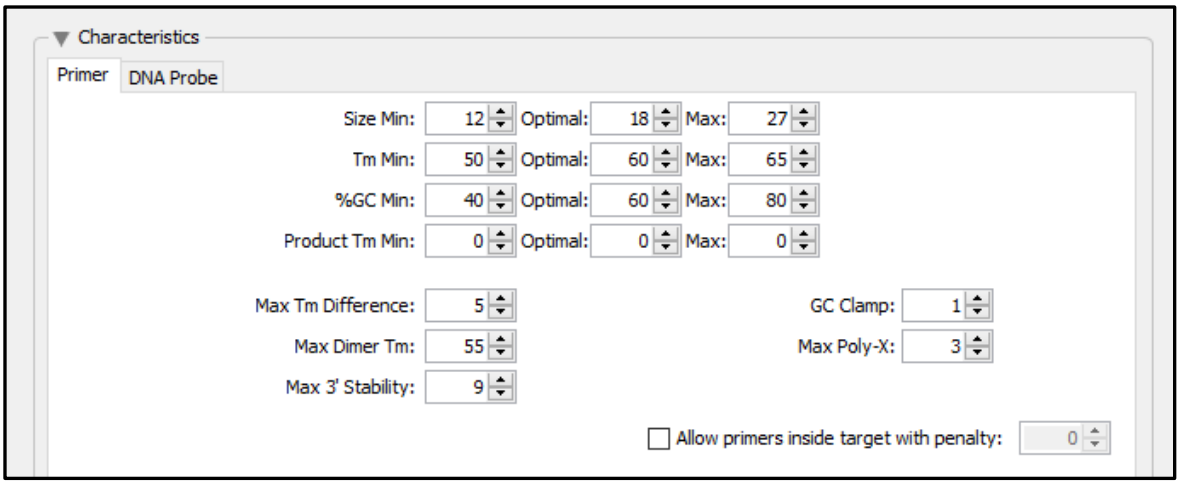

Figure 19: Optimization of the primer's properties.

Moreover, the degenerate primer pair was designed manually using Geneious software version 8.0.4 (Biomatters Ltd., Auckland, New Zealand) by following the procedure that mentioned on Geneious website [43]. Firstly, the file with the aligned sequences was selected. Then the consensus bottom was selected, and the threshold was set to 50 % at which the bases matching was at least 50% of the sequences. The highlighting of the sequences was adjusted as depicted in Figure 20 for easy observation of the differences.

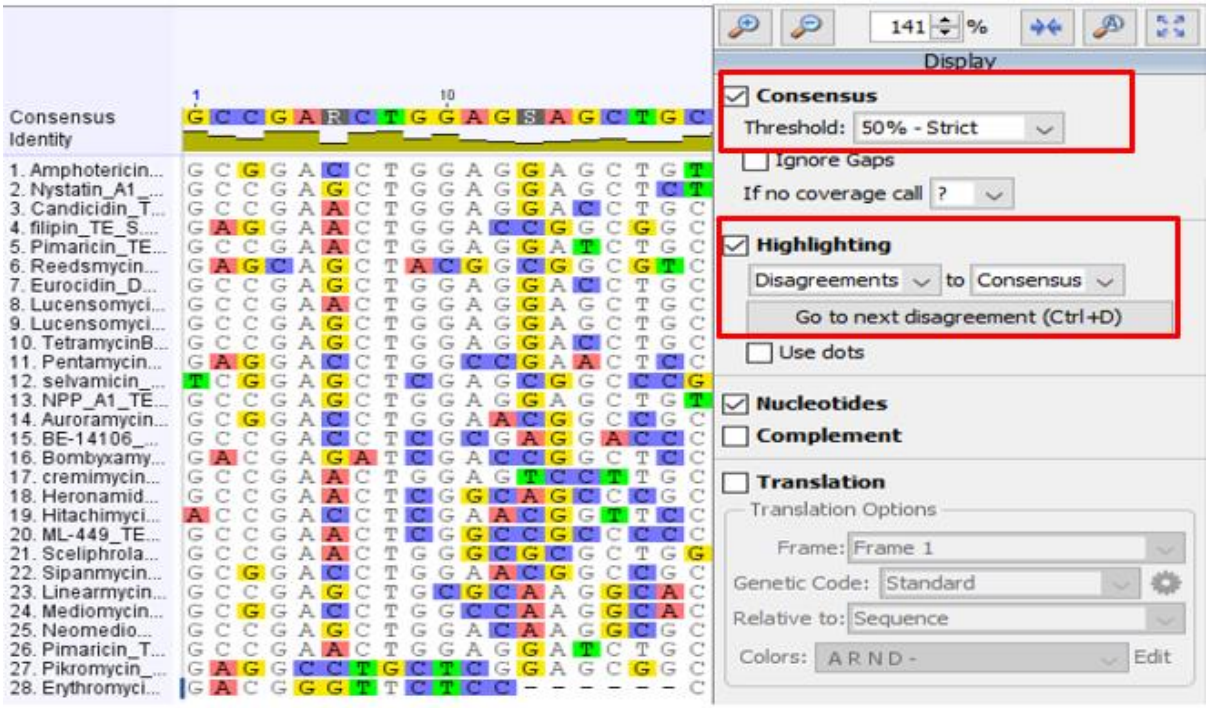

Figure 20: Screenshot illustrating the settings for designing degenerate primers.

The regions of the best option out of the 10 primer pairs that were designed before were selected on the consensus sequence in the alignment. For the forward primer, the region was selected from left to right while the region of the reverse primer was selected from right to left. The Add

Annotation button was selected and the calculated characteristics of the primer, including the Tm range based on the degeneracy were checked. Then the name of the primer was entered, and the primer was added to the consensus as an annotation by clicking Ok.

3.7.3. *In silico* PCR

In silico PCR, also known as virtual PCR, is a computational method used to simulate the polymerase chain reaction (PCR) process. An online tool called "*In silico* PCR amplification" (http://insilico.ehu.es/PCR/index.php?mo=Streptomyces, accessed in October 2023) was used for testing the efficiency and specificity of the designed primer pair [44]. The primer pair was uploaded and tested against all available *Streptomyces* strains allowing up to 2 mismatches, but in 1 nucleotide in 3' end. The maximum lengths of the bands were adjusted to 600 nucleotides (Figure 21).

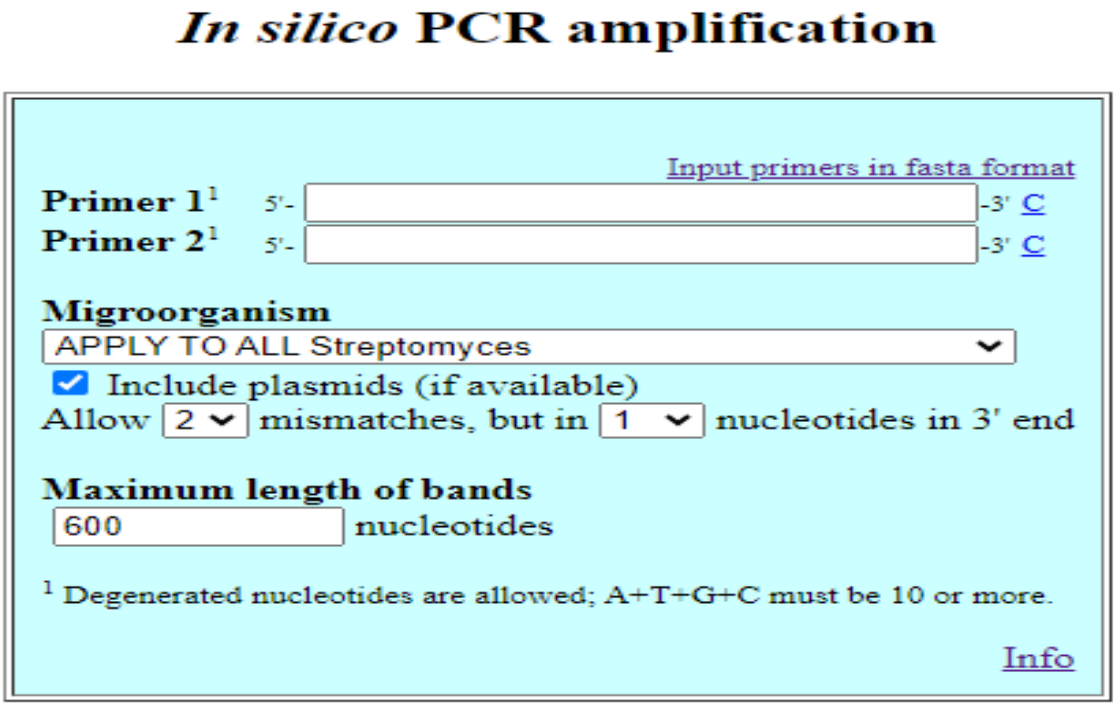

Figure 21: Screenshot illustrating the settings for testing the primer pair.

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4. Results

4.1. Bacterial isolation and purification

The isolation and purification of *Streptomyces*-like strains from samples that contain either adults or larva and detritus, yielded a total of 44 isolates. The results of the isolation process for the first four samples, PA1Da (S1), QR3Da (S2), PA2Da (S3) and QR1Da (S4) indicated the presence of a few promising colonies (*Streptomyces*-like) alongside many non-actinomycetes colonies. Specifically, no promising colonies were isolated from S1 or S3 while four promising isolates were obtained from S2, and one promising isolate was obtained from S4. Moreover, the results of the modified procedure for the isolation of S5-S19 revealed the presence of many promising colonies as different actinomycetes-like strains, specifically many *Streptomyces* strains were isolated. A comprehensive overview of the results is provided in Table 16.

Table 16: Summary table for the isolation and purification of the *Streptomyces*-like strains.

Sample number	**Sample code**	**Code of isolate**	**No. promising isolates (*Streptomyces*-like)**	**Are there some different types?**	**Glycerol stocks**	**Isolation media**
S1	PA1Da	-	X	-	-	-
S2	QR3Da	S2/1 S2/2 S2/3 S2/5	4	No	4	ATCC (10^{-3},10^{-4})
S3	PA2Da	-	X	-	-	-
S4	QR1Da	S4/1	1	No	1	MBS (10^{-4})
S5	QR3Db1	-	X	-	-	
S6	PA1Db	S6/6	1	No	1	Chitin (10^{-1})
S7	TC1D	-	X	-	-	-
S8	PA1 Adult	-	X	-	-	-
S9	AA2 Adult	-	X	-	-	-
S10	AA2D	S10/1-20 S10/25 S10/34	22	No	22	Chitin and ATCC (10^{-1})
S11	PS1D	-	X	-	-	-
S12	PS1 Adult	S12/3	1	No	1	ATCC (10^{-1})
S13	PS2D	-	X	-	-	
S14	AA1D	S14/1	1	-	1	ATCC (10^{-2})
S15	FE1D	-	x	-	-	-
S16	QR2D	S16/2	2	No	2	Chitin (10^{-1})
S17	PA1D	-	X	-	-	-
S18	TC2D	(S18/1-S18/5)	12	yes	12	Chitin (10^{-1}) and ATCC (10^{-3})

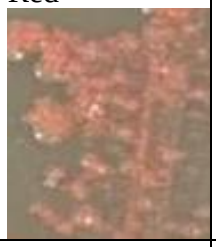
		S18/8 S18/9 S18/11 (S18/13- S18/15) S18/17				
S19	QR3DB2	-	X	-	-	-

4.2. Morphological characterization of the isolates

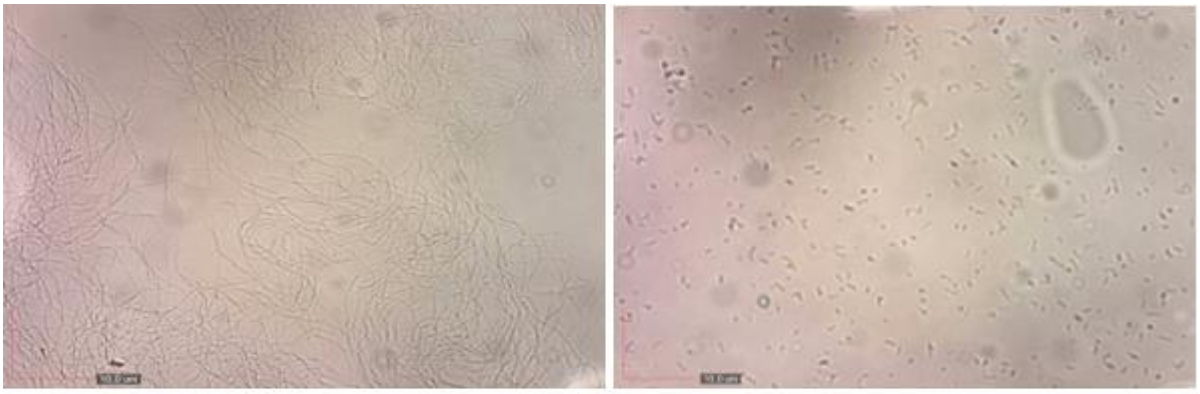
Based on the morphological characteristics, such as filamentous growth and formation of aerial mycelium (Figure 22), it was confirmed that samples S2, S6, S10, and some of the S18 isolates were *Streptomyces*-like strains. Isolate S16/2 was identified as an actinomyces-like strain. However, S4/1, S12/3, S14/1, S18/8 and S18/9 isolates exhibited red colonies with atypical morphology, which did not resemble actinomycetes (table 17). Furthermore, it was observed that the streptomyces-like isolates obtained from S2 were replicates of one strain, while the isolates obtained from S18 were replicates of a different strain. Most of S10 isolates had the same color of substrate mycelium, whereas only two isolates had different colors. Moreover, samples S6, S10 and S18 showed similar morphological characteristics.

Table 17: The appearance of the aerial mycelium and Reverse side of plate showing substrate mycelium of the promising isolates.

Sample number	**Sample code**	**Code of isolate**	**Color of aerial mycelium (AM)**	**Color of substrate mycelium (SM)**	**Pigment produced**
S2	QR3Da	S2/1 S2/2 S2/3 S2/5	White (Wa) 	Pale yellow (Co4a) 	Yes
S4	QR1Da	S4/1	Red	Red	No
S6	PA1Db	S6/6	White-light gray (Wa GY2ih)	Yellowish brown (Coo2m)	Yes

					
S10	AA2D	S10/1 S10/2 S10/4-19 S10/25 S10/34	Gray (GY2fe) 	Ochre (Coo4r) 	Yes
		S10/3 S10/20	Gray (GY2fe) 	Greenish brown (Co4s) 	Yes
S12	PS1 Adult	S12/3	Red 	Red 	No
S14	AA1D	S14/1	Red	Red	No
S16	QR2D	S16/1 S16/2	Pale orange 	Pale orange 	No

S18	TC2D	(S18/1-S18/5) S18/11 (S18/13-S18/15) S18/17	White-light gray (Wa GY2ih) 	Brown (Coo3r-Co3r) 	Yes
		S18/8 S18/9	Red 	Red 	No



S18/17 Isolate S4/1 Isolate

Figure 22: The spore chains morphology of *Streptomyces sp*.(S18/17) versus the spores of non-actinomyces strain (S4/1) observed using light microscopy.

4.3. Genotyping of the promising strains

4.3.1. DNA extraction

The concentration and purity of the DNA of each isolate was measured spectrophotometrically to make sure that the DNA extraction of the isolates has taken place successfully and to adjust the concentration of the DNA used for the PCR as too high concentration may lead to inhibition of the DNA amplification.

The results showed that the A_{260}/A_{280} ratio of the DNA samples ranged from 1.87 to 1.97 all samples had A_{260}/A_{230} ratio around 2, except for isolates S10/25 and S18/5, which had relatively low absorbance at around 1.2 (Table 18).

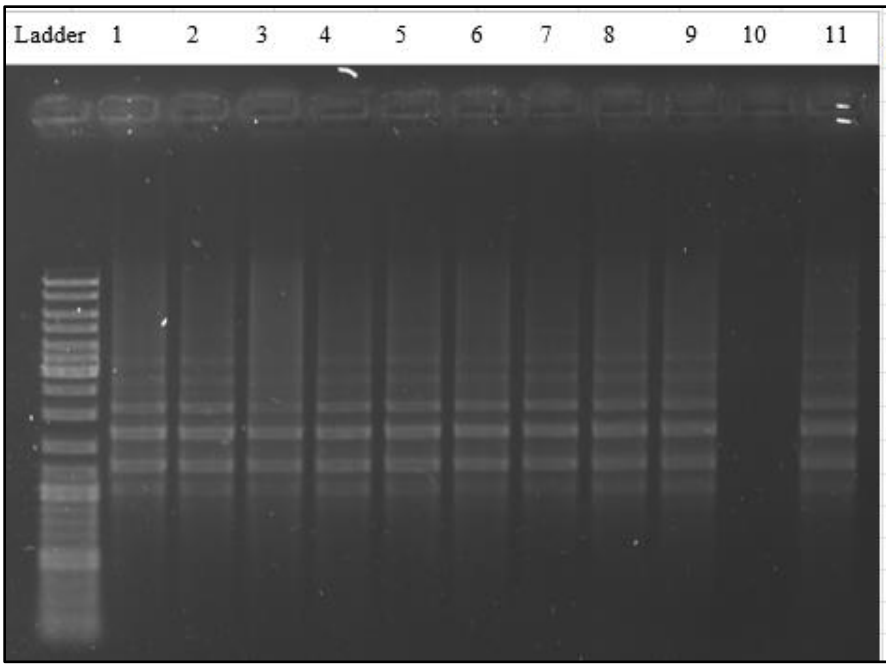
Table 18: The concentration and the absorbance of DNA samples.

Code of isolate	**Concentration of DNA (ng/μL)**	**Absorbance (A_{260}/A_{280})**	**Absorbance (A_{260}/A_{230})**
(S2/1	104.3	1.97	2.23
S6/6	377.0	1.93	2.09
S16/2	288.7	1.93	2.12
S10/3	232.8	1.92	1.87
S10/15	174.3	1.92	1.95
S10/20	115.7	1.91	1.62
S10/25	122.3	1.90	1.21
S18/1	137.2	1.90	1.66
S18/5	62.8	1.87	1.21
S18/8	202.6	1,90	1.96
S18/15	245.4	1.92	1.95
S18/17	104.5	1.89	1.73

4.3.2. Polymerase chain reactions and gel electrophoresis

- **BOX-PCR for morphologically similar strains**

Gel electrophoresis was used to represent the BOX-PCR results of morphologically similar isolates as the banding patterns can be analyzed to determine the genetic relatedness and diversity among the isolates. Each lane on the gel corresponds to a different isolate. Figure 23 shows that the banding patterns of amplified DNA fragments of the isolates are identical. PCR amplification was confirmed by successfully amplifying the DNA of the positive control. Moreover, no bands were observed for the negative control, indicating the absence of contamination in the master mix.

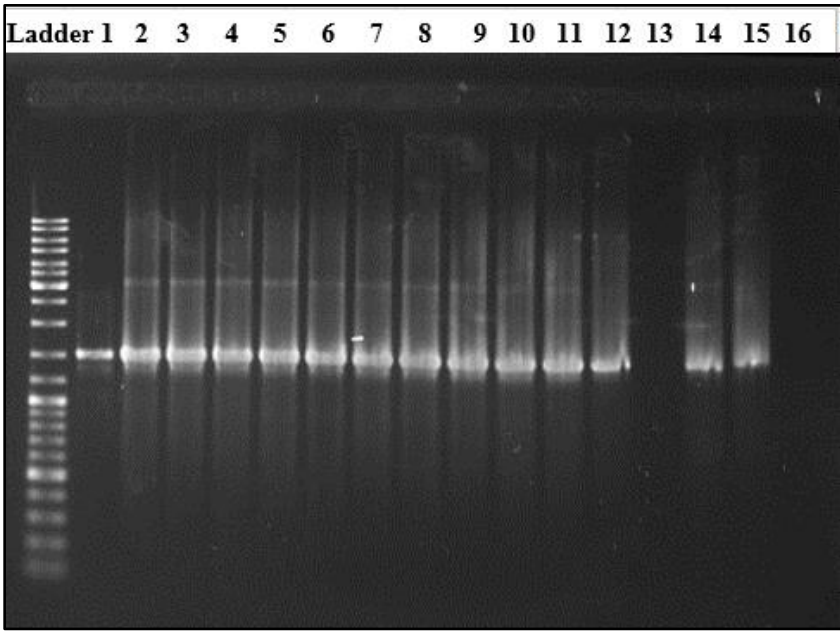


Well number	Code of isolate
1	S6/6
2	S10/3
3	S10/15
4	S10/20
5	S10/25
6	S18/1
7	S18/5
8	S18/15
9	S18/17
10	Negative control
11	Postitive control

Figure 23: The amplified DNA fragments of the morphologically similar isolates visualized on a 1% agarose gel.

- **16S rDNA PCR**

Gel electrophoresis was performed to confirm the successful amplification of 16S rDNA of the samples before sending them for sequencing. As depicted in Figure 24, the gel image demonstrates the presence of the bands at 1550 base pairs (bp), indicating that the amplification of the 16S rDNA has taken place successfully.



Well number	Code of isolate
1	S2/1
2	S6/6
3	S16/2
4	S10/3
5	S10/15
6	S10/20
7	S10/25
8	S18/1
9	S18/5
10	S18/15
11	S18/17
12	Positive control
13	Negative control
14	S18/8
15	Positive control
16	Negative control

Figure 24: The amplified 16S rDNA fragments are visualized on a 1% agarose gel.

4.3.3. Identification of the strains using 16S rRNA

The sequences of the bacterial isolates were analyzed by nucleotide BLAST (blastn) to search for closely related species and the results are represented in Table 19. The table includes the code of isolate, the closest matching species identified by blastn, the percentage of sequence similarity, the accession number, and the E-value. The top matches for each isolate are listed, providing valuable information for the identification of the bacterial isolates.

Table 19:Results of blastn analysis for identification of bacterial isolates.

Code of Isolate	**Closest Matching Species**	**Identity %**	**Accession number**	**E-value**	**type of isolate**
S2/1	*Streptomyces flavogriseus* strain CBS 101.34 Or *Streptomyces flavovirens* strain NBRC 3716	99.86% 99.86%	NR_028988.1 NR_112509.1	0.00 0.00	Filamentous *Actinobacteria*
S6/6	*Streptomyces flavogriseus* strain CBS 101.34 Or *Streptomyces flavovirens* strain NBRC 3716	99.65% 99.65%	NR_028988.1 NR_112509.1	0.00 0.00	Filamentous *Actinobacteria*
S16/2	*Gordonia sputi* strain 3884 Or *Gordonia aichiensis* strain E9028	99.79% 99.64%	NR_037031.1 NR_037030.1	0.00 0.00	Non-filamentous *Actinobacteria*
S10/3	*Streptomyces flavogriseus* strain CBS 101.34 Or *Streptomyces flavovirens* strain NBRC 3716	99.65% 99.65%	NR_028988.1 NR_112509.1	0.00 0.00	Filamentous *Actinobacteria*
S10/15	*Streptomyces flavogriseus* strain CBS 101.34	99.72%	NR_028988.1	0.00	Filamentous *Actinobacteria*

	Or *Streptomyces flavovirens* strain NBRC 3716	99.72%	NR_112509.1	0.00	
S10/20	*Streptomyces flavogriseus* strain CBS 101.34 Or *Streptomyces flavovirens* strain NBRC 3716	99.79% 99.79%	NR_028988.1 NR_112509.1	0.00 0.00	Filamentous *Actinobacteria*
S10/25	*Streptomyces flavogriseus* strain CBS 101.34 Or *Streptomyces flavovirens* strain NBRC 3716	99.93% 99.93%	NR_028988.1 NR_112509.1	0.00 0.00	Filamentous *Actinobacteria*
S18/1	*Streptomyces flavogriseus* strain CBS 101.34 Or *Streptomyces flavovirens* strain NBRC 3716	99.71% 99.71%	NR_028988.1 NR_112509.1	0.00 0.00	Filamentous *Actinobacteria*
S18/5	*Streptomyces flavogriseus* strain CBS 101.34 Or *Streptomyces flavovirens* strain NBRC 3716	99.72% 99.72%	NR_028988.1 NR_112509.1	0.00 0.00	Filamentous *Actinobacteria*
S18/15	*Streptomyces flavogriseus* strain CBS 101.34 Or *Streptomyces flavovirens* strain NBRC 3716	99.86% 99.86%	NR_028988.1 NR_112509.1	0.00 0.00	Filamentous *Actinobacteria*
S18/17	*Streptomyces flavogriseus* strain CBS 101.34 Or *Streptomyces flavovirens* strain NBRC 3716	99.91% 99.91%	NR_028988.1 NR_112509.1	0.00 0.00	Filamentous *Actinobacteria*

S18/8	*Methylobacterium tardum* strain RB677	99.64%	NR_041443.1	0.00	Non-*Actinobacteria* (Proteobacteria)

Based on the results of the blastn analysis, it can be observed that most of the bacterial isolates have the same closest matching *Streptomyces* species, but with different percentages of identity. Out of all the isolates, only two do not belong to the *Streptomyces* species. Isolate S16/2 is classified as a member of the *Gordonia* genus, which is gram-positive *Actinobacteria*, and isolate S18/8 is identified as a gram-negative Proteobacteria known as *Methylobacterium tardum*.

4.4. Screening of isolates for antimicrobial activity

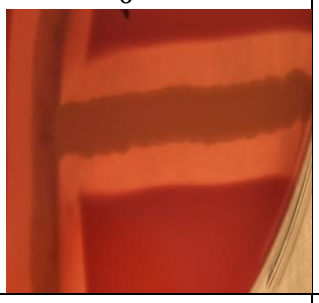
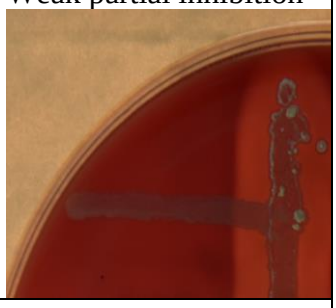
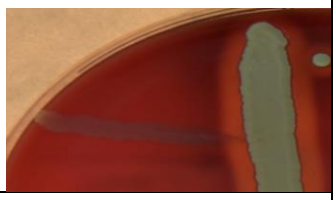
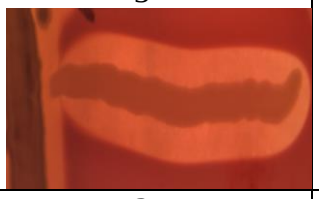
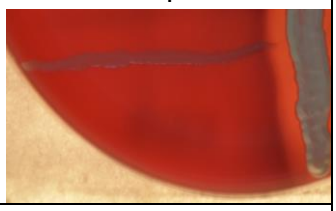
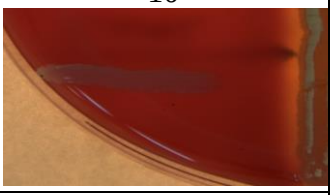
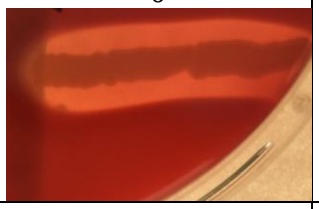
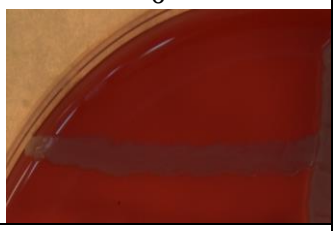
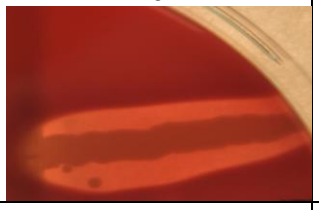
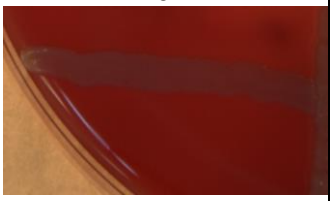
To assess the potential for bioactive compound production, the six BCCO strains and the confirmed *Streptomyces* strains isolated in this study were screened for antimicrobial activity against different bacteria and fungi.

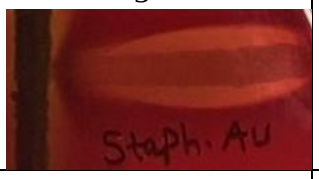
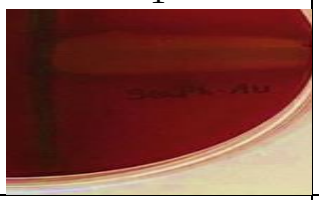
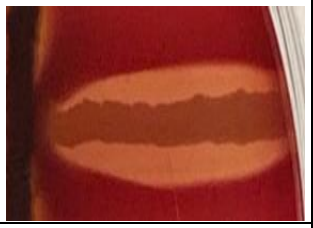
4.4.1. Bioassay against Indicator microorganisms

The sporulation of the strains, hemolytic zone size and pathogen inhibitory zone size were recorded for each sample tested in the bioassay and represented in table 20.

Table 20:Results of the bioassays of *Streptomyces* isolates and BCCO strains after 72 hours.

Code of Isolate	**Sporulation**	**Area of hemolytic zone (mm)**	**Size of inhibitory zone of β-hemolysis of *Staphylococcus aureus* (mm) Promising isolate (Vertical Line) *Staphylococcus aureus* (Horizontal Line)**	**Size of inhibitory zone of *Candida albicans*. (mm) Promising isolate (Vertical Line) *Candida albicans* (Horizontal Line)**

BCCO 10_1092	1	5	0 	Weak partial inhibition 
BCCO 10_1093	3	3	3 	2 
BCCO 10_1095	3	4	3 	4 
BCCO 10_1099	2	3	3 	10 
BCCO 10_1102	0	0	0 	0 
BCCO 10_1105	0	0	0 	0 
S2/1	0	0	0	0
S2/2	0	0	0	0

S2/3	0	0	0	0
S2/5	0	0	0	0
S6/6	3	2	3 	1 
S10/3	3	2	1 	1 
S10/15	3	1	1	Partial inhibition
S10/20	3	1	1	Partial inhibition
S10/25	3	1	1 	1 
S18/15	3	2	3 	1 
S18/1	3	1	2	Partial inhibition

S18/5	3	1	2	Partial inhibition
S18/17	3	1	2	Partial inhibition

Based on the results of the bioassay, it was observed that all strains exhibited a hemolytic zone except BCCO 10_1102, BCCO 10_1105 and S2 isolates. The size of the hemolytic zone varied among the isolates, with isolate BCCO 10_1092 showing the largest zone (5 mm).

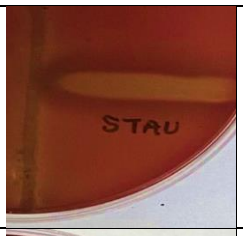
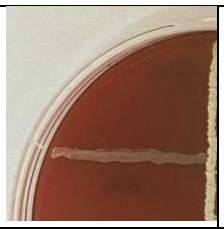
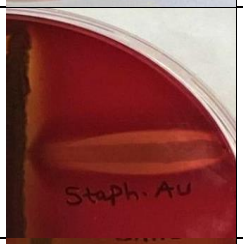
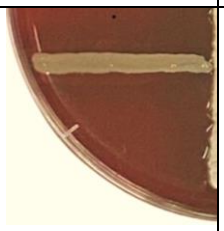
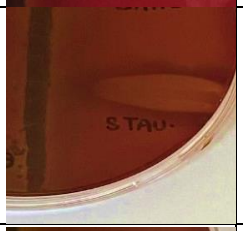
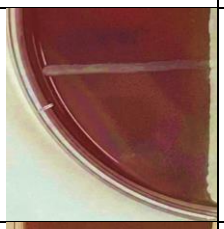
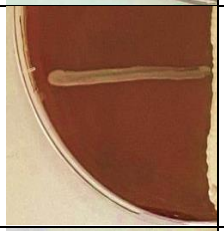
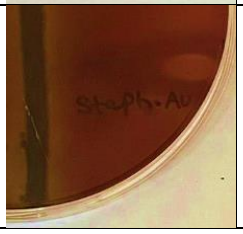
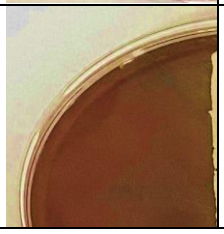
Moreover, the results showed that although all strains are not affecting the growth of *Staphylococcus aureus*, some of them exhibited inhibitory activity against β-hemolysis of *Staphylococcus aureus*.

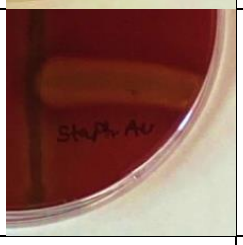
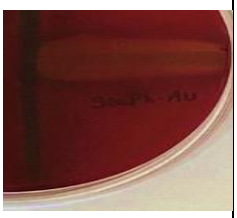
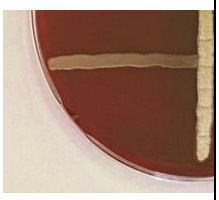
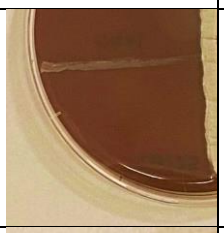
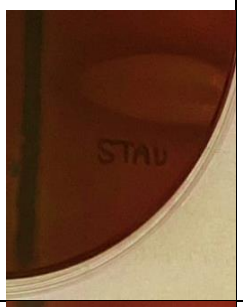
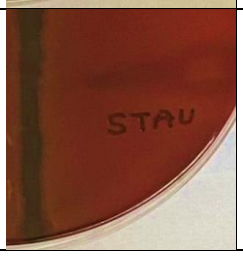
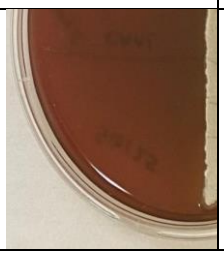
Furthermore, none of the tested isolates exhibited any inhibitory effects against *Pseudomonas aeruginosa* (gram-negative bacteria). The results also show that all strains, except BCCO 10_1102, BCCO 10_1105, and S2 isolates, have inhibitory activity against *Candida albicans*. It was observed that the isolates with a hemolytic zone also exhibited an inhibitory effect against *Candida albicans*, whereas BCCO 10_1102, BCCO 10_1105, and S2 isolates did not have a hemolytic zone or inhibitory effect.

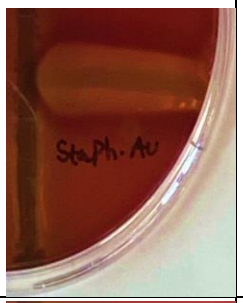
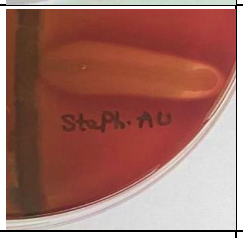
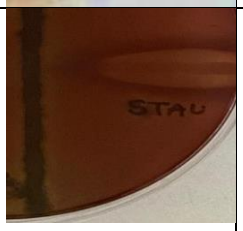
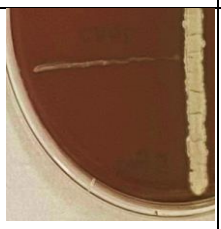
The most promising isolates (S6/6, S10/3, S10/25, and S18/15) were selected, and the bioassay was repeated at different incubation periods of the isolates to determine the optimum time required for the potential isolates to produce secondary metabolites.

Table 21: Table 21: Results of the bioassays of the potential Isolates after 72 hours. The strains were incubated for 3-6 days before adding *Staphylococcus aureus* and *Candida albicans* (same plate).

Code of Isolate	**Incubation period of the isolates**	**Size of hemolytic zone (mm)**	**Promising isolate (Vertical Line) *Staphylococcus aureus* (Horizontal Line)**	**Size of inhibitory zone of β-hemolysis of Staphylococcus aureus (mm)**	**Promising isolate (Vertical Line) Candida albicans (Horizontal Line)**	**Size of the inhibitory zone of CAAL. (mm)**

S6/6	2 days	1		2		Weak partial inhibition
S6/6	3 days	2		4		1
S6/6	4 days	3		9		Partial inhibition
S6/6	5 days	3		12		12
S6/6	6 days	4		Complete inhibition		Complete inhibition
S10/3	2 days	4		2		Partial inhibition
S10/3	3 days	4		1		1
S10/3	4 days	5		23		Complete inhibition

S10/3	5 days	5		13		20
S10/3	6 days	6		Complete inhibition		Complete inhibition
S10/25	2 days	1		0		0
S10/25	3 days	2		0		0
S10/25	4 days	3		6		Partial inhibition
S10/25	5 days	3		6		Partial inhibition
S10/25	6 days	3		23		Complete inhibition

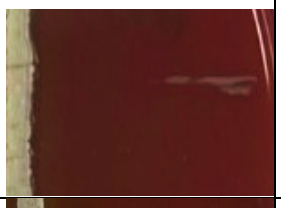
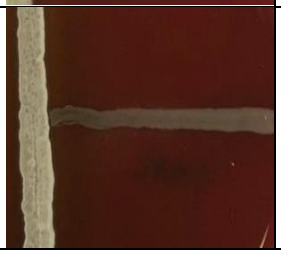
S18/15	2 days	2		0		Partial inhibition (Weak inhibition)
S18/15	3 days	2		0		Partial inhibition
S18/15	4 days	3		6		Partial inhibition
S18/15	5 days	3		11		Partial inhibition
S18/15	6 days	3		Complete inhibition		Complete inhibition

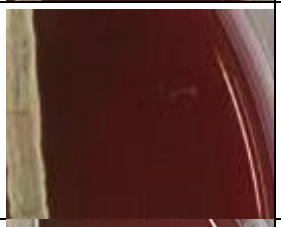
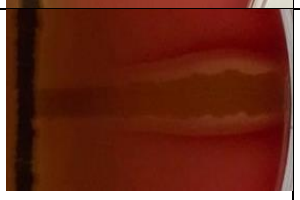
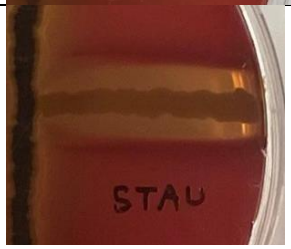
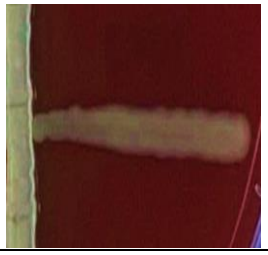
The results showed that the isolates have a significant inhibitory effect against *Candida albicans* and inhibition of the β-hemolysis of *Staphylococcus aureus* after 4 days of incubation and the highest inhibitory effect was observed on day 6.

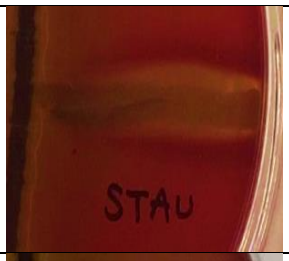
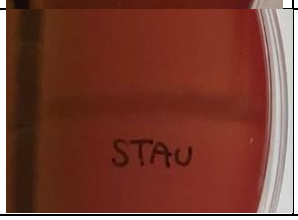
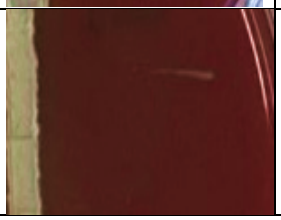
To test if the inhibitory effect is due to the secondary metabolite produced by the *Streptomyces* strains only or it is a synergistic effect, the bioassay will be repeated at which both pathogens (CAAL & STAU) will be inoculated separately against S10/3 after 3-6 days of incubation and

STAU only will be inoculated against the other isolates (S6/6, S10/25 & S18/15) after 3-6 days of incubation.

Table 22: Results of the bioassays of the potential Isolates after 72 hours. The strains were inoculated on blood agar and incubated for 3-6 days before adding *Staphylococcus aureus* and *Candida albicans* (different plates).

Code of Isolate	**Incubation period of the isolates**	**Size of hemolytic zone(mm)**	**Promising isolate (Vertical Line) *Staphylococcus aureus* (Horizontal Line)**	**Size of inhibitory zone of β-hemolysis of STAU. (mm)**	**Promising isolate (Vertical Line) *Candida albicans* (Horizontal Line)**	**Size of inhibitory zone of CAAL (mm)**
S6/6	3 days	2		1		Weak partial inhibition
S6/6	4 days	3		6		Partial inhibition
S6/6	5 days	4		12		4
S6/6	6 days	5		20		16
S10/3	3 days	4		4		Weak Partial inhibition

S10/3	4 days	5		16		19 (Partial inhibition)
S10/3	5 days	5		18		21
S10/3	6 days	6		Complete Inhibition		23
S10/25	3 days	3		1		Weak partial inhibition
S10/25	4 days	3		6		1
S10/25	5 days	4		Complete inhibition		5
S10/25	6 days	4		10		15
S18/15	3 days	2		1		Weak partial inhibition

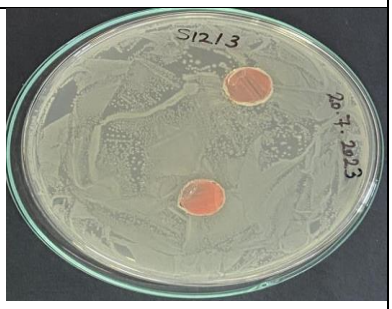
S18/15	4 days	3		6		1
S18/15	5 days	3		12		3
S18/15	6 days	4		Complete inhibition		15

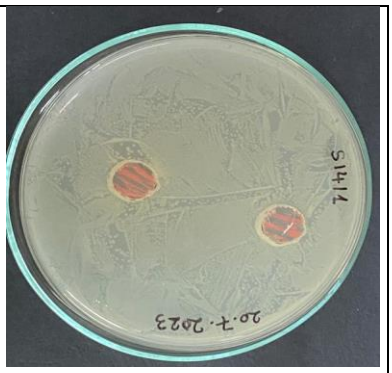
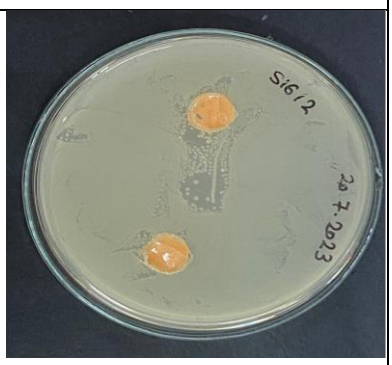
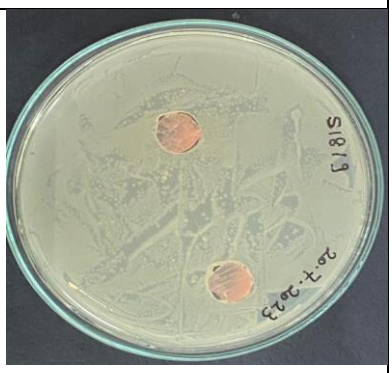
The results demonstrated that the inhibitory zone of *Candida albicans* is significantly larger when both *Streptomyces* and *Staphylococcus aureus* were present on the same plate, compared to when only *Streptomyces* was inoculated.

4.4.2. Bioassay against *Bacillus subtilis*

The results showed that none of the tested isolates exhibited any inhibitory effects against *Bacillus subtilis*, as evidenced by the absence of a halo zone (Table 23).

Table 23: Results of inhibitory activity against *Bacillus subtilis*.

Code of Isolate		**Code of Isolate**	
Control		**S12/3**	

S2/5		**S14/1**	
S6/6		**S16/2**	
S10/3		**S18/9**	
S10/25		**S18/15**	

4.4.3. Antifungal activity test

The inhibition zone of fungal strain CCF 4450 was measured and recorded after three weeks, while the inhibition zones of the other fungal strains were measured after four weeks. This difference in measurement time was due to the relatively faster growth rate of CCF 4450 compared to the other fungal strains.

Table 24: Results of the inhibitory activity against fungi obtained from different areas.

Code of fungal strain	**Strain Identification**	**Strains B1, B2 and B3**	**Strains B4 and B5**	**Strains B6 and B7**
CCF 4450	*Ophiostoma minus*	**Inhibition zone** B1: 2 mm B2: 13 mm B3: 0 mm	**Inhibition zone** B4: 1mm B5: 3 mm	**Inhibition zone** B6: 1mm B7: 2 mm
CCF 6041	*Akanthomyces muscarium*	**Inhibition zone** B1: 3 mm B2: 6 mm B3: 2 mm	**Inhibition zone** B4: 2 mm B5: 2 mm	**Inhibition zone** B6: 2 mm B7: 2 mm
CCF 3535	*Graphium asporum*	**Inhibition zone** B1: 6 mm B2: 8 mm B3: 0 mm	**Inhibition zone** B4: 2 mm B5: 2 mm	**Inhibition zone** B6: 2 mm B7: 2 mm
CCF 3546	*Ophiostoma piceae*	**Inhibition zone** B1: 3 mm B2: 7 mm B3: 0 mm	**Inhibition zone** B4: 6 mm B5: 2 mm	**Inhibition zone** B6: 2 mm B7: 2 mm

The results show that BCCO 10_1099 (B1) and BCCO 10_1104 (B2) strains, which were previously collected from Papua New Guinea, demonstrated moderate and strong inhibitory effects, respectively, against the four fungal strains.

The BCCO 10_1106 (B3) strain that was collected from Morocco, exhibited a weak inhibitory effect against fungal strain (CCF 6041), while it has no inhibitory activity against the other three fungal strains.

Moreover, the four isolates S6/6 (B4), S10/3 (B5), S10/25 (B6) and S18/15 (B7) that were collected from the Czech Republic, have a weak inhibitory effect against all fungal strains.

4.5. Genome Screening of antifungal polyenic secondary metabolites.

4.5.1. Data mining from polyene databases

Robust dataset of 26 linear and cyclic polyenes (macrolactone and macrolactam compounds) was created and used for the analysis of cytochrome P450 (CYP 450) and thioesterase type I (TE I) genes. They are listed in groups based on their structures together with bacterial strain, classification, database, and biological activity (Table 25).

Table 25: List of polyene secondary metabolites produced by *Streptomyces sp*. and phylogenetically close actinomycetes that were used in this study.

Compound	Bacterial strain	Classification	Biological activity	Database	Used for analysis of CYP 450	Used for analysis of TE
Macrolactone						
Amphotericin	*Streptomyces nodosus*-ATCC 14899	Heptaenes	Antifungal	JDB	✓	✓
Candicidin	*Streptomyces sp.* FR-008	Heptaenes	Antifungal	Mibig	✓	✓
Filipin	*Streptomyces avermitilis* MA-4680	Pentaenes	Antifungal and low anti-bacterial activity	Mibig	✓	✓
Nystatin	*Streptomyces noursei* ATCC 11455	Tetraenes	Antifungal	JDB	✓	✓
Pimaricin	*Streptomyces Natalensis ATCC-27448*	Tetraenes	Antifungal and anti-protozoal	JDB	✓	✓
Rimocidin	*Streptomyces diastaticus 108*	Tetraenes	Antifungal	JDB	✓	
Reedsmycins	*Streptomyces* Sp. OUC6819	Pentaenes	Antifungal	Mibig		✓
Eurocidin-D	*Streptomyces eurocidicus* ATCC 27428	Pentaenes	Antifungal	Mibig		✓
Lucensomycin	*Streptomyces achromogenes_ NBRC*-14001 *Streptomyces cyanogenus*	Tetraenes	Antifungal and antiviral activity.	Mibig		✓

Tetramycin	*Streptomyces hygrospinosus* CGMCC 4.1123	Tetraenes	Antifungal	Mibig		✓
NPP	*Pseudonocardia -sp.* AL041005-10	Tetraenes	Antifungal	NCBI		✓
pentamycin	*Streptomyces sp.* S816	Pentaenes	Antifungal	NCBI		✓
Macrolactam						
Auroramycin	*Streptomyces filamentosus* *strain* NRRL 15998	Pentaenes	Antifungal, Anti-MRSA	Mibig NCBI		✓
ML-449	*Streptomyces sp.* MP39-85	Tetraenes	Anti-bacterial, Cytotoxicity	Mibig		✓
BE-14106	*Streptomyces sp.* DSM-21069	Tetraenes	Anti-bacterial, Cytotoxicity	Mibig		✓
Sipanmycin	*Streptomyces sp.* CS149	Pentaenes	Cytotoxicity	Mibig		✓
Hitachimycin	*Streptomyces scabrisporus* CMB-0406	Triene	Anti-protozoal antifungal	Mibig		✓
Heronamide_C	*Streptomyces sp.* CMB-0406	Tetraenes	No anti-bacterial activity and cytotoxicity	Mibig		✓
Sceliphrolactam	*Streptomyces sp.*SD85	Pentaenes	Antifungal	NCBI		✓
Bombyxamycin A	*Streptomyces sp.*SD53	Pentaenes	Anti-bacterial, cytotoxicity	NCBI		✓
cremimycin	*Streptomyces sp.* MJ635-86F5	Triene	Anti-bacterial (G+), cytotoxicity	NCBI		✓
Linear						
Linearmycin A	*Streptomyces sp. Mg1*		Antifungal	Mibig		✓
Mediomycin A	*Streptomyces blastmyceticus*		Antifungal	Mibig		✓
Neomediomycin	*Streptomyces sp. RK95-74*		Antifungal	Mibig		✓

4.5.2 Analysis of Cytochrome P450

Amino acid sequences of cytochrome P450 from 6 polyene biosynthetic gene clusters (BGCs) were used for multiple sequence alignment as depicted in Figure 25. The majority of those polyene BGCs contain two cytochrome P450 genes, with the exception of Candicidin and Rimocidin BGCs.

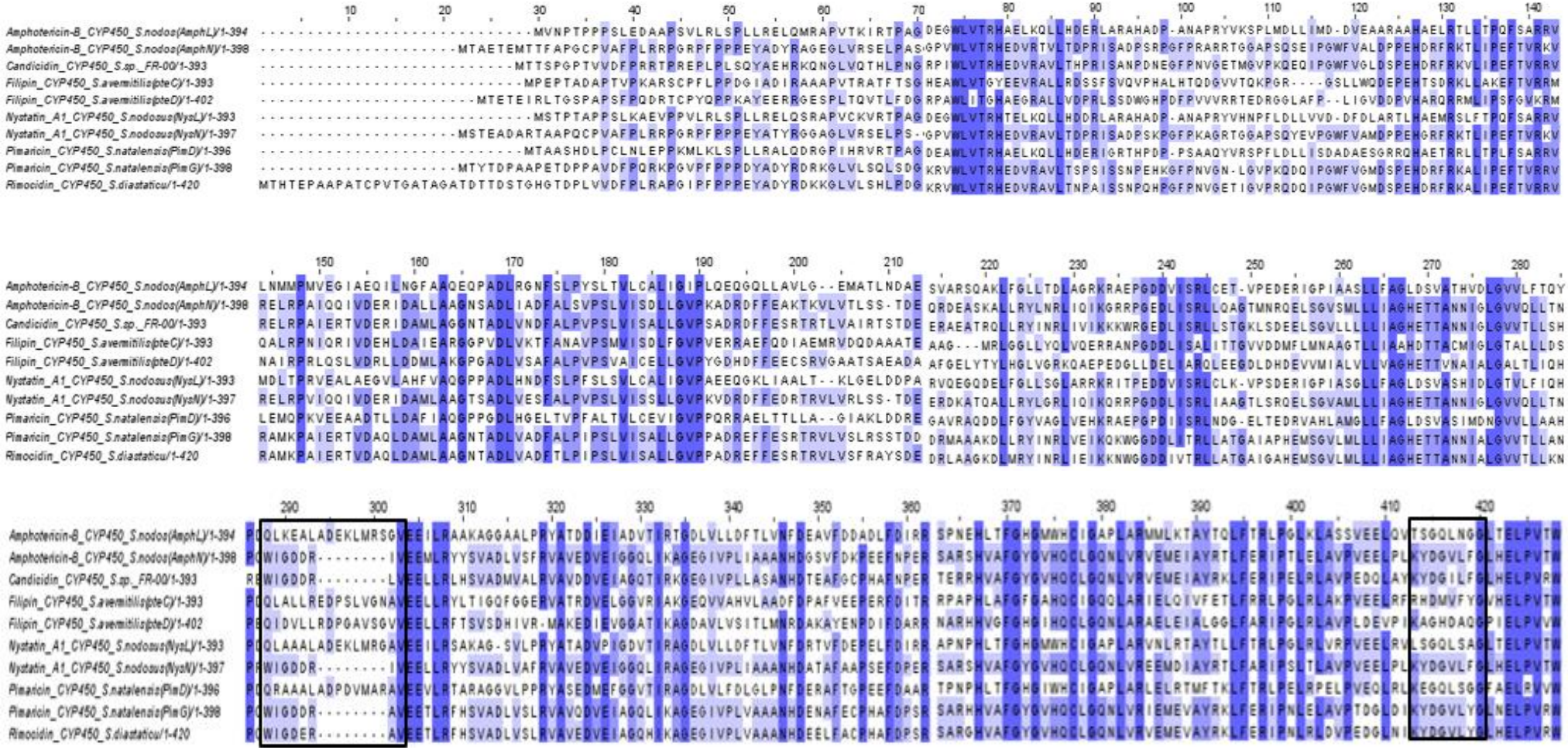


Figure 25: Multiple sequence alignment of the amino acid sequences of cytochrome P450. The highly conserved amino acid residues were highlighted in dark blue, and the sequences used for designed primers in a previous study are indicated with black boxes.

The results demonstrate the presence of the regions where the amino acid residues are highly conserved across the CYP sequences of the six-polyene BGCs. Based on the alignment, a phylogenetic tree was constructed to determine the similarity of the sequences among the CYPs from different BGCs (Figure 26).

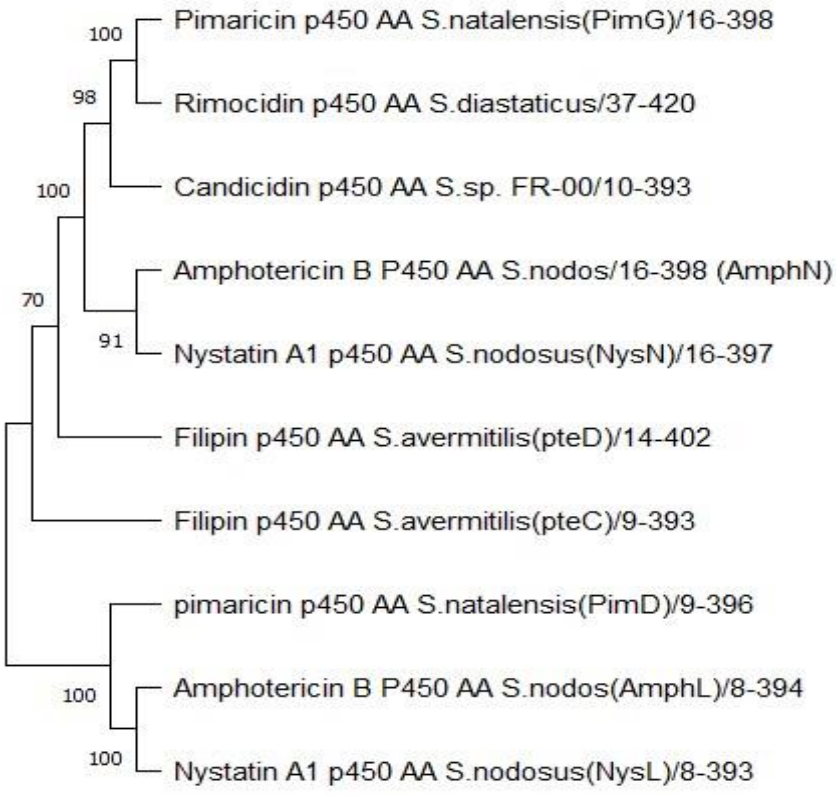


Figure 26: Phylogenetic tree of CYP450 gene extracted from BGCs coding for the 6 different polyenes.

This analysis showed that cytochrome P450 genes encoded by the polyene clusters can be classified into two distinct similarity groups within the constructed tree. As shown in Figure 26, the first group consists of PimG, AmphN, NysN, PteD, RimG and fscP whereas the second group includes PimD, AmphL, PteC and NysL.

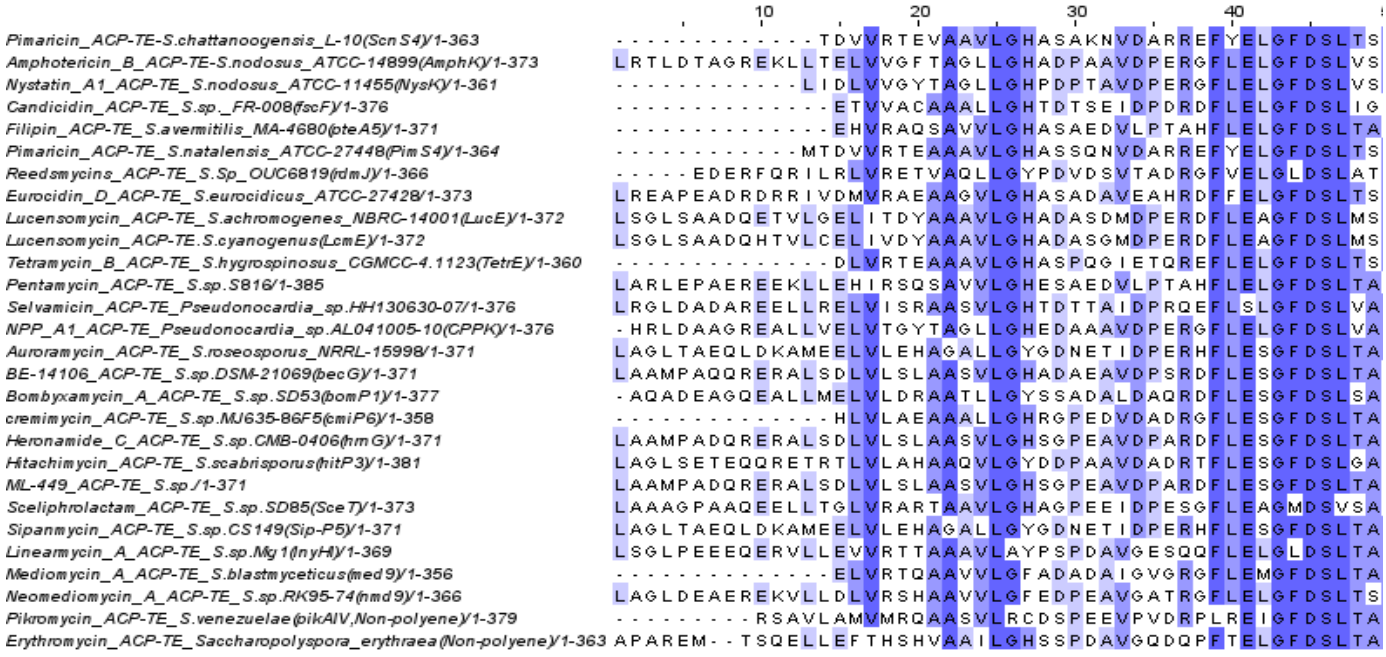
Upon searching for more polyene biosynthetic gene clusters (BGCs) for further analysis of cytochrome P450 (CYP) sequences, it has been discovered that certain polyenes, such as Reedsmycin (a cyclic polyene), and linear polyenes like Linearmycin-A, Mediomycin-A, and Neomediomycin-B, do not possess the genes encoding for cytochrome P450 as well as ferredoxin.

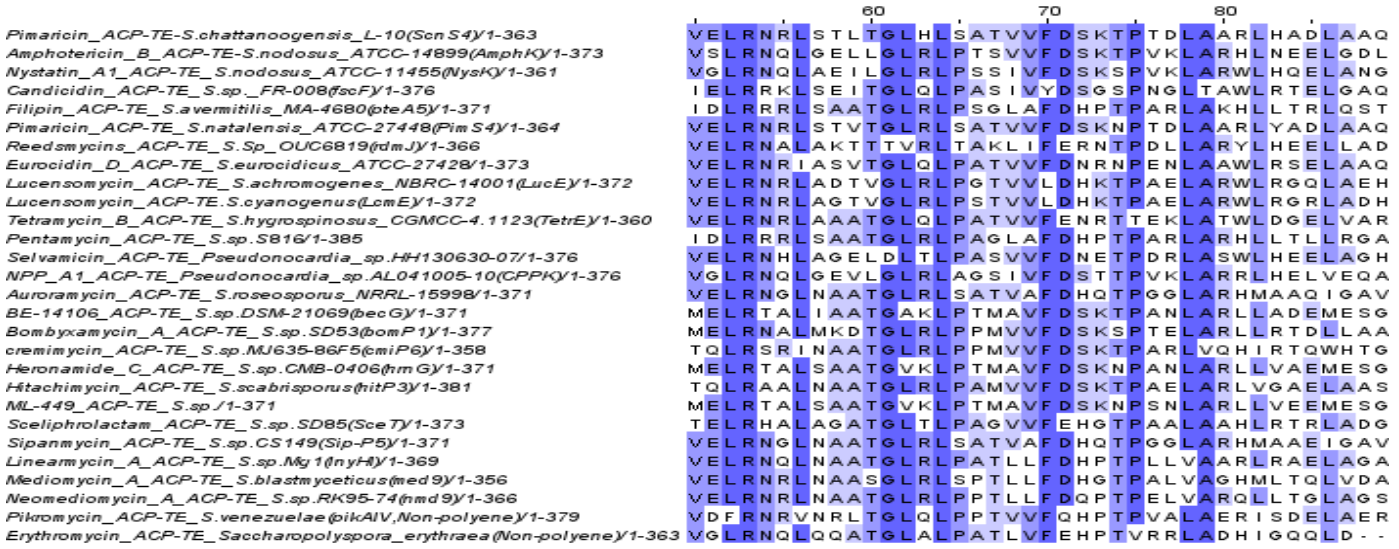
Therefore, the alternative hypothesis was formulated, and the dataset was expanded by including an additional 20 polyene and 2 non-polyene biosynthetic gene clusters (BGCs).

4.5.3 Analysis of Thioesterase type I

Since thioesterase type I is associated with the acyl carrier protein (ACP) of the last module of type I polyketide synthase, the nucleotide sequence of the whole module was downloaded. The analysis of the nucleotide sequences of the last module of type I polyketide synthase that associated with thioesterase type I revealed that there is only one starting codon and one stop codon, which means that the genes within the module cannot be synthesized individually but rather as a whole module. The nucleotide sequences of the last module of 26 polyenes and 2 non-polyenes were translated and the amino acid sequences were used for multiple sequence alignment. Then the aligned amino acid sequences of thioesterase type I (TE I) and acyl carrier protein (ACP) were extracted.

Then the hypothesis was tested by analyzing the sequence similarity of the gene responsible for encoding TE I within the dataset. The results of the multiple sequence alignment of thioesterase gene were compared to the results provided by Zhou, Yucong, et al [29].

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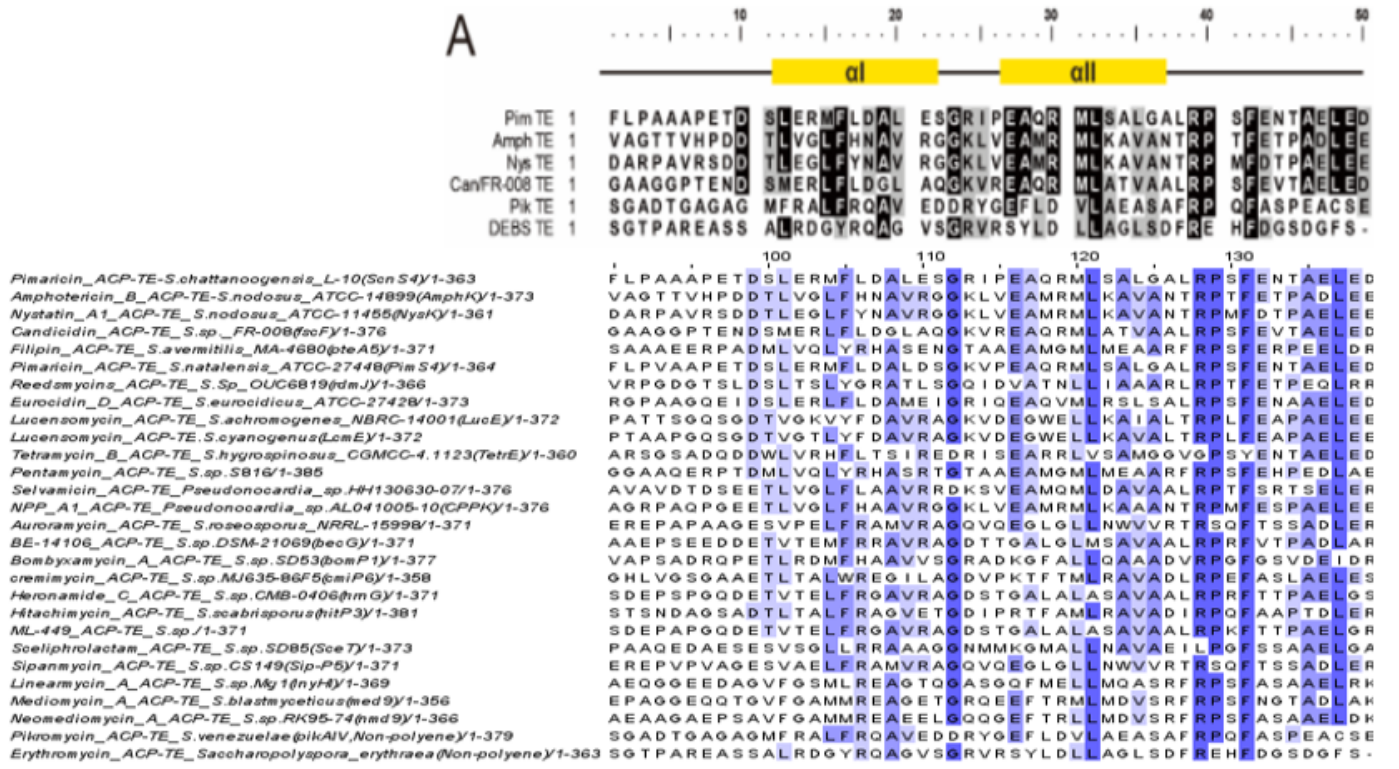
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Tetramycin_B_ACP-TE_S.hygrospinosus_CGMCC-4.1123(TetrE)Y1-360
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cremimycin_ACP-TE_S.sp.MJ635-86F5(cmiP6)Y1-358
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Neomediomycin_A_ACP-TE_S.sp.RK95-74(nmd9)Y1-366
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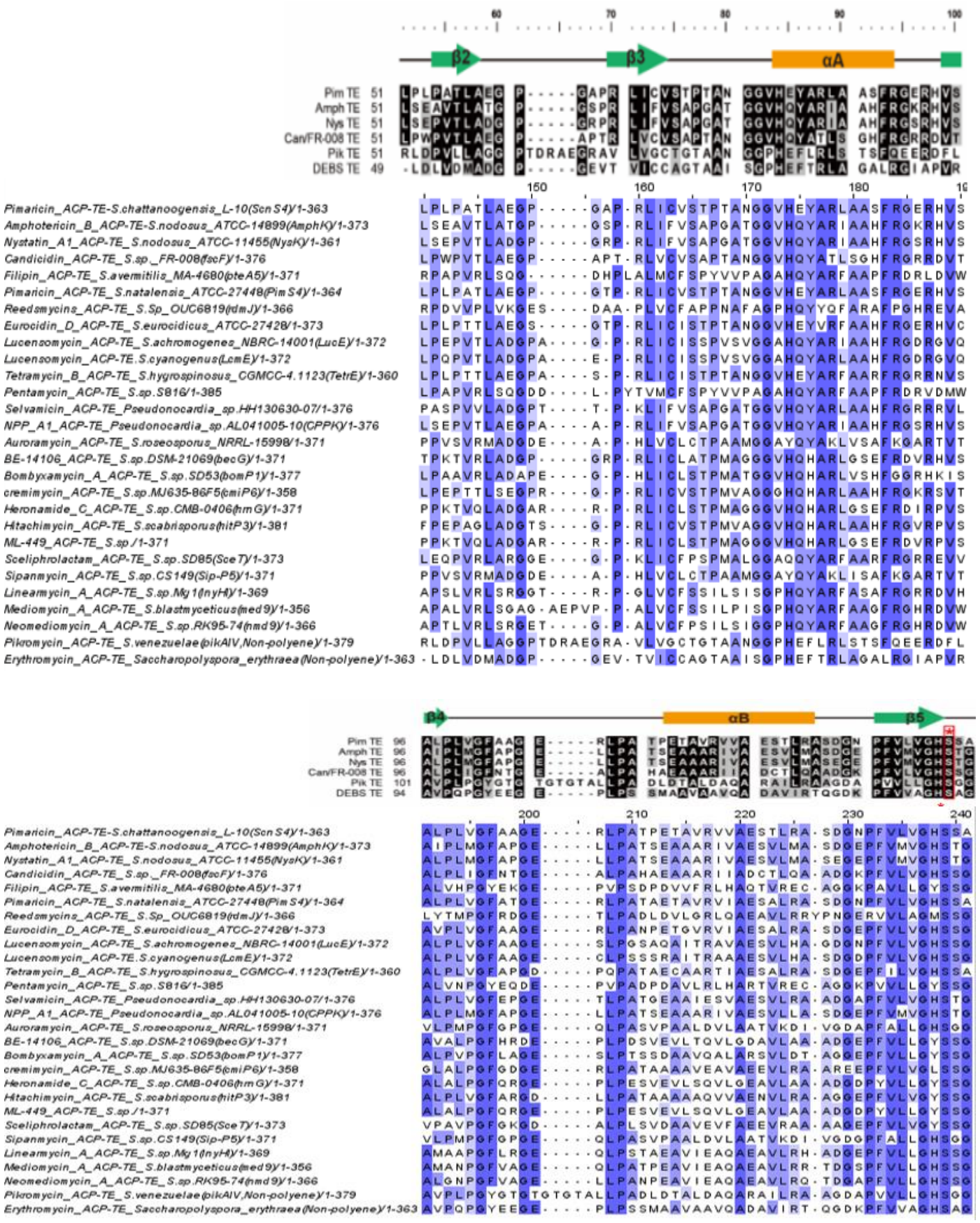
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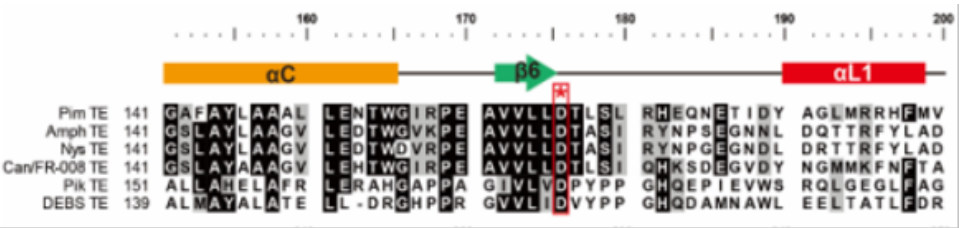
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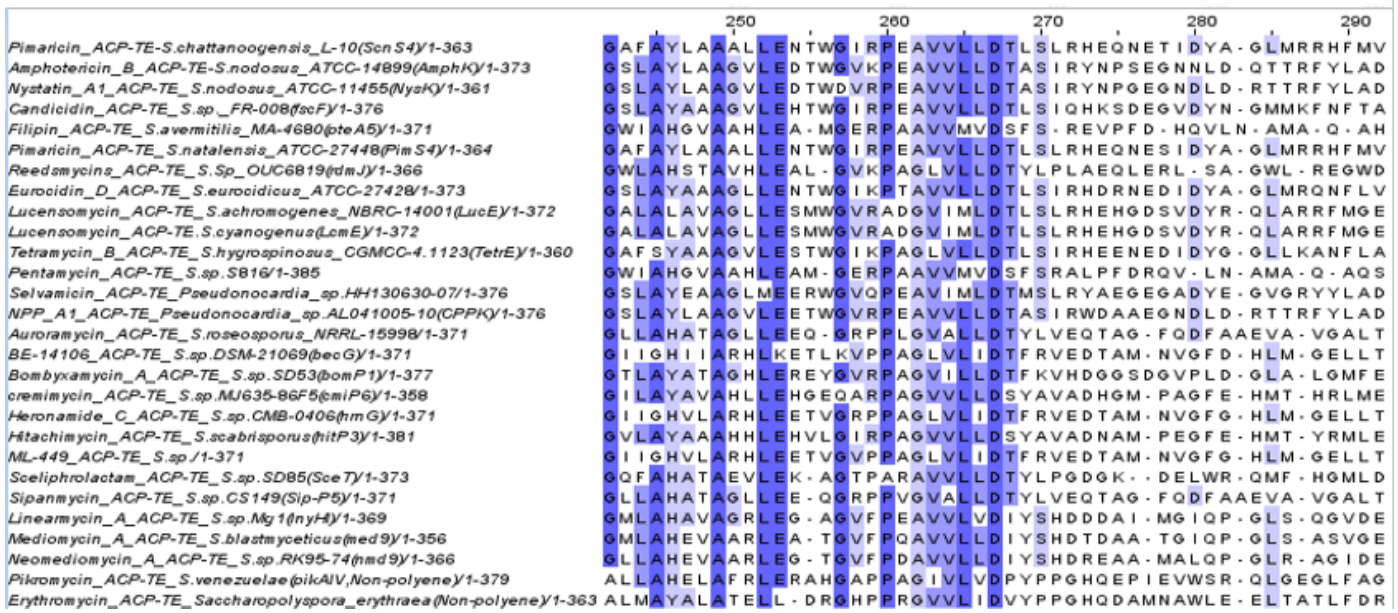
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Pimaricin_ACP-TE_S.natalensis_ATCC-27448(PimS4)1-364 GAFAYLAAALLENTWGIRPEAVVLLDTLSLRHEQNESIDYA-GLMRRHFMV
Reedsmycins_ACP-TE_S.Sp_OUC6819(rdmJ)1-366 GWLAHSTAVHLEAL-GVKPAGLVLLDTYLPLAEQLERL-SA-GWL-REGWD
Eurocidin_D_ACP-TE_S.eurocidicus_ATCC-27428/1-373 GSLAYAAAGLLENTWGIKPTAVVLLDTLSIRHDRNEDIDYA-GLMRQNFLV
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β7
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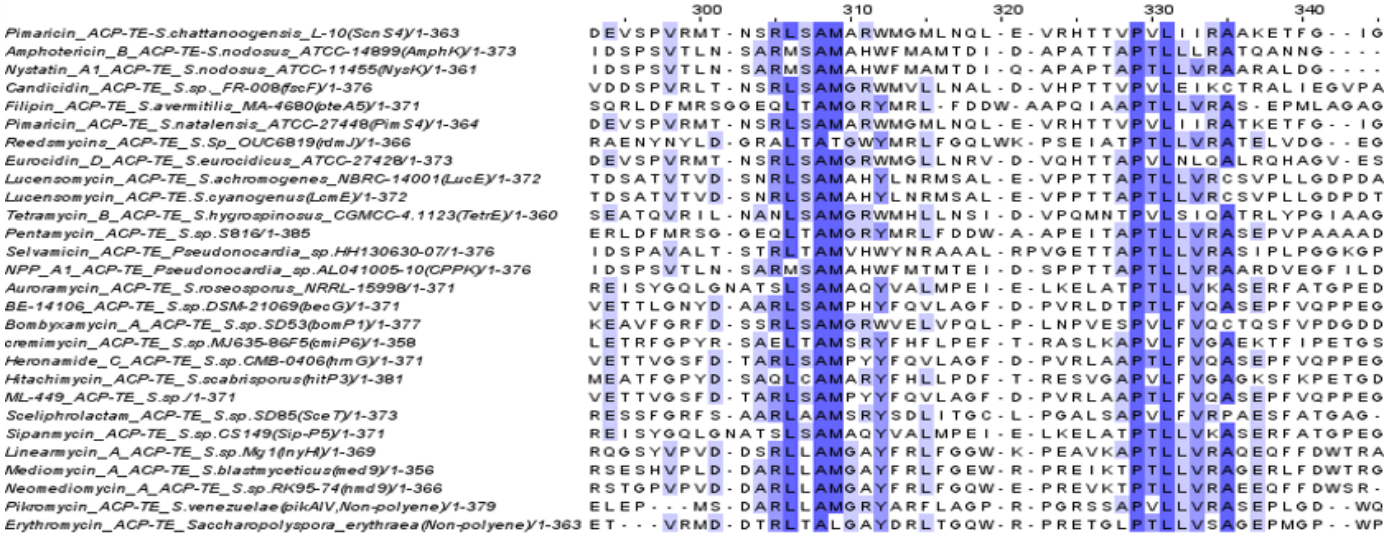
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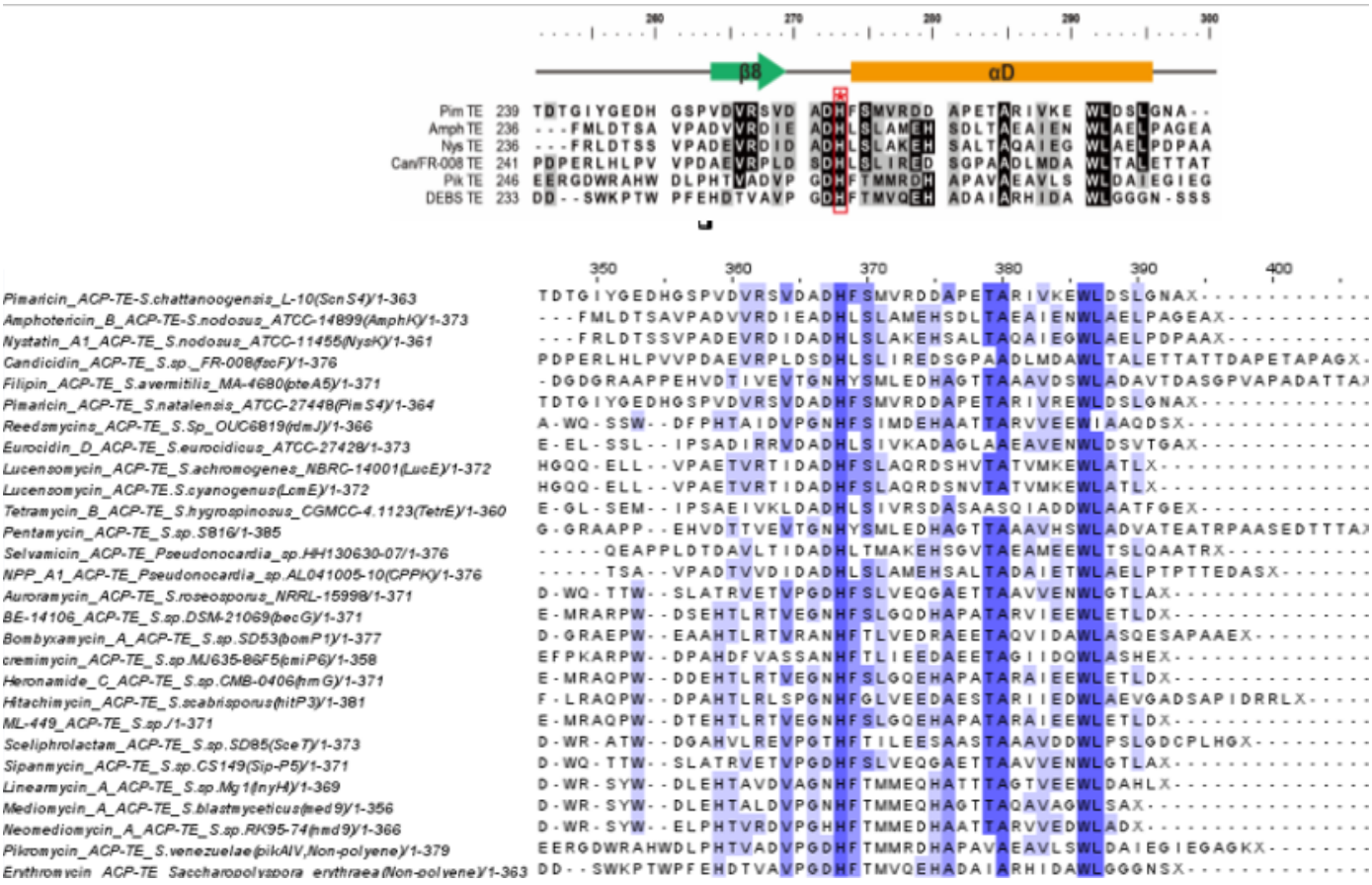
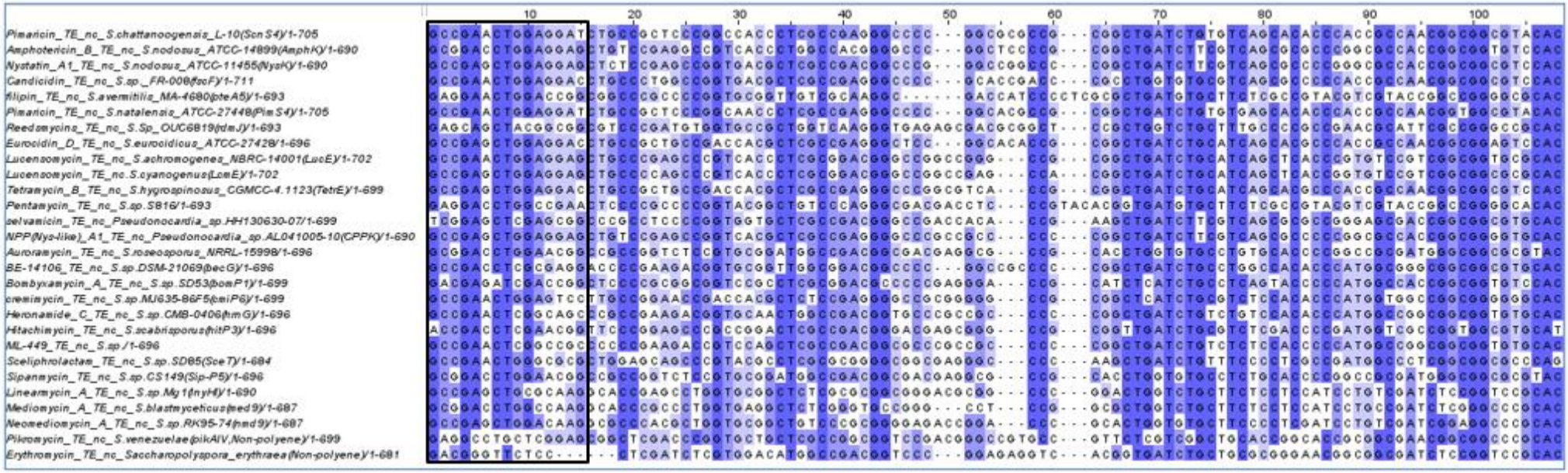


Figure 27: Multiple sequence alignment of amino acid residues of TE type I gene associated with ACP of the last module of polyketide synthase (PKS). The result of the alignment of thioesterase gene was compared to the results of the previous study [29]

The results demonstrated that the ACP gene is highly conserved among both polyene and non-polyene polyketide synthases, since it codes for important functional domains that are crucial for its activity. Moreover, the analysis of the multiple sequence alignment of the comprehensive dataset and its comparison to the aligned sequence in previous literature indicated that the cyclic (macrolactone and macrolactam) and linear polyenes share conserved domains with some variability in this region. Although different amino acids may be present within the conserved regions, these variations do not alter the electric charges and the overall geometry of the conserved domain.

Then the highly conserved region of the thioesterase for the polyenes from amino acid 45 to 387 was extracted and translated back into nucleotide sequences for further analysis at the nucleotide level and for the purpose of designing primers.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Lucensomycin_TE_nc.S.cyanogenus(LcmE)/1-702
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selvamicin_TE_nc_Pseudonocardia_sp.HH130630-07/1-699
NPP(Nys-like)_A1_TE_nc_Pseudonocardia_sp.AL041005-10(CPPK)/1-690
Auromycin_TE_nc_S.roseosporus_NRRL-15998/1-696
BE-14106_TE_nc_S.sp.DSM-21069(becG)/1-696
Bombyxamycin_A_TE_nc_S.sp.SD53(bomP1)/1-699
cremimycin_TE_nc_S.sp.MJ635-86F5(cmiP6)/1-699
Heronamide_C_TE_nc_S.sp.CMB-0406(hmG)/1-696
Hitachimycin_TE_nc_S.scabrisporus(hitP3)/1-696
ML-449_TE_nc_S.sp./1-696
Sceliphrolactam_TE_nc_S.sp.SD85(SceT)/1-684
Sipanmycin_TE_nc_S.sp.CS149(Sip-P5)/1-696
Linearmycin_A_TE_nc_S.sp.Mg1(nyH)/1-690
Mediomycin_A_TE_nc_S.blastmyceticus(med9)/1-687
Neomediomycin_A_TE_nc_S.sp.RK95-74(nmd9)/1-687
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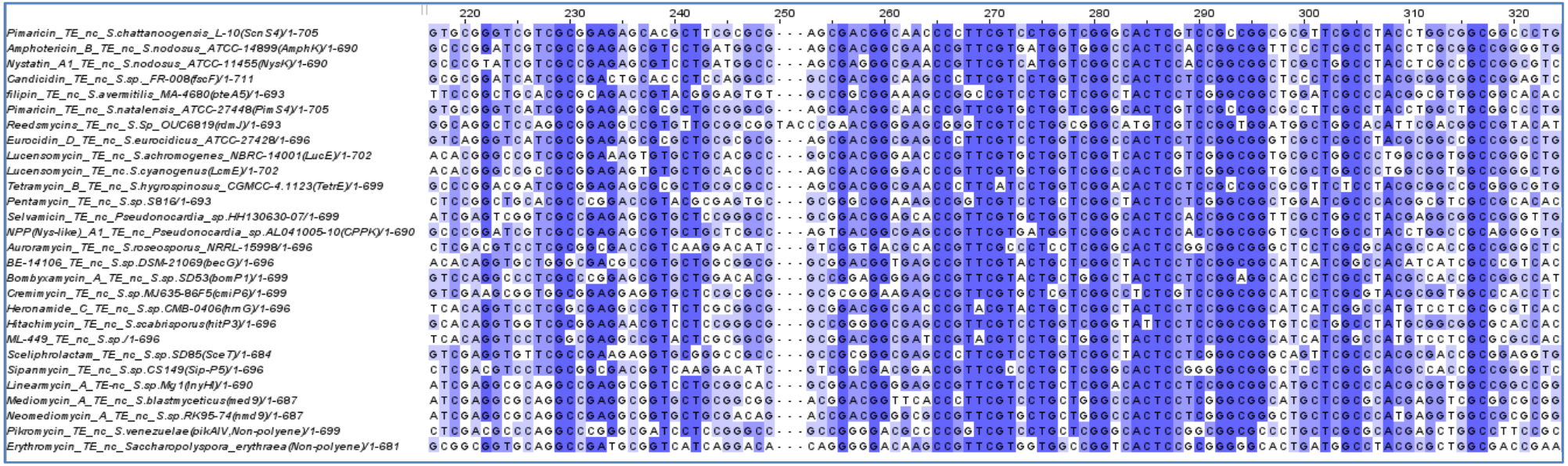
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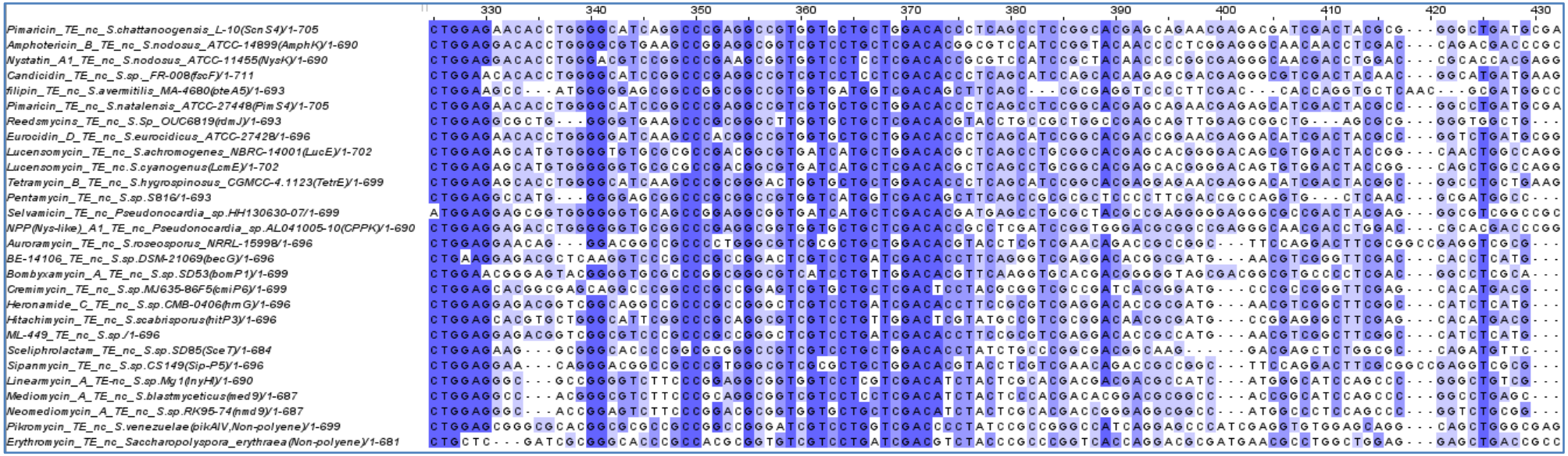
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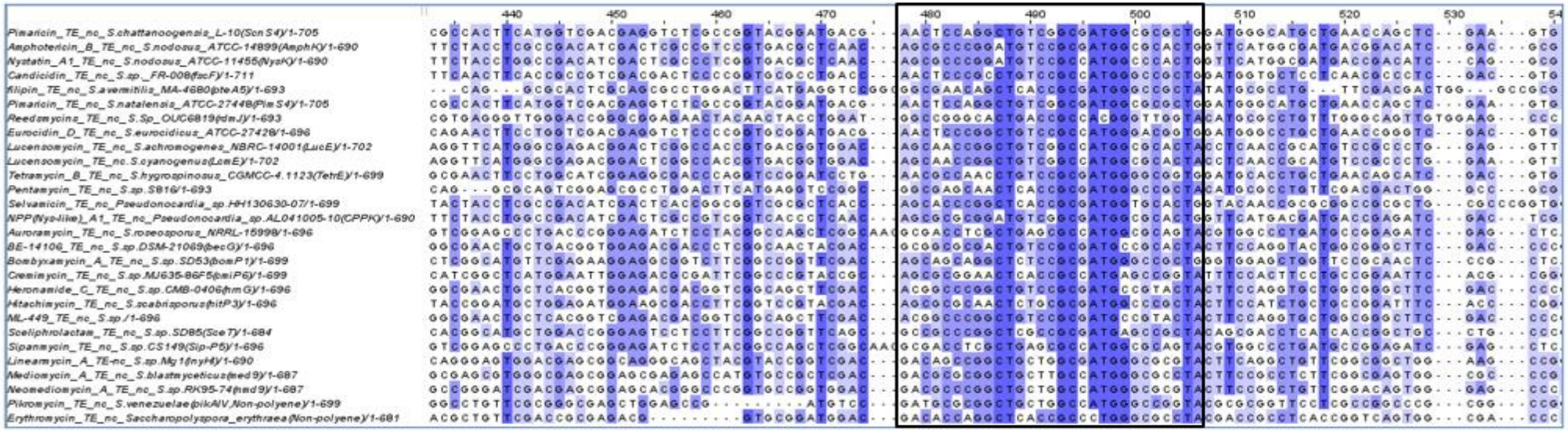
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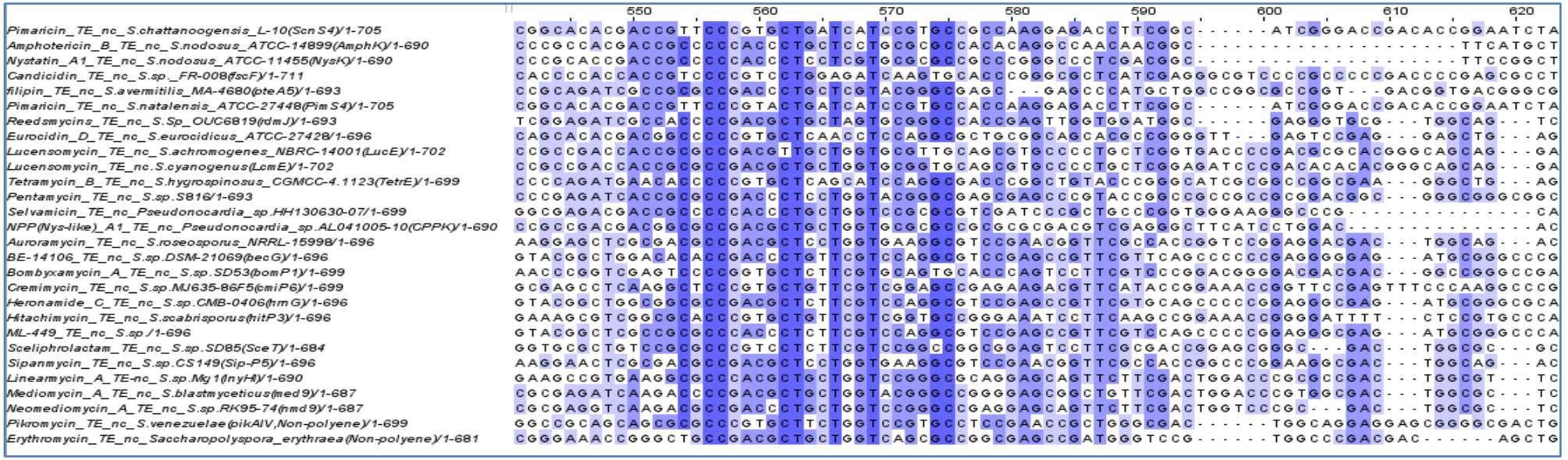
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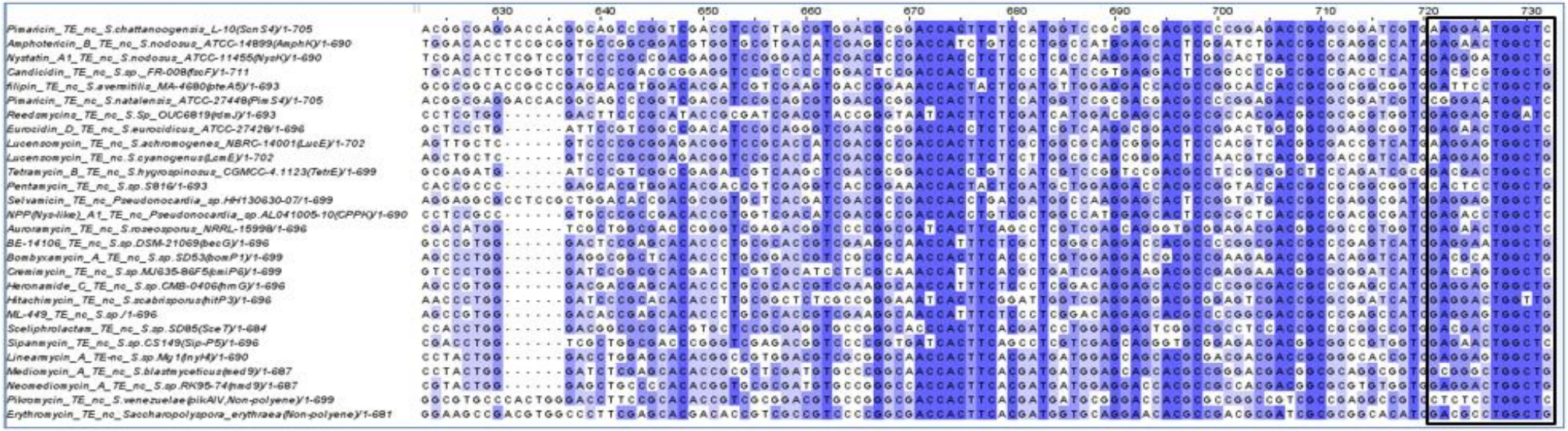

Figure 28: Multiple sequence alignment of the conserved region of TE type I nucleotide sequences. The regions of interest that could be used for designing the primers are indicated with black boxes.

The analysis of the coding region of the thioesterase gene showed that there is a sequence variability at the level of nucleotides so that the degenerative positions needed to be used.

4.5.4. Designing of primers

Factors such as G+C content, length, melting temperature (Tm) and degeneracy should be considered during designing of primers as they provide optimal specificity and efficiency in PCR amplification.

The results of the designed primers are listed in table 26, analyzed and compared. Then the best option for the primer pairs was selected based on the factors mentioned above.

Table 26: List of the ten primers that were designed by Geneious software version R8.1.9. The best primer pair is highlighted in green.

Primer pairs	Sequences	length	Interval	%GC	Tm	Hairpin Tm	Dimer Tm	Product size
1	**TE-Forward** 5`GCCGAGCTGGAGGAGC3`	16	1-16	75.0	59.5	49.6	6.4	501
	TE-Reverse 5`GCCCATCGCGGACAG3`	15	501-487	72.0	62.9	None	None	
2	**TE-Forward** 5`GCCGAGCTGGAGGAGC3`	16	1-16	75.0	59.5	49.6	6.4	499
	TE-Reverse 5`CAGCAGGTGGAAGTAGCGC3`	19	499-481	63.2	61.1	None	None	
3	**TE-Forward** 5`GCCGAGCTGGAGGAGC3`	16	1-16	75.0	59.5	49.6	6.4	499
	TE-Reverse 5`CAGCAGGTGGAAGTAGCG3`	18	499-482	61.1	57.8	None	None	
4	**TE-Forward** 5`GGCCCGCGGCTGATC3`	15	51-65	80.0	60.3	49.6	19.3	500
	TE-Reverse 5`GGCCCGGACGAGCAG3`	15	550-536	80.0	59.4	None	2.8	
5	**TE-Forward** 5`GGCCCGCGGCTGATC3`	15	51-65	80.0	60.3	49.6	19.3	499
	TE-Reverse 5`GCCCGGACGAGCAGC3`	15	549-535	80.0	60.2	None	None	
6	**TE-Forward**	16	53-68	75.0	60.7	43.7	22.9	500

	5`CCCGCGGCTGATCTGC3` **TE-Reverse** 5`GTGGCCCGGACGAGC3`	15	552-538	80.0	59.7	None	None	
7	**TE-Forward** 5`TGATCTGCGTCAGCACCC3` **TE-Reverse** 5`GAACGGCTCGGTGGCC3`	18 16	61-78 562-547	61.1 75.0	59.7 60.5	50.4 47.9	2.7 5.1	502
8	**TE-Forward** 5`TGATCTGCGTCAGCACCC3` **TE-Reverse** 5`ACGGCTCGGTGGCCC3`	18 15	61-78 560-546	61.1 80.0	59.7 61.6	50.4 48.8	2.7 8.6	501
9	**TE-Forward** 5`TGATCTGCGTCAGCACCC3` **TE-Reverse** 5`AACGGCTCGGTGGCC3`	18 15	61-78 561-547	61.1 73.3	59.7 59.2	50.4 47.9	2.7 5.1	500
10	**TE-Forward** 5`TGATCTGCGTCAGCACCC3` **TE-Reverse** 5`AACGGCTCGGTGGCCC3`	18 16	61-78 561-546	61.1 75.0	59.7 62.2	50.4 48.8	2.7 8.6	501

The analysis of the results indicated that primer pair 1 is the optimal choice. This is because not only does it fulfill the required criteria, but upon examining the binding regions for this primer pair, it was observed that these regions were highly conserved. Consequently, designing a less degenerate pair was possible.

Then the sequences of the best primer pair were utilized to design degenerate primers with the objective of targeting a minimum of 50% of the polyenes present in the dataset.

Table 27: Degenerate PCR primer pair properties.

Sequence	**Length**	**Tm**	**%GC**	**Hairpin Tm**	**Self-Dimer Tm**	**Degeneracy**
TE-Forward 5`GCCGARCTGGAGSAGC3`	16	52.5 - 56.6	66.7-73.3	45.2	6.4	4
TE-Reverse 5`SCCCATCGCGGACAG3`	15	56.3 - 57.1	73.3	36.6	None	2

4.5.5. *In silico* PCR

The specificity of the primer pair against the *Streptomyces* genomes was assessed by conducting *in silico* PCR amplification.

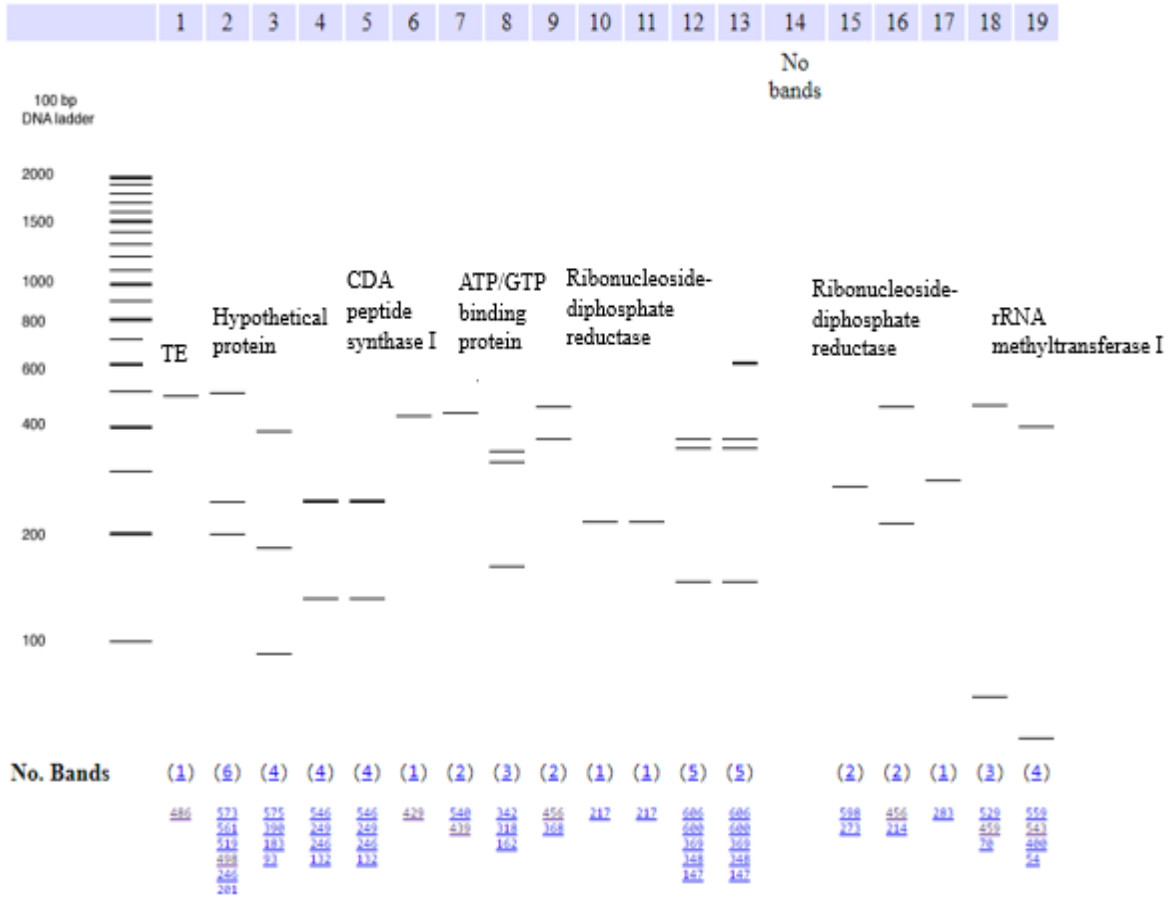

Figure 29: *In-silico* PCR gel illustrating the amplified genes.

The results of the analysis showed that the primer pair, which was designed to target the thioesterase gene, exhibited some degree of non-specific binding. This non-specific binding resulted in the amplification of the intended target as well as other genes with the same band size (around 500 bp).

5. Discussion

The results of the bacterial isolation indicated that chitin and ATCC media were the most efficient media for promoting the growth of *Streptomyces* strains from studied samples. Most of the *Streptomyces*-like strains were isolated from chitin medium. This confirmed that chitin medium enhances the growth of *Streptomyces*, which have a slower growth rate compared to many other common bacteria [8]. One possible explanation is that *Actinobacteria* (e.g. *Streptomyces sp*.) have the ability to utilize chitin as a carbon and nitrogen source by secreting chitinases that degrade chitin into smaller molecules, which can then be taken up and utilized by the bacteria for growth and metabolism. This adaptation allows *Streptomyces* to grow in environments rich in chitin [47].

Moreover, the results of the morphological characterization of the isolates showed that isolates from samples S6, S10 and all of sample S18 except for isolates S18/8 and S18/9 exhibited similar morphological characteristics. Sample S2 appears to resemble *Streptomyces*, although it exhibits distinct morphology compared to samples S6, S10 and S18. Isolate S16/2 exhibited pale orange colonies that resemble actinomyces strain. However, isolates S18/8 and S18/9 exhibited unusual morphological features that were not typical of actinomycetes. Further analysis as BOX-PCR and 16S rRNA identification were done for confirmation of their identity after the DNA extraction of the isolates. A high-quality DNA sample will have a ratio of absorbance values, known as the A_{260}/A_{280} ratio, ranging from 1.7 to 2.0 [45] and the optimal A_{260}/A_{230} ratio is approximately 2 [46]. The results of measuring the purity of the DNA extracts showed that the A_{260}/A_{280} ratio of the DNA samples ranged from 1.87 to 1.97, indicating that they are of good quality. However, the DNA samples of isolates S10/25 and S18/5 had relatively low $A_{260/230}$ indicating that the samples may contain some organic compounds. The results of the BOX-PCR showed that isolates S6/6, S10/3, S10/15, S10/20, S10/25, S18/1, S18/5, S18/15, and S18/17 shared the same genomic fingerprint on the 1% agarose gel. This suggests that although they are collected from different trees, it is very likely that they share the same BGCs, indicating that they belong to the same strain. Furthermore, the results of 16S rRNA identification via blastn analysis revealed that only two isolates (S16/2, S18/8) do not belong to *Streptomyces* species and the majority of the bacterial samples (S2, S6, S10, and S18 except for isolates S18/8 and S18/9) share the closest relatives within *Streptomyces* genus, in particular *Streptomyces flavogriseus* or *Streptomyces flavovirens*, but with variable percentage of identity. This suggests that these samples S6, S10 and S18 except for isolates S18/8 and S18/9 may belong to the same *Streptomyces* strain. On the other hand, sample S2 may belong to different species or strain. However, the identification of bacterial species based solely on 16S rRNA gene sequence

analysis has its limitations as the resolution to distinguish between strains at the species level is too low. Moreover, even if strains show more than 99% similarity, there is no guarantee that they belong to the same species [48]. To obtain a more comprehensive understanding of the taxonomic classification and relationships among these isolates, further analyses using additional analysis would be necessary. These could include whole-genome sequencing or multi-locus sequence analysis (MLSA), which provide a more detailed characterization of bacterial isolates at both the species and strain levels [48].

Furthermore, the bioassay results indicated that all strains, except for BCCO 10_1102, BCCO 10_1105, S2/1, S2/2, S2/3, and S2/5 isolates, exhibited a hemolytic zone. The presence of a hemolytic zone indicates that the strains have the potential to induce hemolysis, which involves the rupture of erythrocytes and serves as a virulence factor [19]. Hemolysins not only affect erythrocytes but can also damage other eukaryotic cells. They are typically lytic proteins, such as enzymes, channel-forming porins or, less frequently, polyene antibiotics [19]. In addition, the results demonstrated that the strains (BCCO 10_1102, BCCO 10_1105, S2/1, S2/2, S2/3, and S2/5) that have no hemolytic activity, possess no inhibitory activity against *Candida albicans*. On the other hand, the strains that showed a β-hemolytic activity also exhibited inhibitory effects against *Candida albicans.* This suggests a possible correlation between hemolysis and antifungal activity. Moreover, some samples (BCCO 10_1093, BCCO 10_1095, BCCO 10_1099, S6, S10, S18) inhibited the β-hemolysis of *Staphylococcus aureus*. The β-hemolysis exhibited by *Staphylococcus aureus* is attributed to the production of an enzyme called beta-hemolysin or sphingomyelinase [49]. The exact mechanism by which *Streptomyces* inhibits the beta hemolysis of *Staphylococcus aureus* is still unclear. However, it is known that *Streptomyces species* are well-known producers of various secondary metabolites, including antibiotics and other bioactive compounds so one possible mechanism could be the production of antimicrobial substances by *Streptomyces* as actinomycin D that directly target and inhibit the production of virulence factors of *Staphylococcus aureus* [50]. Another suggested mechanism could involve the production of enzymes by *Streptomyces* that degrade the β-hemolysin produced by *Staphylococcus aureus*.

Based on the results of the bioassays at different incubation periods, the isolates exhibit a significant inhibitory effect against *Candida albicans* as well as inhibition of the beta hemolysis of *Staphylococcus aureus* after 4 days of incubation. Furthermore, the highest inhibitory effect was observed on day 6, indicating that the cultivation of *Streptomyces* for 4 - 6 days prior to the inoculation of indicator strain is the optimal time for the production of tested antimicrobial activity. The growth of the *Streptomyces* can be related to the inhibitory effects observed against

Candida albicans and the β-hemolysis of *Staphylococcus aureus*. Bacterial growth is divided into four phases: lag, exponential, stationary, and death [51]. The results suggested that the studied *Streptomyces* strains reached the late exponential phase, which is characteristic by production of secondary metabolites with antimicrobial properties after cultivating these strains for 4-6 days on Muller-Hinton agar with sheep blood (5%) (KHM agars).

Moreover, the results of the bioassays indicate that the presence of both *Streptomyces* and *Staphylococcus aureus* on the same plate leads to a significantly larger inhibitory zone against *Candida albicans*, compared to when only *Streptomyces* is inoculated. This suggests that there is a synergistic effect of these two bacteria leading to enhanced production of antimicrobials targeting *Candida albicans*. One possible explanation for this enhanced antifungal activity is that *Streptomyces* may produce secondary metabolites, that have antifungal properties. In addition to that *Staphylococcus aureus* may produce volatile metabolites (e.g. N-(2,5-Dicyano-3,4-dihydro-2H-pyrrol-2-yl)-acetamide and Benzyl methyl ketone) that possess antifungal properties [52]. Therefore, when both bacteria are present together, their combined action of releasing antimicrobial compounds could potentially enhance the overall antifungal activity against *Candida albicans*. Another explanation is that *Staphylococcus aureus* is known for its ability to synthesize compounds that act as elicitors. These elicitor compounds play a crucial role in stimulating the biosynthesis of various antimicrobial secondary metabolites, such as antibiotics and antifungal agents in *Streptomyces* [53] .

The results of the bioassay against *Bacillus subtilis* indicated the absence of halo zone, showing that none of the tested isolates demonstrated any inhibitory effects against *Bacillus subtilis*. This suggests that these isolates do not produce any compound with antimicrobial properties against this bacterium.

The results of the antifungal activity test showed that both BCCO 10_1099 (B1) and BCCO 10_ 1104 (B2) strains exhibited an inhibitory effect against the four fungal strains that were obtained from different areas (Czech Republic, Poland, and Hungary). This suggests that the antifungal compounds produced by these strains have a broad spectrum of activity and are not restricted to targeting only the fungi found in their local environment (Papua New Guinea). Moreover, BCCO 10_1106 (B3) demonstrated a weak inhibitory effect against the fungal strain *Akanthomyces muscarium*. However, it did not exhibit any inhibitory activity against the other three fungal strains (*Graphium asporum, Ophiostoma piceae,* and *Ophiostoma minus*). This suggests that the antifungal compound is relatively specific to certain fungal species. Additionally, the four isolates S6/6 (B4), S10/3 (B5), S10/25 (B6), and S18/15 (B7) that were collected from the Czech

Republic, demonstrated a weak inhibitory effect against all fungal strains. Further tests are needed to determine if changing the incubation period of *Streptomyces* could potentially enhance its antifungal activity or if it only prevents fungal growth near its vicinity.

Moreover, two hypotheses were formulated to identify a specific gene marker crucial for the biosynthesis of polyenes in *Streptomyces* and evolutionarily related actinomycetes. The first hypothesis was that Cytochrome P450 could serve as a reliable gene marker for polyene production. The multiple sequence alignment results revealed that the CYP sequences of the six-polyene BGCs exhibited shared regions where the amino acid residues are highly conserved. This suggests that these conserved regions may correspond to important catalytic sites or binding domains involved in the biosynthesis of polyene antifungal compounds [30]. Furthermore, apart from the Candicidin and Rimocidin BGCs, the other 4 polyene BGCs possess two cytochrome P450 genes. The phylogenetic tree analysis revealed the presence of two distinct groups within the constructed tree. The possible explanation is that each group is involved in regiospecific oxidation [54]. The first group in figure 26, including PimG, AmphN, NysN, PteD, RimG and fscP are catalyzing the oxidation of the exocyclic methyl branch to the carboxyl group, whereas the second group including PimD, AmphL, PteC and NysL, are involved in oxidative modifications of the polyol segment. Example for the regiospecific oxidation by PimG and Pim D is illustrated in Figure 30 [55].

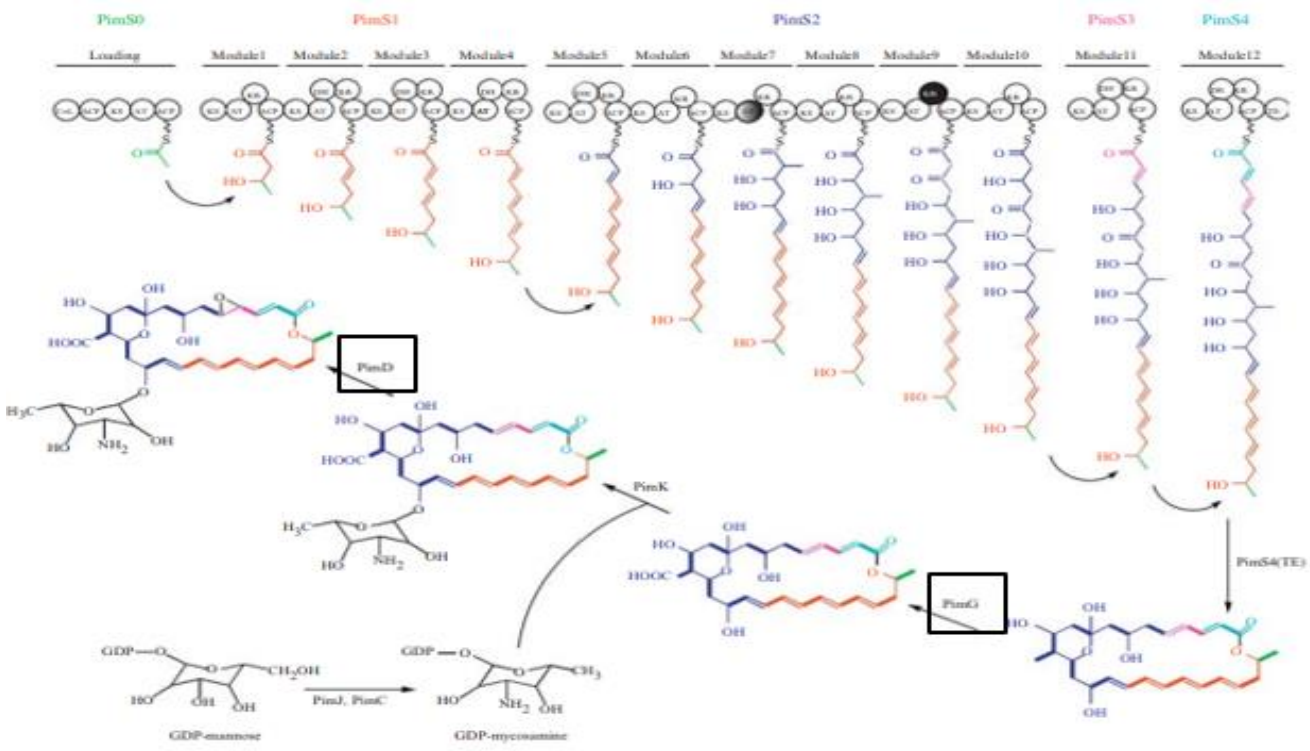

Figure 30: The biosynthesis of Pimaricin. The genes involved in regiospecific oxidation are marked with black boxes.

However, the results of searching for more polyene BGCs indicated that certain polyenes such as Reedsmycin [56], Linearmycin-A, Mediomycin-A, and Neomediomycin-B are devoid of cytochrome P450 genes. The biosynthetic gene clusters of those polyenes are listed in the appendix. This means that cytochrome P450 cannot serve as a reliable gene marker for polyene production as it is not a gene marker for all polyene biosynthetic gene clusters. Therefore, the hypothesis was rejected, and we have focused on alternative gene. The second hypothesis was that thioesterase type I can be used as an effective gene marker for identifying polyene-producing actinomycetes, particularly streptomycetes. Then the BGCs of 20 polyenes and 2 non-polyenes were included to the dataset

The results of multiple sequence alignment of thioesterase type I within the biosynthetic gene clusters (BGCs) of 26 polyenes and 2 non-polyenes were compared to the aligned sequences of TE type I of the cyclic (macrolactone) polyenes in previous literature [29]. The analysis revealed that the results in our study are similar to the results in the literature. Despite including additional sequences of cyclic (macrolactone and macrolactam) and linear polyenes, they still exhibited conserved domains with some degree of variability in these regions. However, these variations in the amino acids that present within the conserved regions do not significantly impact the electric charges or overall geometry of the conserved domain for example, at position 47 (Figure 27), both aspartate (D) and glutamate (E) amino acids have negative charge. These domains share low sequence similarity with non-polyenes at the amino acid level, suggesting that these domains are specific for cyclization and offloading of the polyhydroxylated intermediates with continuous conjugated double bonds [29]. As a result, the amino acid sequences of these domains are translated back to nucleotide sequences and used for designing the primers. The analysis of the domains at the nucleotide level indicated that those different amino acids, that have the same properties, are represented by different codons, and even identical amino acids have multiple possible codons. This poses a challenge when designing primers for thioesterase type I.

Based on the circumstances, 10 primer pairs could be designed. The results showed that primer pair 1 is the best option as both forward and reverse primers have the optimum GC %. This indicates the stability and specificity of primer binding to the target DNA sequence [42]. Furthermore, the melting temperatures (Tm) of the primers are close to each other. Having closely matched Tm values for the forward and reverse primers is important so that they are likely to anneal to the target DNA sequence at a similar temperature during PCR amplification [42].

Moreover, formation of dimers and hairpins are possible only for the forward primer, unlike primer pairs 7-10. This difference in behavior lowers the probability of the primers annealing effectively. The most important advantage of this primer pair over the others is that the analysis of the binding regions for this primer pair revealed a high degree of conservation for polyenes thus designing less degenerate primer pair.

Then the sequences of the best primer pair were used to design the degenerate primers. Degenerate primers provide flexibility in primer design, enabling them to bind to target regions with slight variations. However, degeneracy of the primers was kept low to ensure specific binding. *In silico* PCR was used to evaluate the specificity of the degenerate primer pairs by testing these primers against 19 *Streptomyces* strains. The results show that not only the thioesterase type I gene was amplified, but also other genes. The lack of specificity observed with this primer pair suggests that the sequences of the primer pair may have similarity with other genes within the genome. Moreover, the relatively short length of the primer pair (15 bp) may increase the probability of non-specific binding to other regions of the genome as shorter primers have a higher likelihood of encountering sequences with partial complementarity elsewhere in the genome, leading to unintended amplification during PCR. Therefore, the hypothesis was rejected. To enhance primer specificity and minimize non-specific amplification, it is recommended to design longer primers (18-30 bases) [42] that are more unique to the target gene sequence. Due to time constraints, the primer pair was only tested using *in silico* PCR. However, it is preferable to conduct experimental testing in order to facilitate further analysis, such as sequencing the amplicons.

6. Conclusion

Nowadays, the emergence of multi drug-resistant fungi has become a silent crisis which affects the prevention and treatment of fungal infections. Consequently, discovering novel antifungals has become a priority for the researchers. Among these compounds, polyenes are the 2nd most frequently used antifungals. However, they have drawbacks including drug resistance, low bioavailability, and significant cytotoxicity that limited their uses in the market.

Therefore, this thesis contributed to investigating unexplored niches, specifically bark beetles in order to discover new potential producers of polyenes or other antifungal agents that exhibit low cytotoxicity. Other aspects were to determine whether the antifungal effects exhibited by streptomycetes were specific to certain fungi or occurred randomly and to search for a specific gene marker, which is essential for synthesis of polyenes.

This thesis focused on isolating, characterizing, and identifying streptomycetes obtained from adult bark beetles, larvae, and detritus. The bioactivity of these strains was evaluated. The promising strains were also tested against fungi from different areas. Moreover, phylogenetic analyses of CYP 450 and TE type I enzymes were conducted to identify potential gene markers for polyene biosynthesis. Then a degenerate primer pair was designed for TE type I gene and tested against *Streptomyces* strains using *in-silico* PCR.

The results of the morphological characteristics showed that some isolates were *Streptomyces*-like strains, while others exhibited atypical morphology that did not resemble actinomycetes. The majority of *Streptomyces* strains exhibited inhibitory activity against *Candida albicans* and β-hemolysis of *Staphylococcus aureus*. Cultivating *Streptomyces* strains for 4-6 days before inoculation resulted in a significant inhibitory effect, indicating that *Streptomyces* strains reached the late exponential phase at that time and produced the tested antimicrobial secondary metabolites. Moreover, the co-cultivation of *Streptomyces* and *Staphylococcus aureus* on the same plate resulted in a significantly larger inhibitory zone against *Candida albicans* compared to when only *Streptomyces* was present. This suggests a synergistic effect between the two bacteria, potentially due to the production of antifungal secondary metabolites by *Streptomyces* and *Staphylococcus aureus*. Additionally, *Staphylococcus aureus* may act as an elicitor, stimulating the biosynthesis of antimicrobial secondary metabolites in *Streptomyces*. The four most promising isolates only had a weak inhibitory effect on fungal strains from different areas. Furthermore, the analysis of cytochrome P450 sequences in the polyene biosynthetic gene clusters revealed two different groups. Each group leads to regiospecific oxidation. However, some polyene biosynthetic gene clusters did not have cytochrome P450 enzymes, making it an

unreliable gene marker. Furthermore, the thioesterase type I gene was found in all polyene BGCs but was not effective as a gene marker due to the lack of specificity of the designed primer pair. Therefore, testing the primer pair in the lab and sequencing the products can provide additional information about the amplified regions.

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8. Appendix

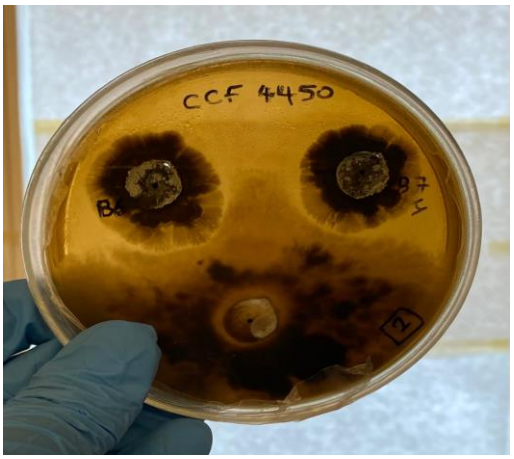
Photo documentation for the results of antifungal test (Photos are taken from the bottom of plates)

- **Results of the inhibitory activity against fungal strain CCF 4450**

Control

Bacterial strains (B1, B2 and B3)

Bacterial strains (B4 and B5)



Bacterial strains (B6 and B7)

- **Results of the inhibitory activity against fungal strain CCF 6041**

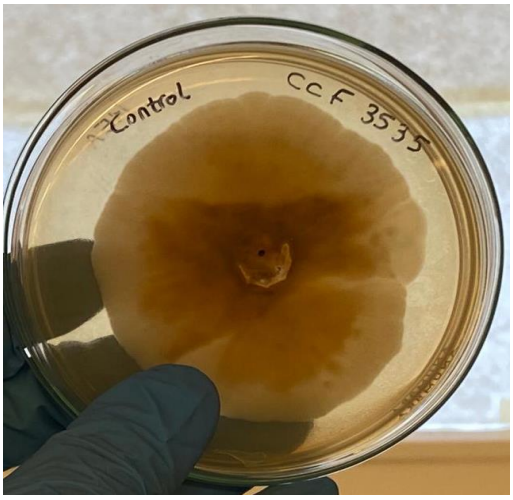
Control

Bacterial strains (B1, B2 and B3)

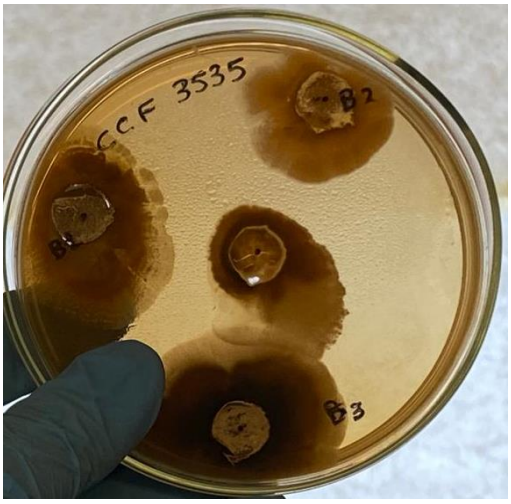
Bacterial strains (B4 and B5)

Bacterial strains (B6 and B7)

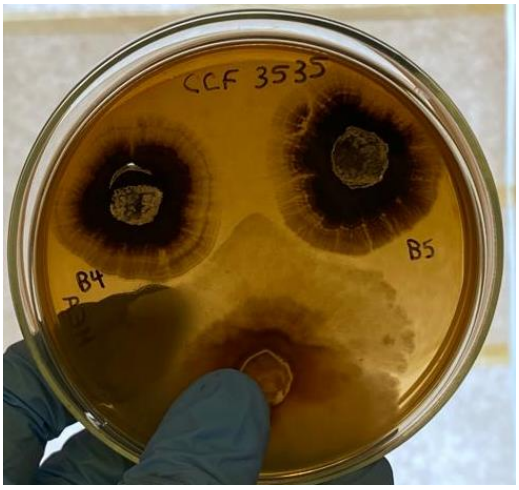
- **Results of the inhibitory activity against fungal strain CCF 3535**



Control



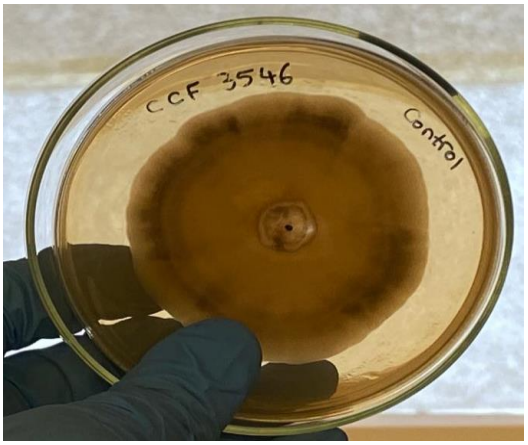
Bacterial strains (B1, B2 and B3)



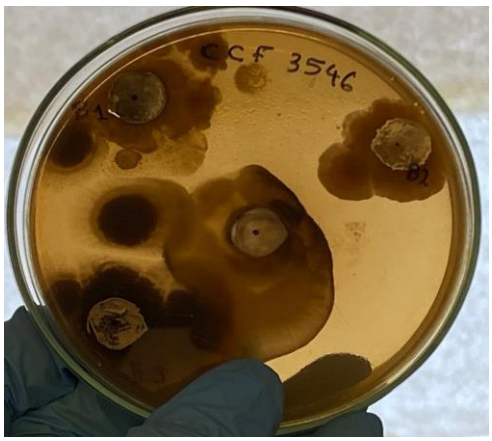
Bacterial strains (B4 and B5)

Bacterial strains (B6 and B7)

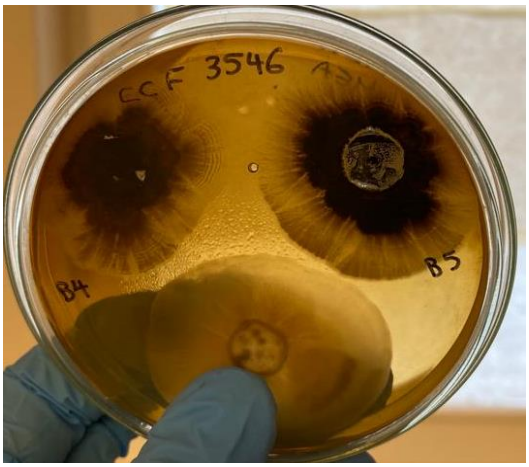
- **Results of the inhibitory activity against fungal strain CCF 3546**



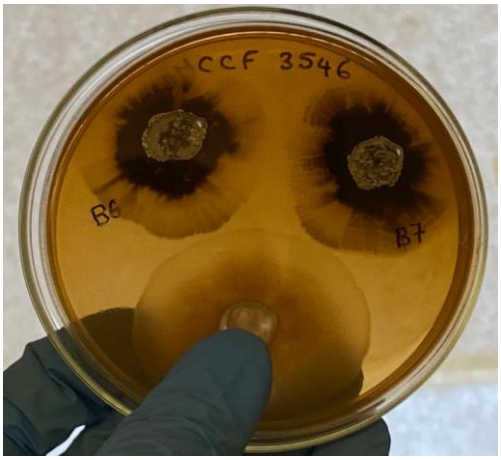
Control



Bacterial strains (B1, B2 and B3)



Bacterial strains (B4 and B5)



Bacterial strains (B6 and B7)

Table 28: Reedsmycin biosynthetic gene cluster from *S. youssoufensis* OUC6819 [53].

Protein	Size (aa)	Proposed function	Homologs	
			Protein/organism	Accession no. (identity/similarity %)
Orf(−3)	213	TetR/AcrR family transcriptional regulator	IF52_RS0105620/*Streptomyces ruber*	WP_030357119.1 (66/74)
Orf(−2)	418	FAD-binding monooxygenase	BCAV_RS14010/*Beutenbergia cavernae*	WP_015883264.1 (59/69)
Orf(−1)	267	Methyltransferase	H299_RS35255/*Streptomyces* sp. CNH287	WP_051262671.1 (68/80)
RdmA	286	XRE family transcriptional regulator	ASD26_RS06115/*Streptomyces* sp. Root1319	WP_056788122.1 (57/65)
RdmB	256	Methyltransferase	AB852_RS08905/*Streptomyces uncialis*	WP_073785832.1 (63/73)
RdmC	218	LuxR family two-component system response regulator	KasW/*Streptomyces kasugaensis*	BAF79686.1 (84/91)
RdmD	111	Two-component system sensor kinase	KasX/*S. kasugaensis*	BAF79687.1 (66/81, the 475th–585th aa)
RdmE	410	Two-component system sensor kinase	KasX/*S. kasugaensis*	BAF79687.1 (68/75, the 31st–413rd aa)
RdmF	233	PAS-LuxR regulator	AOM46_RS26735/*Streptomyces* sp. NBRC 109706	WP_062215992.1 (83/89)
RdmG	4996	Type I polyketide synthase	AOM46_RS26740/*Streptomyces* sp. NBRC 109706	WP_078857234.1 (70/78)
RdmH	6440	Type I polyketide synthase	AOM46_RS26745/*Streptomyces* sp. NBRC 109706	WP_078857210.1 (71/80)
RdmI	9533	Type I polyketide synthase	SHXM_01039/*Streptomyces hygroscopicus* XM201	AQW47576.1 (52/63)
RdmJ	7252	Type I polyketide synthase	AOM46_RS26755/*Streptomyces* sp. NBRC 109706	WP_062216000.1 (74/82)
RdmK	498	MFS transporter	AOK24_RS06280/*Streptomyces niveiscabiei*	WP_055719056.1 (76/83)
RdmL	398	Ferritin	AOK24_RS06275/*S. niveiscabiei*	WP_055719055.1 (84/91)
RdmM	172	Paal family thioesterase	AOK24_RS06270/*S. niveiscabiei*	WP_055719054.1 (75/85)
RdmN	82	Acyl carrier protein	AOK24_RS06265/*S. niveiscabiei*	WP_055719053.1 (54/75)
RdmO	576	Acyl-CoA synthetase	AOK24_RS06260/*S. niveiscabiei*	WP_055719052.1 (66/76)
Orf1	176	Hypothetical protein	AMJ94_18775/*Deltaproteobacteria bacterium* SM23_61 AOK24_RS06255/*S. niveiscabiei* AOK24_RS06250/*S. niveiscabiei*	KPK85730.1 (32/53) WP055719051.1 (78/84, the 1st–79th aa) WP055719050.1 (82/89, the 81st–174th aa)
Orf2	207	DNA-binding response regulator	AOK24_RS06245/*S. niveiscabiei*	WP_063799404.1 (61/74)
Orf3	361	Hypothetical protein	AOK24_RS06240/*S. niveiscabiei*	WP_079056694.1 (56/67)

Table 29: linearmycin-A biosynthetic gene cluster from *Streptomyces sp.* Mg1 [57]

Identifiers	Position	Product
M444_04750 AKL64821.1 lnyT	1061319 - 1062308 (-)	agmatinase
M444_04755 AKL64822.1 lnyS	1062415 - 1063971 (-)	methylmalonyl-CoA carboxyltransferase
M444_04760 AMB20391.1 lnyR	1064457 - 1067375 (+)	transcriptional regulator
M444_04765 AKL64823.1 lnyQ	1067356 - 1068252 (+)	metallophosphoesterase
M444_04770 AKL64824.1 lnyP	1068249 - 1068992 (+)	4'-phosphopantetheinyl transferase
M444_04775 AKL64825.1 lnyO	1069184 - 1070845 (+)	amine oxidase
M444_04780 AKL64826.1 lnyN	1070939 - 1072351 (-)	acyl--CoA ligase
M444_04785 AKL69762.1 lnyM	1072435 - 1073028 (-)	thioesterase
M444_04790 AMB20392.1 lnyL	1073166 - 1073738 (-)	glucose-1-phosphate thymidylyltransferase
M444_04795 AKL64827.1 lnyK	1074063 - 1075127 (+)	daunorubicin ABC transporter ATPase
M444_04800 AKL69763.1 lnyJ	1075226 - 1075996 (+)	ABC transporter
M444_04805 AKL64828.1	1076160 - 1077197 (+)	ACP S-malonyltransferase

Identifiers	Position	Product
M444_04750 AKL64821.1 lnyT	1061319 - 1062308 (-)	agmatinase
M444_04755 AKL64822.1 lnyS	1062415 - 1063971 (-)	methylmalonyl-CoA carboxyltransferase
M444_04760 AMB20391.1 lnyR	1064457 - 1067375 (+)	transcriptional regulator
M444_04765 AKL64823.1 lnyQ	1067356 - 1068252 (+)	metallophosphoesterase
M444_04770 AKL64824.1 lnyP	1068249 - 1068992 (+)	4'-phosphopantetheinyl transferase
M444_04775 AKL64825.1 lnyO	1069184 - 1070845 (+)	amine oxidase
M444_04780 AKL64826.1 lnyN	1070939 - 1072351 (-)	acyl--CoA ligase
M444_04785 AKL69762.1 lnyM	1072435 - 1073028 (-)	thioesterase
M444_04790 AMB20392.1 lnyL	1073166 - 1073738 (-)	glucose-1-phosphate thymidylyltransferase
M444_04795 AKL64827.1 lnyK	1074063 - 1075127 (+)	daunorubicin ABC transporter ATPase
M444_04800 AKL69763.1 lnyJ	1075226 - 1075996 (+)	ABC transporter
M444_04805 AKL64828.1	1076160 - 1077197 (+)	ACP S-malonyltransferase

M444_04810 AMB20393.1 lnyHI	1077302 - 1094107 (-)	polyketide synthase
M444_04815 AKL64829.1 lnyHH	1094175 - 1116644 (-)	polyketide synthase
M444_04820 AKL64830.1 lnyHG	1116707 - 1126318 (-)	polyketide synthase
M444_04825 AKL64831.1 lnyHF	1126413 - 1142603 (-)	polyketide synthase
M444_04830 AMB20394.1 lnyHE	1142689 - 1158345 (-)	polyketide synthase
M444_04835 AKL64832.1 lnyHD	1158599 - 1163497 (-)	polyketide synthase
M444_04840 AKL64833.1 lnyHC	1163620 - 1183653 (-)	polyketide synthase
M444_04845 AKL69764.1 lnyHB	1183678 - 1193985 (-)	polyketide synthase
M444_04850 AKL64834.1 lnyHA	1194150 - 1228586 (-)	polyketide synthase
M444_04855 AKL64835.1 lnyG	1228902 - 1230452 (-)	membrane protein
M444_04860 AKL69765.1 lnyF	1230449 - 1230949 (-)	membrane protein
M444_04865 AKL64836.1 lnyE	1231012 - 1231545 (-)	LnyE

M444_04870 AKL64837.1 lnyD	1231711 - 1233006 (+)	histidine kinase
M444_04875 AKL69766.1 lnyC	1233071 - 1233631 (+)	LuxR family transcriptional regulator
M444_04880 AKL69767.1 lnyB	1233701 - 1236565 (-)	transcriptional regulator

Table 30: Mediomycin-A biosynthetic gene cluster from *Streptomyces blastmyceticus* [58].

Identifiers	Position	Product
BAW35626.1 medA	149 - 739 (-)	
BAW35627.1 medB	899 - 2029 (-)	sulfotransferase
BAW35628.1 medC	2543 - 5371 (+)	putative LuxR family transcriptional regulator
BAW35629.1 medD	5503 - 7134 (+)	putative L-arginine mono-oxygenase
BAW35630.1 medE	7211 - 8617 (-)	putative 4-guanidinylbutanoate:CoA ligase
BAW35631.1 medF	8681 - 9322 (-)	putative type II TE
BAW35632.1 medG	9842 - 10786 (+)	putative 4-guanidinylbutanoyl-CoA:ACP acyltransferase
BAW35633.1 med9	10945 - 22446 (-)	modular polyketide synthase
BAW35634.1 med8	22528 - 39621 (-)	modular polyketide synthase
BAW35635.1 med7	39677 - 49276 (-)	modular polyketide synthase
BAW35636.1 med6	49365 - 70760 (-)	modular polyketide synthase
BAW35637.1 med5	70824 - 86309 (-)	modular polyketide synthase
BAW35638.1 med4	86533 - 91488 (-)	modular polyketide synthase
BAW35639.1 med3	91616 - 116827 (-)	modular polyketide synthase
BAW35640.1 med2	116853 - 126989 (-)	modular polyketide synthase
BAW35641.1 med1	126996 - 151358 (-)	modular polyketide synthase
BAW35642.1 medH	151633 - 153189 (-)	putative membrane protein
BAW35643.1 medI	153186 - 153668 (-)	putative membrane protein
BAW35644.1 medJ	153755 - 154288 (-)	
BAW35645.1 medK	154456 - 155709 (+)	putative histidine kinase
BAW35646.1 medL	155702 - 156331 (+)	putative two-component system response regulator
BAW35647.1 medM	156389 - 159283 (-)	putative LuxR-family transcriptional regulator
BAW35648.1 medN	159372 - 160022 (-)	putative type II TE
BAW35649.1 medO	160405 - 161190 (+)	putative 4-guanidinobutyramide hydrolase
BAW35650.1 medP	161231 - 161671 (-)	putative transcriptional regulator

Table 31: Neomediomycin-B biosynthetic gene cluster from *Streptomyces sp*. RK95-74 [59].

Identifiers	Position	Product
BAW35598.1 nmdA	1987 - 3483 (+)	putative methylmalonic acid semialdehyde dehydrogenase
BAW35599.1 nmdB	3628 - 4071 (+)	hypothetical protein
BAW35600.1 nmdC	4156 - 6249 (+)	putative integral membrane protein
BAW35601.1 nmdD	6493 - 7251 (+)	putative type II TE
BAW35602.1 nmdE	7340 - 10183 (+)	putative transcriptional regulator
BAW35603.1 nmdF	10205 - 10837 (-)	putative DNA-binding response regulator
BAW35604.1 nmdG	10830 - 12110 (-)	putative two-component system sensor kinase
BAW35605.1 nmdH	12245 - 12820 (+)	hypothetical protein
BAW35606.1 nmdI	12889 - 13389 (+)	putative membrane protein
BAW35607.1 nmdJ	13494 - 14930 (+)	putative membrane protein
BAW35608.1 nmd1	15242 - 54211 (+)	modular polyketide synthase
BAW35609.1 nmd2	54262 - 65442 (+)	modular polyketide synthase
BAW35610.1 nmd3	65469 - 85640 (+)	modular polyketide synthase
BAW35611.1 nmd4	85708 - 90672 (+)	modular polyketide synthase
BAW35612.1 nmd5	90919 - 106437 (+)	modular polyketide synthase
BAW35613.1 nmd6	106489 - 122601 (+)	modular polyketide synthase
BAW35614.1 nmd7	122658 - 132275 (+)	modular polyketide synthase
BAW35615.1 nmd8	132332 - 149440 (+)	modular polyketide synthase
BAW35616.1 nmd9	149501 - 171664 (+)	modular polyketide synthase
BAW35617.1 nmdK	171777 - 172724 (-)	putative acyl transferase
BAW35618.1 nmdL	172765 - 174381 (-)	putative ABC-2 type transporter
BAW35619.1 nmdM	174378 - 175385 (-)	putative ABC transporter
BAW35620.1 nmdN	175705 - 176367 (+)	putative type II TE
BAW35621.1 nmdO	176424 - 177836 (+)	putative acyl CoA ligase
BAW35622.1 nmdP	177948 - 179606 (-)	putative amino oxidase
BAW35623.1 nmdQ	179646 - 182426 (-)	putative LuxR family transcriptional regulator
BAW35624.1 nmdR	183092 - 184135 (+)	putative sulfotransferase
BAW35625.1 nmdS	184328 - 185326 (+)	putative amidinohydrolase