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„Selective isolation and taxonomical characterization of bacteria from the rhizosphere of *Leontopodium nivale* subsp. *alpinum*“

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Abbreviations

2xYT .....twofold Yeast Extract / Tryptone

AHLs .....N-acyl homoserine lactones

BLAST .....Basic Local Alignment Search Tool

bld .....bald

Cass. .....Alexandre Henri Gabriel de Cassini

c-di-GMP ....cyclic di-guanosine-monophosphate

CydCD .....Cytochrome bd oxidase complex

DEHP .....di- (2-ethylhexyl) phthalate

DGGE .....denaturing gradient gel electrophoresis

DMSO.....dimethyl sulfoxide

dNTPs .....deoxy nucleotide triphosphates

EDTA .....ethylene diamine tetra acetic acid

FW .....forward

gDNA .....genomic deoxyribonucleic acid

HTC .....high-throughput cultivation

HV .....humic acid vitamin

ITS .....internal transcribed spacer

IUCN .....International Union for Conservation of Nature

kb .....kilo base

kbp .....kilo base pairs

MEGA .....Molecular Evolutionary Genetics Analysis

NC .....antibiotic combination of Nystatin and Cycloheximide

NCN .....antibiotic combination of Nystatin, Cycloheximide and Novobiocin

nt .....nucleotides

PCR .....polymerase chain reaction

RC .....rehydration and centrifugation

RDP .....Ribosomal Database Project's Classifier

RLA .....rhizosphere of *Leontopodium nivale* ssp. *alpinum*

rRNA .....ribosomal ribonucleic acid

RV .....reverse

SDS .....sodium dodecyl sulfate

SE .....soil extract

tRNA .....transfer ribonucleic acid

TSB .....tryptic soy broth

TBE .....Tris / Borate / EDTA

TMA .....alkaline trimethylamine

Unclass. .....unclassified

VOC .....volatile organic compound

whi ..... white

1. Abstract

Ever increasing resistance of pathogens to antimicrobial agents, prompts humankind to urgently search for novel natural compounds that may be developed into antibiotics. Soil-dwelling bacteria, especially actinomycetes, are known as producers of more than 66 % of all antibiotics of natural origin, which are currently applied clinically (Barka et al., 2016). Actinomycetes are still worthwhile sources of yet unknown compounds. These bacteria have been mostly isolated from soil samples, but rhizospheres of traditional medicinal plants still remain under-investigated.

In this study we focus on the ancient medicinal plant *Leontopodium nivale* subsp. *alpinum* from the native alpine range and its rhizosphere bacteria. Soil samples from the rhizosphere were used to selectively isolate bacteria. Six media selective for rare actinomycetes were combined with soil pre-treatments to eradicate abundant other bacteria, and three antibiotic treatments were applied as additional selective forces. Bacterial colonies were selected based on actinomycetes morphology characteristics and observed bioactivity on agar plates. Isolated bacteria were cultivated and the genomic DNA was extracted. 16S-rDNA was amplified, sequenced and used to construct phylogenetic trees to compare isolates to known type strains.

The majority of the sequenced isolates belongs to the phylum *Actinobacteria*. Rare actinomycetes included the genera *Actinokineospora*, *Asanoa*, *Kitasatospora*, *Microbacterium*, *Micrococcus*, *Mycobacterium* and *Nocardia*. Dominating isolates belonged to the genera *Streptomyces* and *Micromonospora*. In total, bacteria of 15 different genera were isolated in this project. Furthermore, bioactivity in contact with other bacteria was observed and assigned to several *Paenibacillus* species and a few *Bacillus* species.

This work provides a foundation for a future project, aimed at testing these bacteria for the potential production of novel antimicrobial compounds.

2. Zusammenfassung

Die zunehmende Resistenzentwicklung von Krankheitserregern gegenüber Antibiotika ist Anlass für eine intensivierte Suche nach natürlichen Substanzen, die zu Antibiotika weiterentwickelt werden können. Bodenbakterien, vor allem Actinomyceten, produzieren zwei Drittel aller klinisch gebräuchlichen Antibiotika natürlichen Ursprungs. Actinomyceten sind nach wie vor reiche Quellen noch unentdeckter Substanzen. Diese Bakterien wurden bisher hauptsächlich aus Bodenproben isoliert, die Rhizosphäre traditioneller Arzneipflanzen ist hingegen weitgehend unerforscht.

Die traditionelle Arzneipflanze Alpen-Edelweiß aus heimischen Vorkommen auf der Rax sowie die Bakterien aus der Rhizosphäre dieser Pflanze standen im Mittelpunkt dieses Projekts. Zur selektiven Isolation von Bakterien wurden Bodenproben aus der Rhizosphäre des Alpen-Edelweißes verwendet. Sechs selektive Medien, entwickelt für die Isolation seltener Aktinomyceten, wurden mit Vorbehandlungsmethoden zur Reduktion häufig vorkommender Bakterien kombiniert. Zur weiteren Selektion wurden drei antibiotische Behandlungsvarianten eingesetzt. Die Bakterienkolonien wurden nach morphologischen Kriterien der Aktinomyceten sowie nach Beobachtung antimikrobieller Aktivität auf Agarplatten ausgewählt. Isolierte Bakterien wurden kultiviert, deren genomische DNA gewonnen, 16S-rDNA vervielfältigt und sequenziert. Die taxonomische Charakterisierung erfolgte durch Vergleich mit bekannten Typstämmen im Rahmen phylogenetischer Stammbäume.

Die Mehrheit der sequenzierten Isolate gehört dem Stamm der Aktinobakterien an. Zu den isolierten seltenen Aktinomyceten gehören die Gattungen *Actinokineospora*, *Asanoa*, *Kitasatospora*, *Microbacterium*, *Micrococcus*, *Mycobacterium* und *Nocardia*. Die vorherrschenden Gattungen waren *Streptomyces* und *Micromonospora*. Insgesamt wurden in diesem Projekt 15 verschiedene Bakteriengattungen isoliert. Die beobachtete Bioaktivität in Kontakt mit anderen Bakterien war auf einige *Paenibacillus*-Spezies und wenige *Bacillus*-Spezies zurückzuführen.

Diese Arbeit stellt eine Grundlage für ein zukünftiges Projekt dar, welches die potentielle Produktion neuer antimikrobiell aktiver Substanzen untersucht.

3. Aim of the Work

Leontopodium nivale subsp. *alpinum* is a well-known traditional medicinal plant which inhabits mountainous areas, for example the Austrian Alps. The alpine habitat of these plants provides harsh living conditions for all inhabitants. Sparsely examined environments with challenging climate properties are promising areas of prospecting for antimicrobial substances. The rhizosphere is a complex environment where numerous producers of antimicrobial substances may be dwelling. Due to the antimicrobial resistance crisis, there is an urgent need for new compounds that can be developed into antibiotics. The aim of this work was to isolate, applying modern techniques and established knowledge, bacteria from the rhizosphere of *Leontopodium nivale* subsp. *alpinum*, which may have potential to produce novel antibiotics. The focus was on using specialized pre-treatments and selective media for the isolation of rare actinomycete bacteria, which are well-known producers of antimicrobial substances. Further aim was to taxonomically identify these bacteria, which will assist in their future characterization.

4. Introduction

Leontopodium nivale subsp. *alpinum*

Leontopodium species can be found mainly in the mountain regions of Central, temperate Southeastern and Eastern Asia (Safer et al., 2011). *Leontopodium nivale* subsp. *alpinum* (Cass.) Greuter from *Asteraceae* family, syn. *Leontopodium alpinum,* is also known as Alpen-Edelweiss (Fischer et al., 2008; Safer et al., 2011) (Fig.1). Its European distribution covers mountain ranges of Central and Southern Europe, more precisely the Pyrenees, the Carpathians, the Tatra, the Balkan Mountains and the Alps (Meusel and Jäger, 1992; Tauchen and Kokoska, 2016). The distribution area of genus *Leontopodium* is uncommonly interrupted between its Asian and the European habitats (Meusel and Jäger, 1992).

It remains unclear to taxonomists to date, whether there are two species of *Leontopodium* in Europe or if there is one species with two subspecies. Blöch et al. (2010) claim, that two species, *L. nivale* and *L. alpinum*, are established in Europe. Greuter (2003) in contrast divided one species into two subspecies, *L. nivale* ssp. *nivale* and *L. nivale* ssp. *alpinum*. Fischer et al. (2008) treat *L. nivale* ssp. *alpinum* and

Fig.1: Flowering exemplar of *Leontopodium nivale* ssp. *alpinum* (Cass.) Greuter. Reprinted from “The chemistry and pharmacology of Edelweiss: a review.” by Tauchen J. and Kokoska L. (2016) Phytochem Rev, 16(2), 296. With kind permission of Tomas Lichtenberg. © 2016 by Springer Science + Business Media Dordrecht. Reprinted with permission.

L. alpinum as synonyms. Within this work we will further on use *Leontopodium nivale* subsp. *alpinum* to refer to the experimental plant species.

Leontopodium nivale ssp. *alpinum* grows most commonly on rocky limestone and hillside meadows at heights between 1000 m and 3400 m above sea level (Hörandl et al. 2011; Finkenzeller 2014).

This herbaceous perennial plant prefers full sunlight in its habitat (Tauchen and Kokoska, 2016). *Leontopodium nivale* subsp. *alpinum* used to be a popular plant in traditional medicine, especially in the geographic areas of its native distribution range. Extensive wild harvesting led to decreasing populations of Edelweiss. Hence, *L. nivale* ssp. *alpinum* is now listed on the International Union for Conservation of Nature (IUCN) Red List of threatened species in some European countries. Moreover, collection of the wild Edelweiss plants is either prohibited or requires a permission (Khela, 2013).

Extracts of Edelweiss possess healing properties, according to traditional folk medicine. There is evidence, that Edelweiss alleviates abdominal aches, angina pectoris and other cardiac dysfunctions, bronchitis, tonsillitis, pneumonitis, cough, gastritis, colitis, diarrhea, dysentery, diphtheria, rheumatic pain and fever in humans and farm animals (Hegi 1935; Hartwell, 1968; Hoppe 1975; Bitschnau 1991; Kiene 1992; Knechtl 1992; Pickl-Herck 1995; Wieser 1995; Speroni et al. 2006; Hornick et al. 2008; Daniela et al. 2012).

Antimicrobial effects of Edelweiss compounds were inferred from transmitted evidence of several medical indications. Indeed, Dobner et al. (2003) demonstrated antibacterial activities of *L. nivale* ssp. *alpinum* crude extracts. The spectrum of action included Gram-positive bacteria as well as Gram-negative bacteria. Dichloromethane extracts of aboveground plant parts inhibited growth of *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pyogenes* strains. Most effective crude extracts were acquired by extracting herbs and roots with dichloromethane. The observed effects were allocated to bisabolane sesquiterpenes, tricyclic sesquiterpenes as well as sesquiterpene esters and coumarin.

A compound with radical scavenging properties was found in the flowering herb of Edelweiss. Leontopodic acid, which has a complex fully substituted glucaric acid moiety, is supposed to have the potential to protect DNA molecules from damage by alleviating oxidative stress (Schwaiger et al., 2005; 2006; Cicek et al. 2012). Therefore,

leontopodic acid could be applied in cosmetics like sun cream (Tauchen and Kokoska, 2016). Anti-inflammatory effect of leontopodic acid is another subject of interest. Daniela et al. (2012) reported distinctive anti-inflammatory effects of an ethanolic extract of the callus culture, which contained leontopodic acid among other substances. This extract was shown to protect human skin keratinocytes and endothelial cells from UV-induced damage. Further application areas for other indications like chronic inflammatory skin diseases and inflammations of other cause require further investigations.

Utilization of biotechnologically grown callus cultures is an option to make *Leontopodium nivale* ssp. *alpinum* constituents more readily available for cosmetic and therapeutic use (Daniela et al., 2012). Furthermore, Hook (1993) showed successful vegetative plant propagation and breeding from seeds, sourcing from wild plants or cultivars. Edelweiss is already cultivated on a large scale in Switzerland (Schwaiger et al., 2005) and southern Finland (Khela 2013).

Endophytes associated with medicinal plants

Bioactive compounds of natural origin remain highly interesting in the field of drug discovery. Medicinal plants produce a wide range of various bioactive compounds in largely varying contents. The content of bioactive compounds depends on plant species, plant individual genetics and epigenetics, environmental conditions and the individual microbiome (Zhao et al, 2011; Morsy, 2014). Plants are surrounded by and interspersed with living organisms, even their internal tissues and cells are inhabited by bacteria and fungi (Wilson, 1995). Broad definition claimed by Hallmann et al. (1997), suggests that all microorganisms isolated from inside plant tissue after surface sterilization are to be called endophytes. Endophytic bacteria and fungi are either symbiotic organisms or neutral to the plant, and definitely not disadvantageous for the host (Chanway, 1996; Hallmann et al., 1997). The definition of endophytes changed over time (Hardoim et al., 2015). The most current definition was formulated by Hardoim et al. (2015), who claims that endophytes are microorganisms that inhabit interior plant tissues and can be isolated there from.

A vast number of plant species, bacteria and fungi were already exploited for production of bioactive compounds (Demain and Sanchez, 2009). By examining

extraordinary biotopes, new valuable natural products are sought after (Strobel and Daisy, 2003). Endophytes are associated with higher plants and only a minority of them have been investigated for production of bioactive compounds (Strobel et al., 2004). Plant's interior compartments can be interpreted as ecological niches for endophytes, which are less investigated than microbes in soils or microorganisms on the surface of plants (Strobel, 2003). Furthermore, endophytes are influencing secondary metabolite production by their host plants (Zhang et al., 2006). By exploring unique endophytic communities it could be possible to discover new valuable compounds with possible medical and industrial applications, antimicrobial drugs for instance. Additionally, plants with traditional medicinal use and originating from uncommon environments are considered as promising candidates for new discoveries (Strobel and Daisy, 2003).

The rhizosphere as source of microorganisms

The term rhizosphere delimits the immediate soil surrounding the plant roots contrasting to bulk soil (Venturi and Keel, 2016). Bulk soil, however, is distinctly remote from plant roots and is mostly unaffected by them. Even though it is influenced by soil genesis processes in the long term (Richter et al., 2007). Differences between bulk soil and rhizosphere soil concern chemical, physical and biological properties (Wang et al., 2002). Bulk soil is poorer in plant-derived nutrients (Richter et al., 2007), nitrogen for instance (Herman et al., 2006). It was observed that nitrogen mineralization in bulk soil is up to ten times lower than in rhizosphere soil (Herman et al., 2006). Because of completely distinctive environmental conditions, the microbial community of bulk soil and rhizosphere is distinctively different (Garland, 1996).

The rhizosphere differs from bulk soil by inhabiting an intricate microbial community (Smalla et al., 2001, Paterson, 2003), and is directly influenced by plant roots as well as by these microorganisms. Plant roots release compounds into the rhizosphere by root exudation comprising root excretion and secretion (Bais et al., 2006). Within the rhizosphere, numerous players get in contact with each other. Plants roots are ubiquitously confronted with bacteria, fungi, protists, other plant species' roots and animals. All involved organisms are able to affect this habitat by producing substances for communication, nutrition and defense, amongst others. This intense communication comprises manifold interactions spanning symbioses, commensalism and parasitism. Often interaction with microorganisms is beneficial for plants, in many

cases it is beneficial for both sides indeed (Lynch and Whipps, 1990; Barea et al., 2005; Bais et al. 2006; Venturi and Keel, 2016).

Plants are also able to detect harmful interactions by recognizing pathogen-associated molecular patterns, e. g. cellular structures of bacterial and fungal origin. The detection of a suspicious structure initiates several signal cascades of immunity and allows the plant to react appropriately (Oldroyd, 2013).

At the rhizosphere organic carbon is more easily available for microbes compared to bulk soil, because of root exudation (Herman et al., 2006). It can be discussed whether plants are able to attract targeted beneficial bacteria by providing specific exudates or if they are excreting substances by change (Mendes et al., 2013). Plant roots are known to actively influence their environment changing the soil pH. The pH value alters nutrient availability substantially, especially of phosphorus, zinc and iron ions. Moreover plant roots can change soils texture (Marschner et al., 1987).

Microorganisms living in close proximity to roots are referred to as rhizo-microbiome. Numerous contributory factors determine microbial species of the rhizosphere (Bais et al., 2006; Dennis et al., 2010). Additionally, rhizosphere microbiome composition is determined by plant species, cultivars, seasonal aspects (Garland, 1996; Grayston et al., 1998) and plant age. Exudates are substances actively released by plants and comprise protons, inorganic acids, oxygen, water, sugars, amino acids, organic acids and fatty acids as well as growth factors, vitamins and enzymes (Paterson, 2003; Bais et al., 2006; Dennis et al., 2010). Endophytes are most probably recruited from the rhizosphere (Wagner et al., 2016; Kandel et al., 2017; Wemheuer et al., 2017). There is evidence, that organic acids, amino acids and proteins exuded by plant roots can influence recruitment of bacterial endophytes from the rhizosphere (Bulgarelli et al., 2012; Kawasaki et al., 2016; Pétriacq et al., 2017). Additionally, particles released by plant roots, so-called rhizodeposits, like dead plant parts, shed root cells and mucilage are released into the rhizosphere. Such carbon-rich substances are very attractive for soil bacteria, which is a possible explanation for their higher abundance in the rhizosphere compared to bulk soil. (Paterson, 2003; Bais et al., 2006; Dennis et al., 2010).

Compounds of low molecular weight are produced and perceived by diverse species of different kingdoms and allow their communication (Venturi and Keel, 2016). A popular example for important small molecules are N-acyl homoserine lactones

(AHLs). They are produced by different genera of bacteria, for example by Proteobacteria, and serve for quorum sensing. This sensing mechanism enables bacteria to detect population density by cell-cell signaling and they can adapt their population growth consequently. Proliferating cells increasingly occupy available space and aggregate. Hence the amount of produced AHLs is increased until a critical threshold concentration is reached. At this point the AHLs signal leads to a modulation of genes by a transcription factor. Thereby a positive signal cascade is started and perceived by a quorum sensor (Fuqua et al., 2001). Quorum sensing with AHLs also plays a role between plants and bacteria in both, beneficial and pathogenic interactions. On one hand, plants learned to disturb bacterial AHL signals, but are also able to simulate them. On the other hand, AHL signals are able to modify plants defense, development and growth (Venturi and Fuqua, 2013). There is also evidence, that substances involved in quorum sensing can play an important role in colonization of plants by endophytes (Zúñiga et al., 2013).

The rhizosphere environment requires microbes to produce antimicrobially active substances to suppress microbial growth of competitors (Killham and Prosser, 2015). Antibiotic biosynthesis often involves complex biosynthetic pathways that are energy-demanding. There are some hypotheses aiming to explain why bacteria and fungi produce antibiotics. One explanation is to acquire an advantage in the competitive struggle for nutrients in soil. Another possible explanation might be communication by secreting low levels of antibiotics. Antibiotics as signal molecules influence virulence factors, building of biofilms and modulate gene expression. Furthermore, antibiotics may contribute to quorum sensing processes, lantibiotics for instance (Andersson and Hughes, 2014). Czaran et al. (2002) report, that the omnipresence of antimicrobial substances maintains diversity. The diversity of bacteria species in soil is extremely high (Torsvik et al., 1990; Dykhuizen, 1998).

An example for a Streptomycetes sp. inhabiting the rhizosphere producing a substance with antibiotic properties was reported by Arora et al. (2018). A strain of *Streptomyces* sp. was isolated from the rhizosphere of the medicinal plant *Crataegus oxycantha* and was shown to produce the antibiotic nalidixic acid.

Antibiotics and antibiotic resistances

Antimicrobial substances are commonly produced by bacteria and fungi (Reeves, 1972; Bérdy, 1974). The process of inhibition caused by antibiotics was proposed to be called *antibiosis* by Vuillemin in 1889, describing their effect “against life” (Aronson, 1997). Waksman amended the term to *antibiotic* (Radetsky, 1996). Antibiotics inhibit the growth of antagonized microbes or kill them, even in low concentrations (Martindale, 1989; Mutschler et al. 2008).

The discovery of penicillin by Fleming in 1928 constitutes a milestone in the treatment of infectious disease. Fleming observed inhibition of *Staphyloccus* sp. around a colony of fungus contaminating an agar plate (Fleming, 1929). Benzylpenicillin, also referred to as Penicillin G (World Health Organization, 2008), was isolated by Fleming from mold *Penicillium chrysogenum* (De Hoog et al., 2000). Penicillin is a member of the large group of beta-lactam antibiotics, which contains also cephalosporin, carbapenem and structurally divergent monobactams antibiotics (Page, 2012). Beta-lactam-antibiotics act bactericidally to proliferating bacteria (Karow and Lang-Roth, 2015). Beta-lactam-antibiotics target bacterial murein-synthetases, enzymes necessary for cell wall synthesis. Resulting cell wall defects lead to osmotic death of affected bacteria (Stahlmann and Lode, 2013; Karow and Lang-Roth, 2015). Animals lack comparable cell wall synthesis enzymes, therefore they are not affected (Hopwood, 2007). Penicillin G is still used to treat infections caused by Gram-positive and Gram-negative bacteria. Various *Streptococcus* species are susceptible to Penicillin G, although it is often less effective against pneumococci, meningococci and gonococci. This antibiotic can be inactivated by beta-lactamases, produced by bacteria (World Health Organization, 2008), for example by many *Staphylococcus* species. As many as 80 percent of *Staphylococcus* species are able to cleave the beta-lactam-ring hydrolytically, which provides them with resistance towards beta-lactam-antibiotics. Genes providing bacteria with beta-lactamase are either located at the bacterial chromosome or in plasmids. Additionally beta-lactam-antibiotics spare dormant bacteria (Karow and Lang-Roth, 2015). Further mechanisms of resistance towards beta-lactam-antibiotics are mutations concerning the targeted cell structure and changes of the cell membrane (Mutschler et al., 2016).

Streptomycin was the first discovered antibiotic produced by actinomycetes, discovered by Selman Waksman in 1943. Streptomycin from *Streptomyces griseus*

was the first potent cure for tuberculosis (Hopwood, 2007). This aminoglycoside antibiotic is currently applied as drug of second choice for the treatment of infections caused by multiresistant strains of *M. tuberculosis*, combined with other medicinal substances (Stahlmann and Lode, 2013). Aminoglycoside antibiotics inhibit bacterial protein synthesis. They bind to the 30 S ribosomal subunit and inhibit the translation by blocking the binding of initiating formylmethionine-tRNA. As a result of misreading, nonsense-proteins are produced which cause bacteria to deteriorate (Sharma et al., 2007; Stahlmann and Lode, 2013). Frequently used aminoglycoside antibiotics are for instance gentamicin, tobramycin and amikacin (Stahlmann and Lode, 2013). Gentamicin is produced by *Micromonospora purpurea* (Weinstein et al., 1963), tobramycin is produced by *Streptomyces tenebrarius* (Katz and Baltz, 2016) and amikacin is derived from kanamycin, which is produced by *Streptomyces kanamyceticus* (Sneader, 2005). The usage of numerous aminoglycoside antibiotics is limited because of nephro- and ototoxicity. Additionally, resistances towards aminoglycoside antibiotics are increasing (Pucci and Bush, 2013).

Chlortetracycline is the oldest tetracycline antibiotic. It was discovered in 1945 by Duggar (Jukes, 1985). Chlortetracycline is produced by *Streptomyces aureofaciens* (Duggar, 1948). Shortly afterwards tetracycline, which was named terramycin initially, and oxytetracycline were isolated from *Streptomyces rimosus* (Hopwood, 2007). Nowadays, the derivatives doxycycline and minocycline are frequently used representatives of this class. Tetracyclines inhibit bacterial protein synthesis by binding at the 30S ribosomal subunit and blocking the docking of aminoacyl-t-RNA-molecules. Tetracyclines act bacteriostatically by disturbing bacterial cell growth (Dellas, 2011; Stahlmann and Lode, 2013). Tetracyclines are effective against Gram-positive and Gram-negative bacteria, for example against *Propionibacterium acnes*, *Brucella* sp., *Yersinia* sp., *Vibrio cholerae*, cell wall lacking *Mycoplasma* sp. and some *protozoa* species. Antibiotic resistances towards tetracyclines are widespread among *Staphylococcus* sp., *Streptococcus* sp. and *Pneumococcus* sp. Acquired antibiotic resistance towards tetracyline antibiotics is located on plasmids (Stahlmann and Lode, 2013). The resistance mechanism depends commonly on an efflux system, which is defined as the bacterial unloading of xenobiotic substances like antibiotics from cytoplasm (Bay and Turner, 2016). Furthermore, bacteria can protect the ribosomal binding site for tetracyclines and deny antibiotic access thereby (Stahlmann and Lode, 2013).

Erythromycin A was isolated from *Saccharopolyspora erythrea* in 1952, which was previously named *Streptomyces erythrea* (Labeda, 1987a). Erythromycin A is effective against Gram-positive bacteria (Staunton and Wilkinson, 2003) and Gram-negative bacteria. Erythromycin belongs to the macrolides, an important group of antibiotic drugs. Clarithromycin and azithromycin are modern macrolides with improved properties. (Karow and Lang-Roth, 2015). Macrolides are important medicinal drugs for the treatment of respiratory tract infections (Xu et al., 2012). Macrolides act bacteriostatically by inhibition of bacterial protein synthesis. They bind to the 50S subunit of the bacterial ribosome and inhibit the translocation of tRNA. The extension of the peptide chain is hampered and consequently bacterial growth is inhibited (Dellas, 2011; Chellat et al., 2016). Resistance towards macrolides was observed in case of *Streptococcus pneumoniae* (Xu, 2012; Butler et al., 2017).

Antimicrobial drugs significantly improved life conditions and life span of humans (Laxminarayan et al., 2016). Successful development of antibiotics in the first decades of the antibiotic era led to the attitude, that infectious diseases can be completely defeated (Spellberg and Taylor-Blake, 2013). However, resistance development towards applied antibiotics is inevitable (Pillar and Sahm, 2012; Frieden, 2013). Antibiotic resistance results increasingly in difficult-to-treat or impossible to treat infections (Blair et al., 2015). Thus, many antibiotic drugs must be expected to be applicable for a short time span. Insufficiently effective substances must be replaced by other sufficient drugs (Pillar and Sahm, 2012; Frieden, 2013). MRSA for instance, is resistant towards penicillins, cephalosporins and carpapenems (Blair et al., 2015). MRSA can be treated with several substances of different modes of action, e. g. vancomycin, linezolid and daptomycin, but the treatment is not always successful (Klevens et al., 2007). Further threatening bacteria, especially for patients in hospitals, are *Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa* and *Enterobacter* species (Rice, 2008).

Systematic screening for antimicrobial substances between 1940 and 1962 yielded the majority of known classes of antibiotics (Singh and Barrett, 2006; Silver, 2011). Empirical screening was conducted by investigation of fermentation broths and microbial extracts for potential growth inhibition of bacteria (Skipper et al., 1954; Chopra, 2000). The yielded substances were either directly used as antibiotic drugs or served as lead structures for antibiotic development (Silver, 2011). The number of new classes of antibiotics invented distinctively decreased in the last decades, therefore

alternative approaches in drug discovery are in demand (Bérdy, 2005). High throughput screening and combinatorial chemistry were promising tools in drug discovery, however, they did not result in many drugs (Verdine, 1996; Bérdy, 2005). Antibiotics differ from other drug structures concerning physical properties, thus scanning of chemical libraries is inefficient in antibiotic discovery (Singh et al., 2017). Molecular technologies, e. g. structure-based drug design (Blundell, 1996) and genetic engineering (Bérdy, 2005) are potent means in modern antibiotic discovery. New lead structures are necessary for development of new antibiotics, sourcing from traditional and genetic based natural products (Singh et al., 2017). Natural products are invincible concerning structural diversity (Verdine, 1996) and most modern antibiotics are derived from natural products (Brown and Wright, 2016). Antibiotics of bacterial origin are established in medical use (Demain and Sanchez, 2009). Examples are given in tbl.1, structures are given in following tbl.2.

Tbl.1: Substances of bacterial origin with antibiotic or antifungal properties.

Name	Class	Producer	Mode of action
amphothericin B[1) 2)]	macrolide	*Streptomyces nodosus*	impairs cell-wall integrity
cephamycin C[11) 12)]	cephalosporin	*Streptomyces lactamdurans*	impairs cell-wall integrity
chloramphenicol[8) 12)]	chloramphenicol	*Streptomyces venezuelae*	inhibits protein synthesis
chlortetracyclin[1)]	polyketide	*Streptomyces aureofaciens*	inhibits protein synthesis
daptomycin[1) 3)]	lipopeptide	*Streptomyces roseosporus*	inhibits cell-wall synthesis / depolarizes cell membrane
erythromycin[1)]	macrolide	*Saccharopolyspora erythraea*[1)]	inhibits protein synthesis
fosfomycin[4) 6)]	phosphoenolpyruvate analogue	*Streptomyces* spp.	inhibits cell wall synthesis
gentamicin[1)]	aminoglycoside	*Micromonospora purpurea*[1)]	inhibits protein synthesis
kanamycin[1) 8)]	aminoglycoside	*Streptomyces kanamyceticus*	inhibits protein synthesis
neomycin[1) 7)]	aminoglycoside	*Streptomyces fradiae*	inhibits protein synthesis
nystatin[1) 4) 5)]	macrolide	*Streptomyces noursei*	impairs cell-wall integrity
oxytetracycline[1) 8)]	tetracycline	*Streptomyces rimosus*	inhibits protein synthesis
rifamycin[9) 10)]	polyketide	*Amycolatopsis rifamycinica*	inhibits DNA-dependent RNA-Polymerase
tobramycin[1) 8)]	aminoglycoside	*Streptomyces spp.*	inhibits protein synthesis
vancomycin[1) 8)]	glycopeptide	*Streptomyces orientalis*	inhibitis cell wall synthesis

1) Sahm et al. (2013)

2) Hamilton-Miller (1973)

3) Heidary et al. (2017)

4) Hopwood (2007)

5) Dos Santos et al. (2017)

6) Falagas et al. (2016)

7) Kudo and Eguchi (2016)

8) Karow and Lang-Roth, 2015

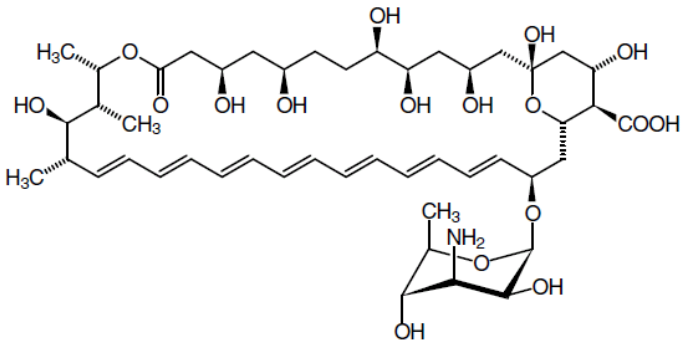
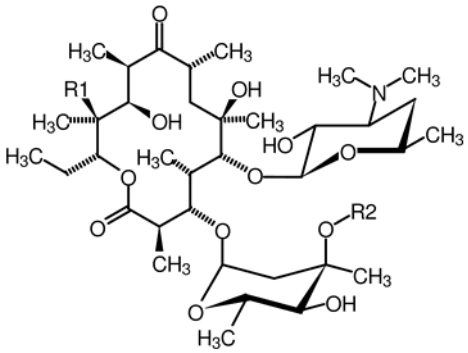
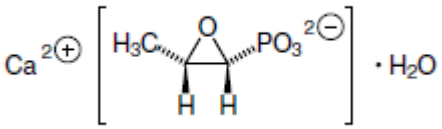
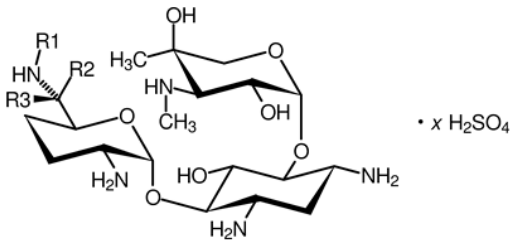
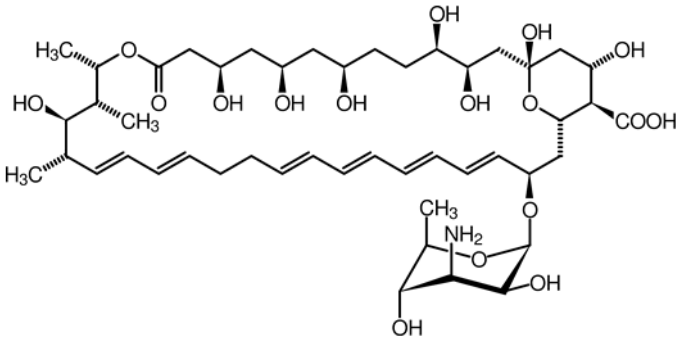
9) Saxena et al. (2014)

10) Mutschler et al. (2016)

11) Oliveira et al. (2015)

12) Solanki et al. (2008)

Tbl.2: Structures of antibiotic and antifungal substances of bacterial origin.

Name	Impact	Structure
amphotericin B	Antifungal	
erythromycin	antibacterial	
fosfomycin	antibacterial	
gentamicin	antibacterial	
nystatin	antifungal	

European Pharmacopoeia (2017)

Actinomycetes, especially *Streptomyces* species and rare actinomycetes, are rich sources of antibiotics (Bérdy, 2005). *Streptomyces* spp. colonies undergo a complex development uncommon for bacteria, referred to as *Streptomyces* life cycle (Hopwood, 2007). The typical process of *Streptomyces* development consists of spore germination, formation of vegetative mycelium and aerial hyphae, which transform to mature endospores. Furthermore “exploration” was observed in several *Streptomyces* spp. recently. Exploration constitutes another form of *Streptomyces* spp. behaviour and occurs in the presence of fungi (Jones and Elliot, 2017). Both processes are initiated by spore germination, the following typical development (illustrated by fig.2A), or explorative growth (illustrated by fig.2B).

Streptomyces spp. spores are able to outlive unfavorable conditions for years. The majority of *Streptomyces* spp. in soil is present in form of dormant spores (Hopwood, 2007). *Streptomyces* spp. resemble filamentous fungi concerning typical growth habits. *Streptomyces* spp. spores germination starts facing beneficial conditions and nutrients. Pursuing the typical *Streptomyces* life cycle, one or two germ tubes are formed which develop by hyphae tip extension and branching into vegetative mycelium. Nutrient depletion initiates the growth of aerial hyphae. Aerial hyphae are coated with a hydrophobic layer, which enables them to overcome the aqueous environment of the vegetative mycelium. Subsequently aerial hyphae transform to spore chains which disseminate mature spores. Moreover, a lack of nutrients induces the production of secondary metabolites in *Streptomyces* spp. (Flärdh and Buttner, 2009).

The switch from growth of vegetative mycelium to formation of aerial hyphae is fulfilled by regulatory proteins, which are products of *bld*-genes. Bld stands for “bald” and refers to the hairless phenotype of *bld*-mutants without aerial hyphae, in contrast to the hirsute phenotype of *Streptomyces* spp. wildtype colonies carrying aerial hyphae (Jones and Elliot, 2017). *Bld* genes play an important role in *Streptomyces* spp. classic development and do furthermore effect antibiotic production (Elliot et al., 2001). A complex of BldD and cyclic di-guanosine-monophosphate (c-di-GMP; a frequently used transmitter substance within bacteria) suppresses *bld* regulator genes, encoding for sigma factor BldN and BldM for instance (Jenal, 2004; Jenal and Malone, 2006; Jones and Elliot, 2018). Moreover, Whi regulator proteins are necessary for the developmental step from aerial hyphae to mature spores, among others. Whi is the abbreviation for “white” and describes the pigment-lacking phenotype of *whi*-gene mutants, which differ from the greyish pigmented phenotype of sporulating

Streptomyces spp. wildtype colonies (Jones and Elliot, 2017). Additionally, BldO regulator protein suppresses WhiB sporulation regulator. When suppression by BldD master regulator protein ends, *Streptomyces* spp. development proceeds. Sigma factor BldN activates BldM-homodimer, BldM-homodimer activates WhiB associated with WhiA and in further consequence WhiG is activated. Further *whi*-genes promote *Streptomyces* spp. sporulation progress (Jones and Elliot, 2018).

Natural habitats of *Streptomyces* spp. are densely colonized with species from various kingdoms. Hence it appears possible, that pure cultures withheld some *Streptomyces* ssp. growth habits. Exploratory growth is an alternative form of Streptomyces spp. development behaviour, observed in several *Streptomyces* spp., *Streptomyces venezuelae* for instance. Exploration occurs when *Streptomyces* spp. colonies are located adjacent to fungi colonies, e. g. yeast, whereby glucose is depleted, but amino acids are still plentiful available. Crucial for initiating exploratory growth is the lack of glucose, which can be induced by glucose-competing fungi or glucose-deficient media. *Streptomyces* spp. exploration starts by growth of vegetative mycelium and aerial spores next to and directly on the yeast colony. Facing glucose depletion *Streptomyces* spp. start to disseminate alkaline trimethylamine (TMA), which constitutes a volatile organic compound (VOC). Thus the local medium pH is increased and exploratory growth is induced in spatially remote *Streptomyces* spp. colonies too. Raising medium pH can eliminate food competitive microorganisms directly. There is also evidence, that TMA effects the susceptibility to antibiotics in bacteria. Hyphae produced during exploratory growth possess a hydrophilic surface and are unbranched. These rapidly growing hyphae allow *Streptomyces* spp. to investigate their environment and enables them to overcome anorganic obstacles. In contrast to the classic *Streptomyces* spp. life cycle *bld*- and *whi*-genes are not involved in genetic control of exploratory growth and responsible genes are unknown yet (Jones and Elliot, 2017; 2018).

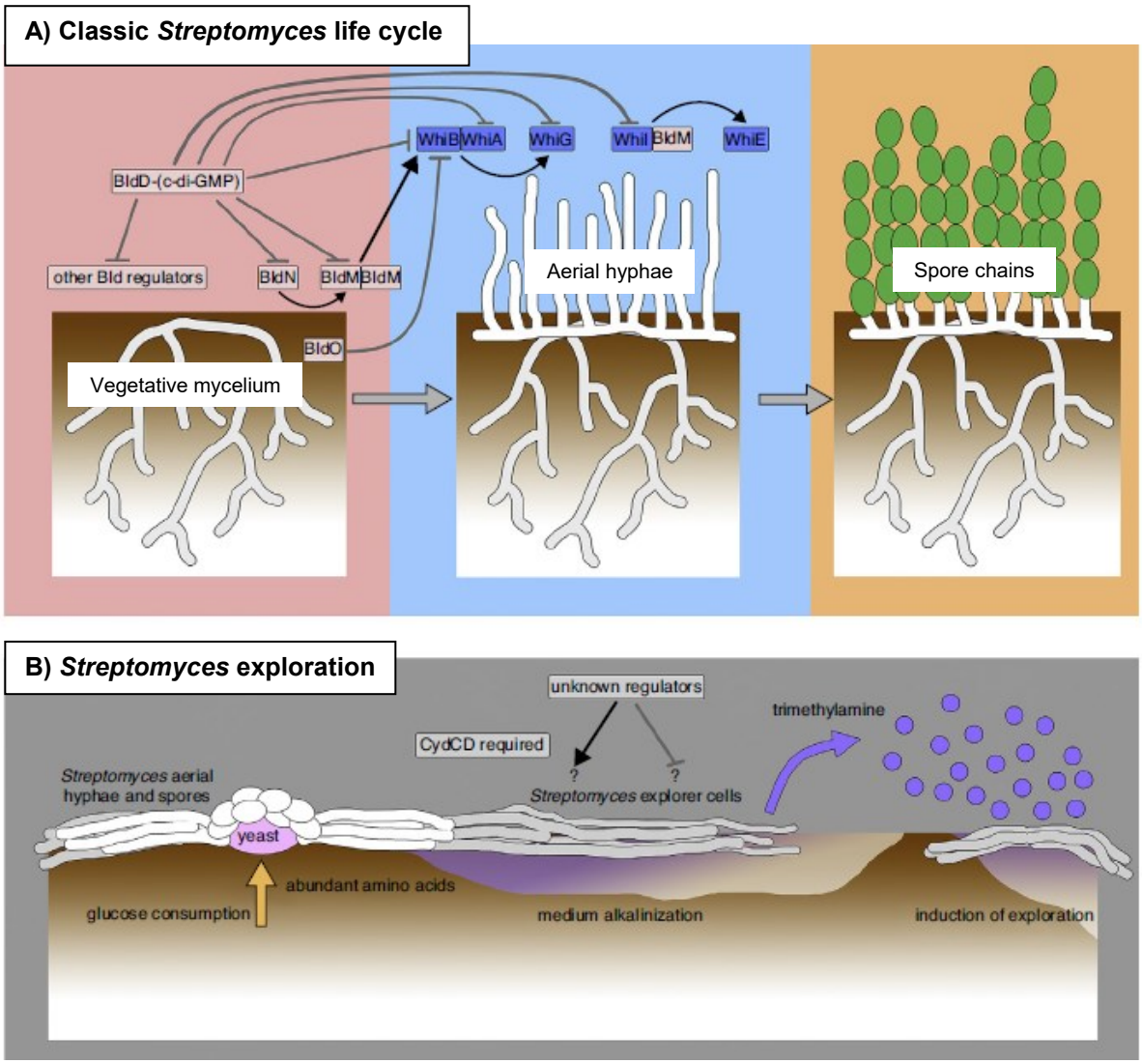


Fig.2 A): The classic *Streptomyces* spp. life cycle consists of growth of vegetative mycelium, formation of aerial hyphae and spore chains. *Bld*- and *whi*-genes, encoding for regulatory proteins, coordinate the transitions between the developmental steps. BldD regulator protein associated with cyclic di-guanosine-monophosphate (c-di-GMP) suppresses sigma factor BldN, BldM-homomer and other Bld regulators as well as regulator proteins WhiB, WhiA, WhiG and WhiI-BldM-heteromer. Repealing of suppression leads to progress in *Streptomyces* spp. development. Sigma factor BldN activates BldM-homodimer, which activates WhiB associated to WhiA. The latter activates regulator protein WhiG. Furthermore WhiI-BldM-heterodimer activates WhiE regulator protein.

Fig.2 B): *Streptomyces* spp. exploratory growth observed in presence of yeast. *Streptomyces* spp. cover the close proximity and the surface of the yeast colony with aerial hyphae and spores. Exploration is initiated by a lack of glucose, whereby amino acids are sufficiently available. Unbranched explorer cells expand and release alkaline trimethylamine (TMA). Cytochrome bd oxidase complex (CydCD) is necessary for exploring *Streptomyces* spp., it eventually provides withstanding of alkaline conditions. Volatile organic compound (VOC) TMA induces exploration in further *Streptomyces* spp. colonies.

Adapted from "'Exploring' the regulation of *Streptomyces* growth and development." by Jones S. E. and Elliot M.A. (2018) Curr. Opin. Microbiol. 42, 26. ©2017 by Elsevier Ltd. Adapted with permission.

The complex life cycle of *Streptomyces* spp. and several other Actinobacteria finds expression in their morphology. Aerial mycelium and spore chains give *Streptomyces* spp. colonies on solid media a furry appearance. Actinobacteria's various spore habits, colorful colonies and pigments among others are considered as helpful for morphological identification (Li et al., 2016).

Morphology of actinobacteria

Colonies of actinobacteria often stand out to the observer by exhibiting a mycotic morphology (Srinivasan et al., 1991). Many actinobacteria form a mycelium, similar to fungi. However, considering cellular organization it is obvious, that actinobacteria are prokaryotes (Barka et al., 2016). The consistency of actinobacteria colonies may be leathery and tough, and they are often able to invade solid media (Srinivasan et al., 1991).

Vegetative mycelium, also referred to as substrate mycelium, anchors the colony in the solid medium and is responsible for nutrient uptake. Lipophilic pigments color the vegetative mycelium in a plethora of different colors, from white, yellow, orange and purple to blue, brown and black, among others. Hydrophilic pigments can be delivered to the surrounding medium. The ability to produce pigments is widespread within actinobacteria and is consulted for morphological description (Li et al., 2016). *Micromonospora* spp. colonies for example exhibit white, orange and brown colors, among others (Barka et al., 2016)

Like *Streptomyces* spp. most actinobacteria produce vegetative mycelium after germination, which develops to aerial mycelium and carries spores afterwards. Reproduction of actinobacteria mainly takes place by producing asexual spores. Within actinobacteria spore formation is organized in morphologically diverse ways. Spores of *Micromonospora* spp., for instance, are carried by vegetative mycelium instead of aerial mycelium. Furthermore the shape of spore chains and the numbers of spores are highly multifarious according to different genera and the surface texture of single spores can give evidence about the genus (Barka et al., 2016). Often not only mycelium but also spores are colored (Li et al., 2016). *Streptomyces* spp. for instance often produce grey pigmented spores (Flärdh and Buttner, 2009).

Morphological description of actinobacteria supports identification efforts, but has to be combined with chemotaxonomic analysis and genomic studies (Barka et al., 2016). Morphological identification facilitates the isolation of targeted rare and less studied actinobacteria. Furthermore methods for isolation of distinct genera are necessary (Li et al., 2016).

Methods for the isolation of bacteria

Soil comprises an enormous plethora of microorganisms. However, microbiology laboratory practice requires pure cultures of bacteria, which are yielded by applying various isolation methods (Harrigan and McCance, 2014; Brooks, 2016). Principal methods for the isolation of bacteria are serial dilution of cell suspensions, pour plate method, streak-out and centrifugation techniques, for instance. Targeted bacteria, e. g. actinobacteria, are intended to be preferably isolated by application of selective measures. Selective isolation methods utilize properties of actinobacteria like nutrition habits, susceptibility to antibiotics and chemical as well as physical influences. Furthermore membrane filter method can be used to isolate genera with filamentous growth style. Combining various selective measures is the most effective approach for the isolation of targeted bacteria (Kumar and Jadeja, 2016).

Especially actinobacteria can easily be outcompeted by other, more quickly expanding bacteria. Therefore selective culture media advantaging actinobacteria are used. Rare actinobacteria are preferably isolated by applying pre-treatment techniques, which are eradicating *Streptomyces* spp. (Hayakawa, 2008). Various pre-treatment methods are based on the assumption, that spores of actinobacteria are more resistant towards physical and chemical treatments than other bacteria (Jiang et al., 2016). Established methods for the reduction of viable frequent and abundant bacteria in environmental samples include treatment with dry or wet heat, ultrasonic waves and microwave irradiation. Pre-treatment of samples with phenol, sodium dodecyl sulfate (SDS), benzethonium chloride, nalidixic acid, leucomycin, tobramycin, chlorhexidine gluconate and chloramine-T, among others, prior to inoculation facilitates isolation of rare actinomycetes (Tiwari and Gupta, 2013; Kumar and Jadeja, 2016).

Moreover, desirable genera of rare actinobacteria are augmented by applying enrichment techniques to original samples. *Pinus* sp. pollen grains can be used to

attract motile actinomycetes from a soil sample suspension. Furthermore rehydration and centrifugation (RC) method is applicable for the selective isolation of motile actinomycetes. Calcium carbonate addition prior to inoculation enhances the number of *Actinokineospora* spp. and various other attractants (e.g. vanillin or γ-collidine) can be applied to facilitate the isolation of certain genera of actinobacteria. (Hayakawa, 2008). Considering, that the vast majority of soil bacteria is uncultivable (Aoi and Epstein, 2016), enrichment techniques are a possible mean to increase the number of cultivable species (Fang et al., 2017).

Nonselective culture media allow growth of a plethora of various bacteria present in the sample (Lagier et al., 2015). Selective isolation of actinobacteria requires culture media, which advantage targeted bacteria towards undesired competitive bacteria (Kumar and Jadeja, 2016). Most actinobacteria have a chemo-organothrophic lifestyle (Goodfellow, 2012). Many actinobacteria are saprophytes (Barka et al., 2016), which are able to metabolize small molecules like glucose as well as complex polysaccharides like agar. *Streptomyces* spp., for instance, are able to exploit a multitude of nutrient sources by producing various enzymes. Available carbon sources effect growth and antibiotic production in bacteria. Abundant glucose leads to affluent growth, but antibiotic production is inhibited. Easily available glucose is consumed first, subsequently more difficult accessible carbon sources are used for producing antibiotics (Sánchez et al., 2010). Hayakawa and Nonomura (1987) designed humic acid vitamin (HV) culture medium, which provides humic acid as exclusive carbon and nitrogen source. Selective culture medium LSV-SE agar contains lignin instead of humic acid. Additionally soy bean flour and soil extract are included (Hayakawa et al., 1996a). Another nutrient-poor selective medium is MGA-SE agar, also containing soil extract (Nonomura and Ohara, 1971). Glycerol, starch, casein, L-arginine, L-asparagine, nitrate, glycine, chitin, B-vitamins and trace salts are further useful ingredients in selective nutrient media for the isolation of actinobacteria (Hayakawa and Nonomura, 1987; Kumar and Jadeja, 2016).

Antibiotics supplementing culture media are further established tools in selective isolation of actinobacteria. Especially fungi inhibiting substances, e. g. nystatin, cycloheximide and pimaricin are important ingredients (Hayakawa, 2008; Kumar and Jadeja, 2016). Furthermore antibiotics inhibiting gram-negative bacteria are helpful. Selecting inhibitors includes considering susceptibility of targeted bacteria towards applied chemicals and antibiotics (Jiang et al., 2016). Substances in use effective

against bacteria are cycloheximide, nalidixic acid, trimethoprim, gentamicin, tunicamycin, leucomycin, faridomycin, kanamycin, josamycin and chlortetracycline (Hayakawa, 2008; Subramani and Aalbersberg, 2013; Kumar and Jadeja, 2016).

The isolation of several extraordinary actinomycetes requires special conditions. Halophilic actinomycetes, for instance, are isolated with salt-supplemented nutrient media and alkaliphilic actinomycetes require nutrient media pH of 10 to 12 for growth. Thermophilic actinobacteria are incubated at 55°C instead of 28°C (Jiang et al., 2016).

Successful selective isolation requires combination of several mentioned means. Combining pre-treatment with antibiotic supplemented selective media is a frequently used approach (Hayakawa, 2008; Kumar and Jadeja, 2016). HV agar supplemented with nalidixic acid and trimethoprim inhibits competitive microorganisms and allows growth of soil actinomycetes (Hayakawa et al., 1996b). Gebreyohannes et al. (2013) applied serial dilutions to sediment samples, dry heat pre-treatment and starch-casein-medium as well as glycerol-yeast extract-agar were supplemented with amphotericin B. Another example of integrated selective isolation is combining serial dilutions of soil samples with microwave irradiation as pre-treatment. Additionally Gause's synthetic agar, Gause's calcium agar and Gause's synthetic nutrient-poor agar were supplemented with antibacterial and antifungal effective potassium dichromate (Wang et al., 2013)

An alternative isolation approach is high-throughput cultivation (HTC) technique, by which microorganisms are grown in microcapsules and detected with flow cytometry. Each microcapsule contains initially a single cell and is cultivated in microtiter plates with nutrient medium. HTC provides nutrients in low concentrations, similar to environmental conditions and targets hitherto uncultivable bacteria (Zengler et al., 2005).

Isolation and cultivation of desirable bacteria is followed by chemotaxonomic and molecular characterization (Barka et al., 2016). Concerning molecular characterization, 16S-rRNA gene sequencing is a well-established method in various disciplines, for identification of bacteria in antibiotic research for instance (Woo et al., 2008).

Identification of bacteria using 16S-rRNA gene sequencing and inferring of phylogenetic trees

Phylogeny discusses the issue of historical evolution of life and depicts it by statistical means (Pagel, 1999). Since evolutionary changes of DNA mostly happen in similar ways (e.g. nucleotide substitution, insertion, deletion or recombination), it is possible to compare organisms using mathematics-based approaches (Nei and Kumar, 2000). Molecular phylogeny is a comparative approach for exploring evolutionary relationships of organisms, for example based on sequence similarities in 16S-rDNA (Woese and Fox, 1977; Woese, 1987). Compiling rooted or unrooted phylogenetic trees or networks is extensively used in many scientific disciplines (Charleston, 2013). Inferring phylogenetic trees is fulfilled by using algorithms (Fuhrman and Hagström, 2008), commonly used examples are maximum likelihood or parsimony. Phylogenetic trees facilitate the visualization of relationships, however, in awareness of highly simplifying realities. (Charleston, 2013).

Ribosomes are cell components responsible for protein biosynthesis, which are rather conserved within species. They constitute high-molecular weight complexes of ribosomal ribonucleic acids (rRNA) and proteins (Müller-Esterl, 2009). The 16S-rRNA gene of 1541 nucleotides (nt) encodes 16S ribosomal RNA, the component of 30S ribosomal subunit (Weisburg et al., 1991; Graw, 2015).

16S-rRNA gene sequencing plays a vital role in taxonomical characterization and determination of phylogenetic relationships of bacteria (Stackebrandt and Goebel, 1994; Woo et al., 2000; Patel, 2001). The number of copies of the 16S-rRNA gene varies from one to 15 within bacteria (Kembel et al., 2012).

16S-rRNA gene is divided in conserved, semi-conserved and hypervariable regions, the latter nine in number (Gray et al., 1984). Because of highly conserved inter- and intra-species properties, the 16S-rRNA gene is proved to be well applicable as a barcoding gene for bacteria (Woese, 1987; Petti, 2007). Highly conserved sequence regions are necessary for resolution of the backbone of the phylogeny and as sites for primer design (Castresana, 2000; Patel, 2001), whereby variable and highly variable regions are allowing a good differentiation of species (Kirschner et al., 1993; Chakravorty et al., 2007). Additionally, the 16S-rDNA is almost ubiquitously present in prokaryotic organisms and its function remains constant (Petti, 2007).

16S-rDNA sequencing is used for many purposes, for example the rapid and affordable identification of bacterial isolates or pathogenic microbes in clinical applications, called barcoding (Woo et al., 2000; Patel, 2001). Even rare bacteria can be identified clearly and reproducibly (Drancourt et al., 2000).

Polymerase chain reaction (PCR) enables quick amplification of certain DNA fragments and sequencing approaches for identification of bacteria are just one of many possible applications (Graw, 2015). 16S-rRNA gene sequencing allows determination of cultured and noncultured microbes (Petti, 2007).

Despite many advantageous properties, 16S-rRNA gene sequencing has also limitations. Determination to genus level is mostly obtained, but determination to species level is often impossible (Drancourt et al., 2000; Mignard and Flandrois, 2006). This method is not applicable for distinguishing *Acinetobacter* species, because of highly similar 16S-rRNA genes. Additionally *Bacillus anthracis* and *B. cereus* possess identical 16S-rRNA genes, however, they differ distinctively by acquiring virulence plasmids (La Scola et al., 2006). DNA-DNA-re-association is an alternative approach describing strain relationships (Stackebrandt and Goebel, 1994). Resulting unknown sequences are compared with sequences from databases. Hence, the reliability of sequencing results stands and falls with the quality of data base entries (Boudewijns et al., 2006). Comparing a sequence to a database by using different programs can result in various similarity data and hence different matches (Drancourt et al., 2000). PCR products of more than one species can be separated by denaturing gradient gel electrophoresis (DGGE) and identified subsequently (Teske et al., 1996).

Summary

Actinobacteria, *Streptomyces* spp. in particular, are prolific sources of antibiotics. Rare actinomycetes are less investigated and subsequently subject of interest. A lot of promise holds the examination of hitherto unexplored environments, endophytic actinomycetes and actinomycetes associated with medicinal plants (Tiwari and Gupta, 2013). Early approaches in drug discovery are exploited again, using nowadays capabilities and expertise (Lewis, 2012).

In this study we aimed to selectively isolate potential producers of antibiotics from the rhizosphere of the folk medicinal plant *Leontopodium nivale* subsp. *alpinum*, originating

from the mountainous area of the Rax. Isolates with promising morphology were cultivated, approximately identified by 16S-rRNA gene sequencing and comparatively analyzed by inferring phylogenetic trees.

5. Results

Rhizosphere soil samples from *Leontopodium nivale* subsp. *alpinum* were collected at the Rax plateau. Soil samples were pre-treated with dry heat, phenol application, microwave exposure and no pre-treatment as a control. Bacteria were isolated using six selective media: AV agar, MC agar, MGA-SE agar, GAC agar, HV agar and LSV-SE agar. Moreover, three different antibiotics (Nystatin, Cycloheximide and Novobiocin) were added to some selective media to reduce the number of frequent and abundant bacteria and fungi. Colonies with morphologies similar to those described for actinomycete bacteria (Labeda, 1987b; Barka et al., 2016; Li et al., 2016) were chosen. Additionally colonies showing visible inhibition zones in contact with other bacteria were gathered. Picked colonies were cultivated, cells were collected from liquid culture, total DNA isolated, 16S-rRNA gene fragments amplified by PCR and sequenced.

In this work we obtained 287 isolates in total, of which 108 isolates were successfully identified to genus level and 179 isolates remained unidentified. 16S-rRNA gene sequence length of at least 450 nt was obtained for 108 isolates. Fig.3 shows the numbers of isolates belonging to 18 different bacterial genera, comprising together these 108 isolates. Of the 108 obtained 16S-rRNA gene sequences 50 possess a length of at least 1300 nt. Those 50 16S-rRNA gene sequences were used for inferring phylogenetic trees. Sequences shorter than 450 nt were not used for further analyses.

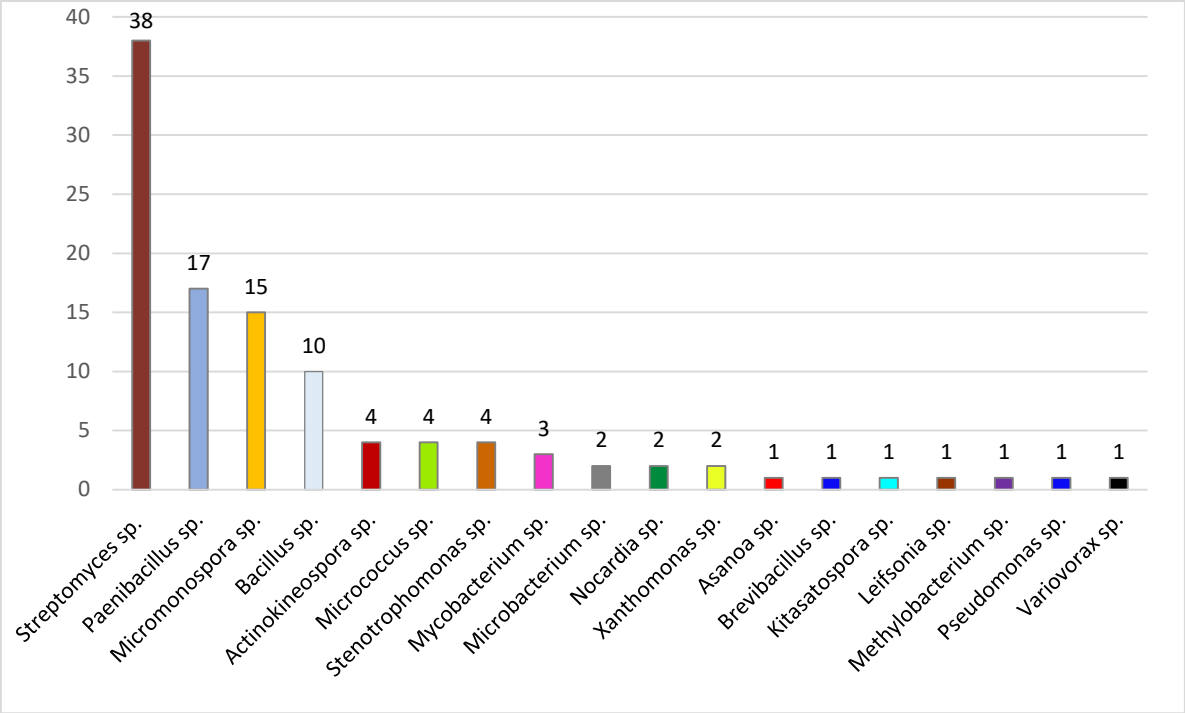


Fig.3: Occurring 18 genera within 108 sequenced isolates sorted by abundance.

13 isolates were disposed after identification through sequencing because of safety concerns based on the “Verordnung biologische Arbeitsstoffe”, provided by the Austrian government (Bundesrecht konsolidiert: Gesamte Rechtsvorschrift für Verordnung biologische Arbeitsstoffe, 2018). That applied to eight isolates of *Bacillus* sp. (thereof four close to *B. cereus*) and two isolates of *Stenotrophomonas* sp. (close to *St. maltophilia*) as well as three isolates of *Mycobacterium* sp. (Fig.4).

Fig.4: Isolation plate showing isolate RLA137, identified as *Mycobacterium* sp. which originates from MGA agar without added antibiotics and control pre-treatment.

Bioactivity in the form of visible inhibition zones in contact with other bacteria (e.g. Fig.5) was observed in case of twelve isolates. Ten bioactive isolates were identified as *Paenibacillus* sp., Fig.7 shows RLA001 (closest match was *Paenibacillus* sp. MBEE99). Two bioactive isolates were identified as *Bacillus* sp.

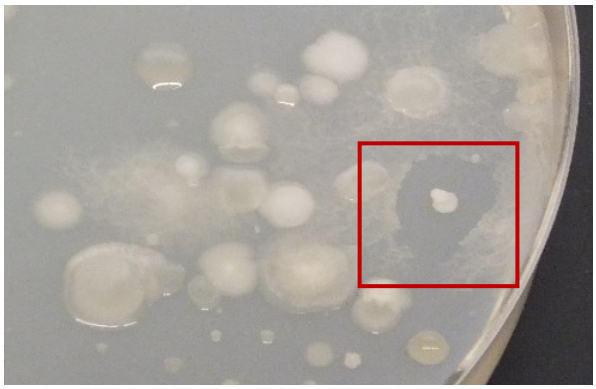


Fig.5: Close-up of a cultivation plate with GAC agar, Nystatin and Cycloheximide added and submitted to microwave pre-treatment. Inhibition zone in contact with other bacteria marked with a red rectangle.

Isolates allocated to media

Selective nutrition media yielded different numbers of isolates. Tbl.3 comprises the total numbers of isolates obtained on different growth media, as well as the number of sequenced isolates therefrom.

Tbl.3: Total numbers of isolates and sequenced isolates allocated to media.

Media	AV	MC	MGA	GAC	HV	LSV	Total
Isolates	42	51	64	62	37	31	287
Sequenced isolates	12	31	18	37	8	2	108

Tbl.4: Yielded genera listed according to the six selective media. This table comprises a total number of 108 isolates identified to genus level.

Genus \ Media	AV	MC	MGA	GAC	HV	LSV
Streptomyces sp.	8	14	4	12	-	-
Paenibacillus sp.	1	2	-	13	1	-
Micromonospora sp.	-	-	12	3	-	-
Bacillus sp.	1	4	-	3	-	2
Actinokineospora sp.	-	4	-	-	-	-
Micrococcus sp.	-	3	-	-	1	-
Stenotrophomonas sp.	1	-	-	-	3	-
Mycobacterium sp.	-	-	1	2	-	-
Microbacterium sp.	-	1	-	-	1	-
Nocardia sp.	-	-	1	1	-	-
Xanthomonas sp.	-	1	-	1	-	-
Asanoa sp.	-	1	-	-	-	-
Brevibacillus sp.	1	-	-	-	-	-
Kitasatospora sp.	-	1	-	-	-	-
Leifsonia sp.	-	-	-	1	-	-
Methylobacterium sp.	-	-	-	-	1	-
Pseudomonas sp.	-	-	-	-	1	-
Variovorax sp.	-	-	-	1	-	-
Total	12	31	18	37	8	2

Streptomyces is the predominantly isolated genus in three out of six selective media. Fig.6 shows *Streptomyces* sp. (closest match was *Streptomyces* sp. RM-27-35). This *Streptomyces* strain was found on GAC agar as well as on MC and AV agar, according to 16S data.

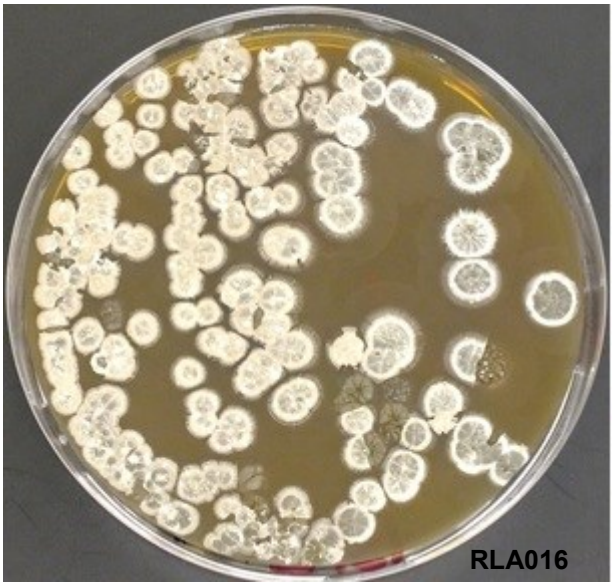


Fig.6: Picture shows isolate RLA016 on ISP2 medium. RLA016 was identified as *Streptomyces* sp. and matches with RLA052 and RLA152, according to 16S data. These isolates arose from control pre-treatment.

Paenibacillus spp. were occurring on AV, MC, GAC and HV agar but were not present on MGA and LSV agar. Fig.7 shows RLA001, identified as *Paenibacillus* sp. (closest match was *Paenibacillus* sp. MBEE99).

Fig.7: High density streak-out plate showing isolate RLA001 on ISP2 medium. RLA001 was identified as *Paenibacillus* sp. (closest match was *Paenibacillus* sp. MBEE99). This strain was obtained on GAC agar (Nystatin and Cycloheximide added) after phenol pre-treatment.

Micromonospora species are abundantly occurring on MGA agar (Fig.8) and on GAC agar they were found too.

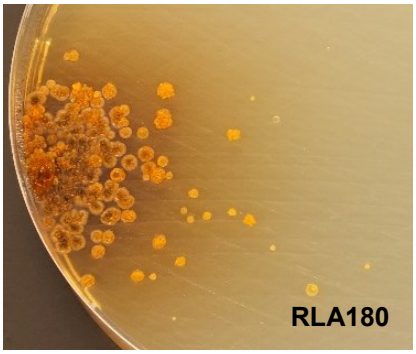


Fig.8: Close-up of a high density streak-out plate with isolate RLA180, identified as *Micromonospora* sp. and most similar to *Micromonospora* sp. HBUC10207. This isolate was originally harvested from MGA agar with Nystatin, Cycloheximide and Novobiocin added after microwave pre-treatment. Most similar to *Micromonospora* sp. HBUC10207 were additionally RLA164, RLA165, and RLA183, all

Bacillus sp. was isolated on four out of six selective media, but was absent on MGA and HV agar. One isolate (RLA112) was identified as *Brevibacillus* sp. and originated from AV agar.

Rare actinomycetes *Actinokineospora* sp., *Asanoa* sp. and *Kitasatospora* sp. appeared exclusively on MC agar. Closest match for four isolates of *Actinokineospora* sp. (RLA067, RLA068, RLA069 and RLA072) was *Actinokineospora diospyrosa* 173276. Fig.9 shows isolate RLA067.

Fig.9: Close-up of isolation plate showing isolate RLA067. *Actinokineospora diospyrosa* 173276 was the closest match for this isolate, which was obtained on MC agar without added antibiotics and control pre-treatment.

Isolate RLA199 (Fig.10) was identified as *Asanoa* sp., most similar to *Asanoa ferruginea* strain 6257-C.

Fig.10: Close-up of isolation plate showing isolate RLA199, identified as *Asanoa* sp., most similar to *Asanoa ferruginea* 6257-C. This strain was originally isolated on MC agar without antibiotics added and after phenol pre-treatment.

Leifsonia sp., which is another rare Actinomycete, was isolated from GAC agar.

Stenotrophomonas spp. (RLA188 and RLA208 similar to *St. maltophilia*, RLA208 and RLA210 similar to *St. rhizophilia*) were isolated on AV and HV agar. *Microbacterium* sp. arose from MC and HV agar. *Micrococcus* sp. was detected on MC and HV agar. *Methylobacterium* sp. arose from HV agar. *Nocardia* sp. from the group *Nocardia soli* as well as *Mycobacterium* sp. were present on MGA and GAC agar. *Pseudomonas* sp. was unique to AV agar. *Variovorax* sp. was only isolated once on GAC medium. *Xanthomonas* sp. was isolated on MC and GAC agar.

AV agar

Tbl.5: Number of isolates originating from AV agar, allocated to dry heat pre-treatment, phenol application, microwave exposure and no pre-treatment as a control.

Pre-treatment / Genus	Dry heat	Phenol	Microwave	No Pre-treatment
Streptomyces sp.	-	-	3	5
Paenibacillus sp.	-	-	-	1
Bacillus sp.	1	-	-	-
Stenotrophomonas sp.	-	-	-	1
Brevibacillus sp.	1	-	-	-

MC agar

Tbl.6: Number of isolates originating from MC agar, allocated to dry heat pre-treatment, phenol application, microwave exposure and no pre-treatment as a control.

Pre-treatment / Genus	Dry heat	Phenol	Microwave	No Pre-treatment
Streptomyces sp.	-	-	-	14
Paenibacillus sp.	-	1	1	-
Bacillus sp.	-	-	-	4
Actinokineospora sp.	-	-	-	4
Micrococcus sp.	3	-	-	-
Microbacterium sp.	-	-	-	1
Xanthomonas sp.	-	-	-	1
Asanoa sp.	-	1	-	-
Kitasatospora sp.	-	-	-	1

MGA agar

Tbl.7: Number of isolates originating from MGA agar, allocated to dry heat pre-treatment, phenol application, microwave exposure and no pre-treatment as a control.

Pre-treatment / Genus	Dry heat	Phenol	Microwave	No Pre-treatment
Streptomyces sp.	-	-	-	4
Micromonospora sp.	-	4	8	-
Mycobacterium sp.	-	-	-	1
Nocardia sp.	-	-	-	1

GAC agar

Tbl.8: Number of isolates originating from GAC agar, allocated to dry heat pre-treatment, phenol application, microwave exposure and no pre-treatment as a control.

Pre-treatment / Genus	Dry heat	Phenol	Microwave	No Pre-treatment
Streptomyces sp.	-	-	1	11
Paenibacillus sp.	-	3	7	3
Micromonospora sp.	-	1	1	1
Bacillus sp.	1	-	1	1
Mycobacterium sp.	-	-	-	2
Nocardia sp.	-	-	-	1
Xanthomonas sp.	-	-	-	1
Leifsonia sp.	-	-	-	1
Variovorax sp.	-	-	-	1

HV agar

Tbl.9: Number of isolates originating from HV agar, allocated to dry heat pre-treatment, phenol application, microwave exposure and no pre-treatment as a control.

Pre-treatment / Genus	Dry heat	Phenol	Microwave	No Pre-treatment
Paenibacillus sp.	-	1	-	-
Micrococcus sp.	1	-	-	-
Stenotrophomonas sp.	-	-	-	3
Microbacterium sp.	-	-	-	1
Methylobacterium sp.	-	-	-	1
Pseudomonas sp.	-	-	-	1

LSV-SE agar

Two isolates of *Bacillus* sp. were obtained on LSV-SE agar from soil samples pre-treated by microwave exposure.

Phylogenetic trees

Unrooted phylogenetic trees were compiled with 16S rDNA sequences of the isolates from the rhizosphere of *Leontopodium nivale* subsp. *alpinum* (RLA). The neighbor joining method was used, combined with maximum likelihood method and bootstrap values were defined. Type strains (T) were ascertained by BLAST search, straininfo.net and GenBank. In several cases no appropriate Type strain was available, hence closely related reference strains were added.

Phylogenetic trees were built according to bacteria's media of origin and based on their affiliation to the groups of Actinobacteria and Bacteria, additionally to the genus *Streptomyces*.

Phylogenetic trees according to media

MGA agar

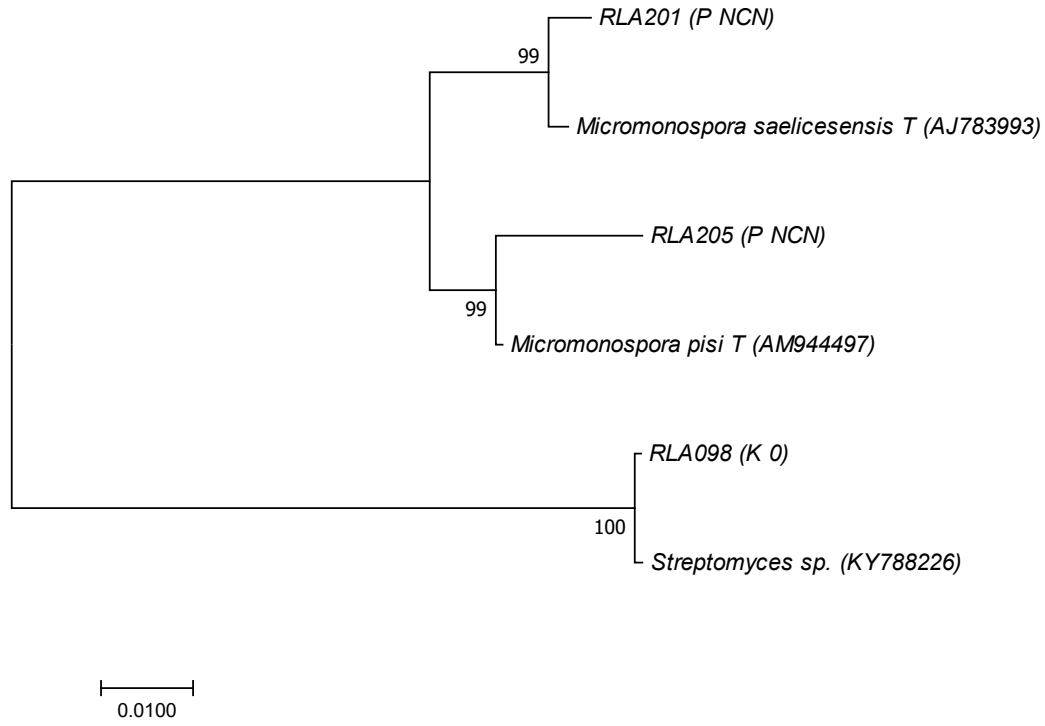


Fig.11: Neighbor joining phylogenetic tree of bacteria allocated to MGA medium was compiled using maximum likelihood method and TN93 model (-lnL = 2778,248513; no. of sites = 1444). Numbers at branch notes are bootstrap percentages based on 1000 resamplings. K = control pre-treatment, P = phenol pre-treatment. 0 = no antibiotics, NC = Nystatin and Cycloheximide, NCN = Nystatin, Cycloheximide and Novobiocin.

MC agar

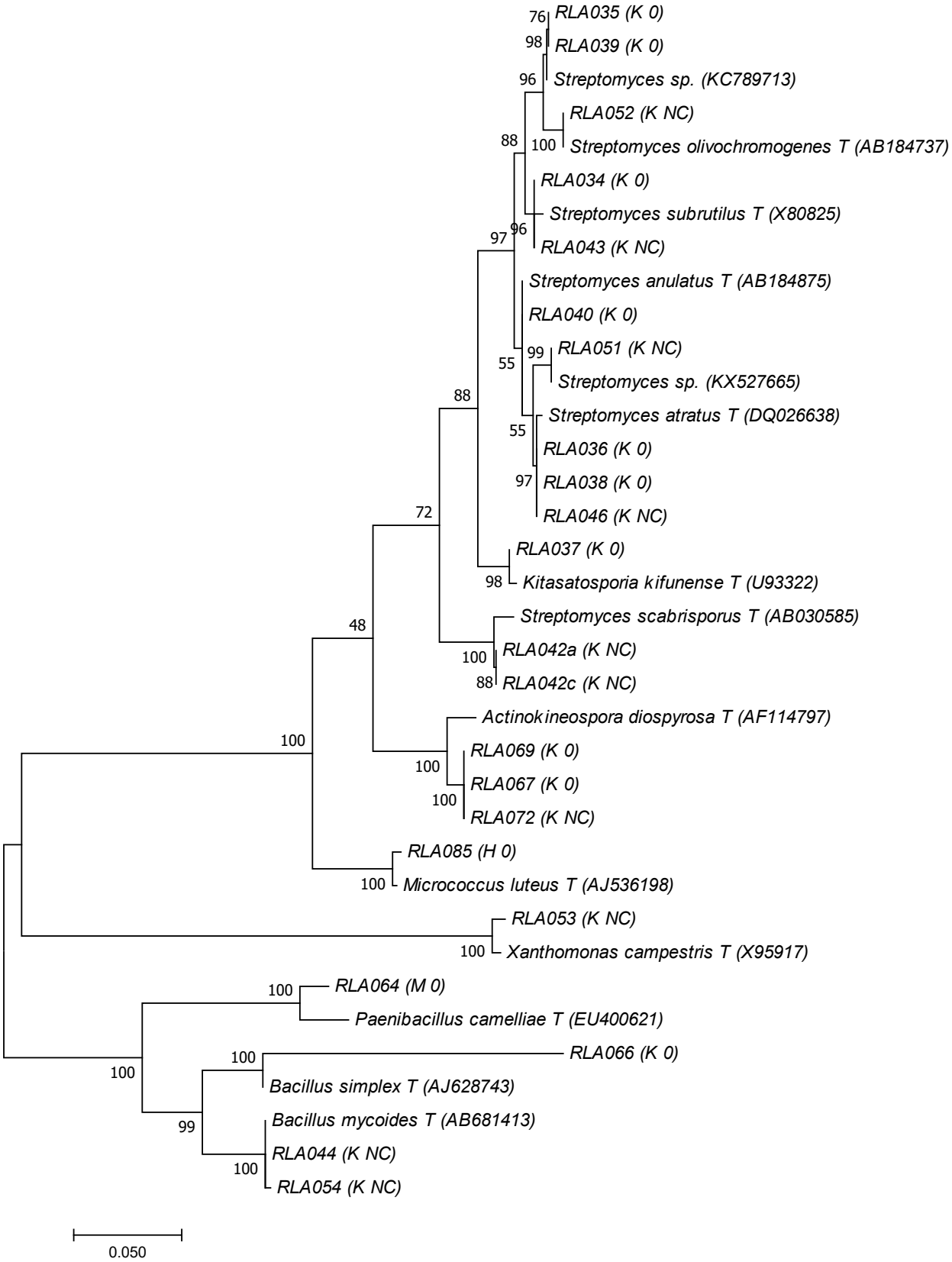


Fig.12: Neighbor joining phylogenetic tree of bacteria allocated to MC medium was compiled using maximum likelihood method and K2+G model (-lnL = 6769,292253; no. of sites = 1530). Numbers at branch notes are bootstrap percentages based on 1000 resamplings. K = control pre-treatment, H = heat pre-treatment, M = microwave pre-treatment. 0 = no antibiotics, NC = Nystatin and Cycloheximide.

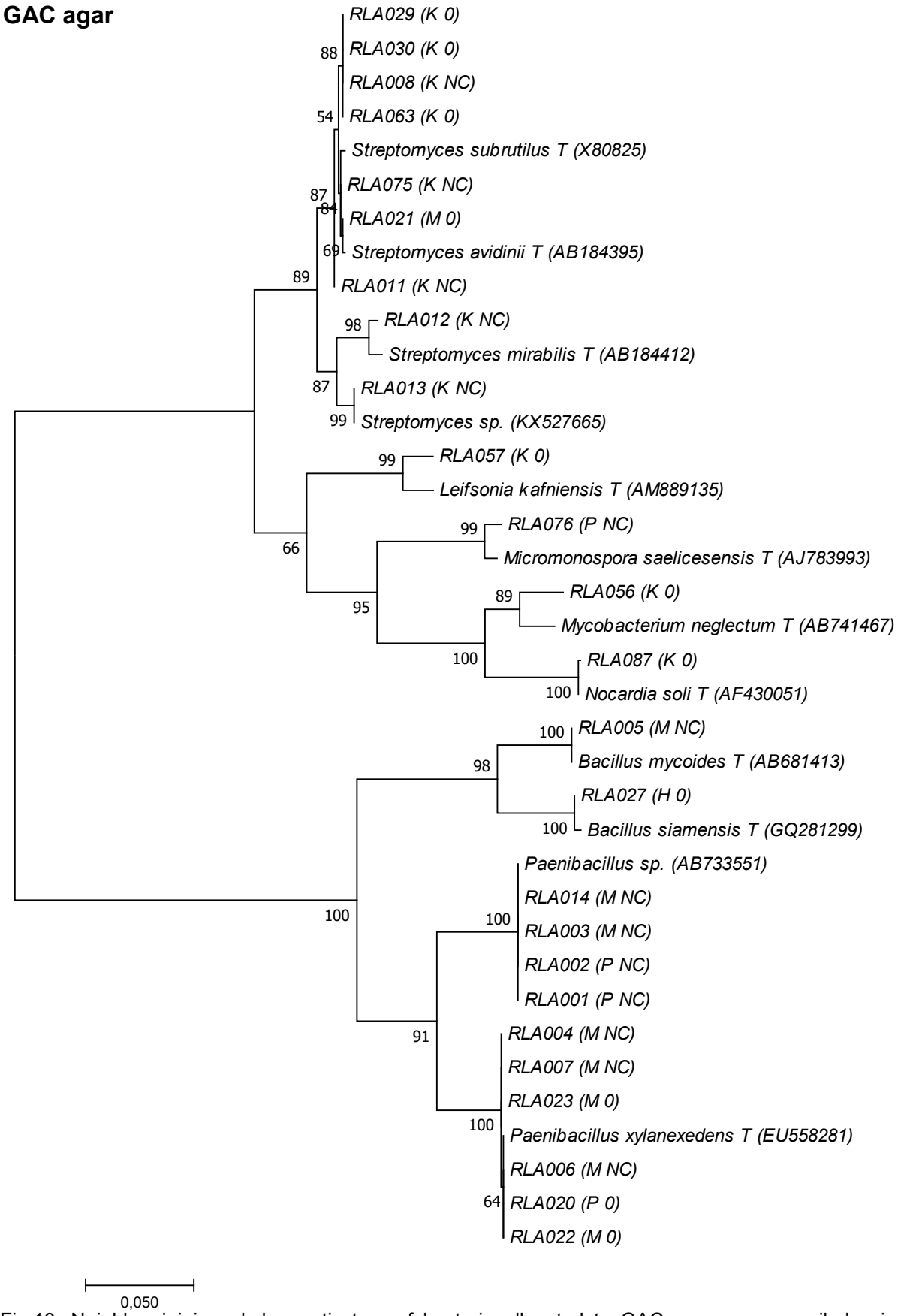


Fig.13: Neighbor joining phylogenetic tree of bacteria allocated to GAC agar was compiled using maximum likelihood method and K2+G model (-lnL = 4820,133495; no. of sites = 1477). Numbers at branch notes are bootstrap percentages based on 1000 resamplings. K = control pre-treatment, H = heat pre-treatment, M = microwave pre-treatment, P = phenol pre-treatment. 0 = no antibiotics, NC = Nystatin and Cycloheximide, NCN = Nystatin, Cycloheximide and Novobiocin.

Phylogenetic trees of Actinobacteria, *Streptomyces* spp. and Bacteria

Actinobacteria except *Streptomyces* species, which are shown in a separate tree, are arranged in a phylogenetic tree (fig.14) with eight clades. Clade one contains two isolates (RLA201 and RLA076) and their type strain *Micromonospora saelicesensis*. RLA201 and RLA076 differ from each other and are distinctively divergent to their type strain. Clade two contains RLA205 and its distantly related type strain *Micromonospora pisi*. Clade three consists of RLA199 and its distantly related type strain *Asanoa ferruginea*. Clade four comprises three ident isolates (RLA072, RLA069 and RLA067) and their distinctively divergent type strain *Actinokineospora diospyrosa*. Clade five consists of RLA056 and distinctively divergent type strain *Mycobacterium neglectum*. Clade six contains isolate RLA087 and is ident with two type strains: *Nocardia soli* and *Nocardia cummidelens*. Clade seven consists of RLA085 and its type strain *Micrococcus luteus*. Clade eight consists of isolate RLA057 and distinctively divergent type strain *Leifsonia kafnienesis*. According to bootstrap values all clades are distinctly distant from each other.

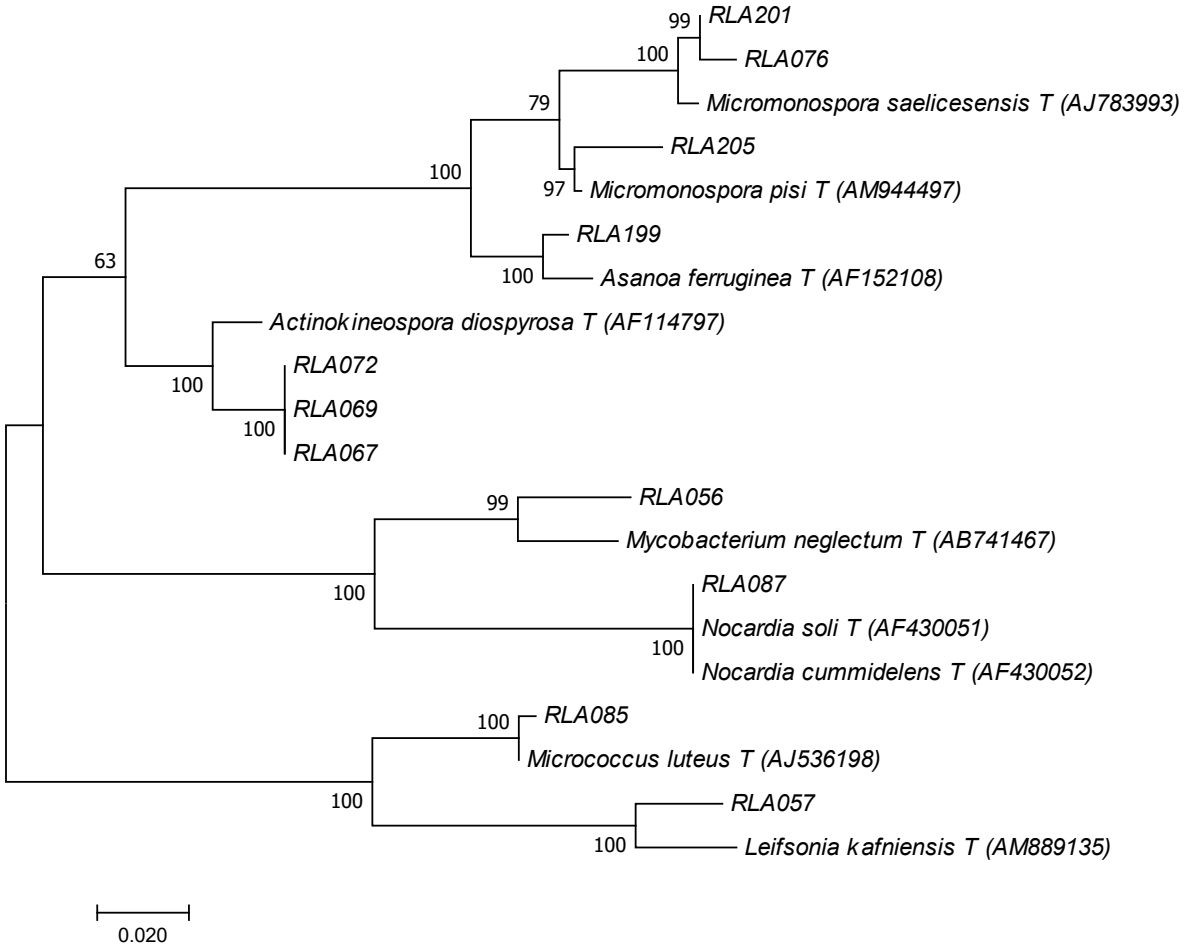


Fig.14: Neighbor joining phylogenetic tree of Actinobacteria (except *Streptomyces* species) was compiled using maximum likelihood method and TN93+G+I model (-lnL = 3189,834; no. of sites = 1452). Numbers at branch notes are bootstrap percentages based on 1000 resamplings.

Streptomyces species are arranged in a phylogenetic tree (fig.15) with ten clades. Clade one consists of ten isolates (RLA156, RLA075, RLA021, RLA011, RLA008, RLA029, RLA030, RLA034, RLA043 and RLA063), two type strains (*S. avidinii* and *S. subrutilus*) and reference strain *Streptomyces* sp. (MG980187). RLA021 is the closest related isolate to type strain *S. avidinii*. RLA156 is ident with RLA075 and reference strain *Streptomyces* sp. (MG980187). Type strain *S. subrutilus* is related to RLA156 and RLA075. Isolate RLA011 is slightly divergent to aforementioned isolates of clade one. Type strain *S. goshikiensis* is too divergent to RLA030 and was therefore excluded. Isolates RLA008, RLA029, RLA030, RLA034, RLA043 and RLA063 are ident and distinctively divergent relatives of other members of clade one. Clade two consists of ident RLA035 and RLA039 and no appropriate type strain was available for them (type strains *S. mirabilis* and *S. olivochromogenes* are far distinctively divergent), but they are related to reference strain *Streptomyces* sp. (KC789713). Clade three consists of RLA012 and distinctively divergent type strain *S. mirabilis*. Clade four consists of RLA052 and type strain *S. olivochromogenes*, which are ident. Clade five consists of RLA040 and type strain *S. anulatus*, which are ident. Clade six consists of RLA051, RLA013 and reference strain *Streptomyces* sp. (KX527665) which is ident with both isolates whereby type strain *S. atratus* is far distinctively divergent. Clade seven contains three ident isolates (RLA036, RLA038 and RLA046) and type strain *S. atratu*s, which is distantly related to the three isolates. Clade eight consists of RLA131, RLA098 and reference strain *Streptomyces* sp. (KY788226). RLA131 is distinctively divergent from RLA098 and the reference strain. Clade nine consists of RLA037 and distinctively divergent type strain *Kitasatospora kifunense* (U93322). Clade ten consists of RLA042a, RLA042c and type strain *S. scabrisporus*. RLA042a and RLA042c are ident. Type strain *S. scabrisporus* is distinctively divergent to both isolates. According to bootstrap values all clades are distinctly distant from each other.

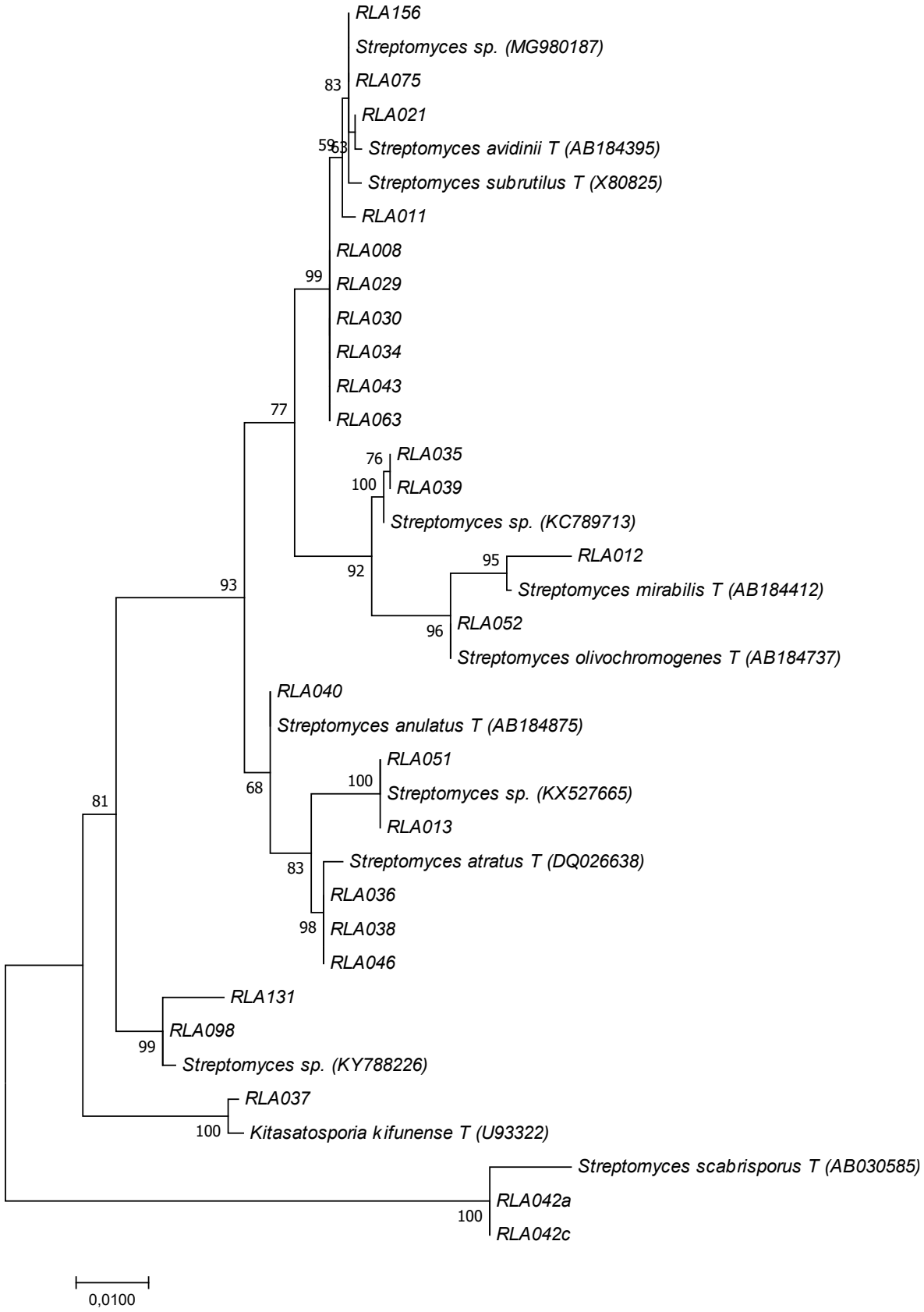


Fig.15: Neighbor-joining phylogenetic tree of *Streptomyces* species was compiled using maximum likelihood method and T92+G+I model (-lnL = 2891,735; no. of sites = 1449). Numbers at branch notes are bootstrap percentages based on 1000 resamplings.

Various Bacteria species are arranged in a phylogenetic tree (fig.16) with seven clades. Clade one consists of six isolates (RLA006, RLA020, RLA022, RLA004, RLA007 and RLA023) and type strain *Paenibacillus xylanexedens*. RLA006, RLA020 and RLA022 are identical with the type strain. RLA004, RLA007 and RLA023 are closely related to the aforementioned clan. Clade two consists of four isolates (RLA001, RLA003, RLA002 and RLA014) and reference strain *Paenibacillus* sp. (AB733551) which are ident. For clade two no appropriate type strain was available. Clade three contains RLA064 and distinctively divergent type strain *Paenibacillus camelliae*. Clade four contains RLA027 and closely related type strain *Bacillus siamensis*. Clade five consists of four isolates (RLA047, RLA054, RLA005 and RLA044) and type strain *Bacillus mycoides*. RLA047, RLA005 and RLA044 are identical with type strain *B. mycoides*. RLA054 is closely related to type strain *B. mycoides*. Clade six contains RLA066 and far distinctively divergent type strain *Bacillus simplex* which is the most appropriate available type strain. Clade seven forms an outgroup to the *Bacillus*- and *Paenibacillus*-clades. Clade seven contains RLA053 and distinctively divergent type strain *Xanthomonas campestris*. According to bootstrap values all clades are distinctly distant from each other.

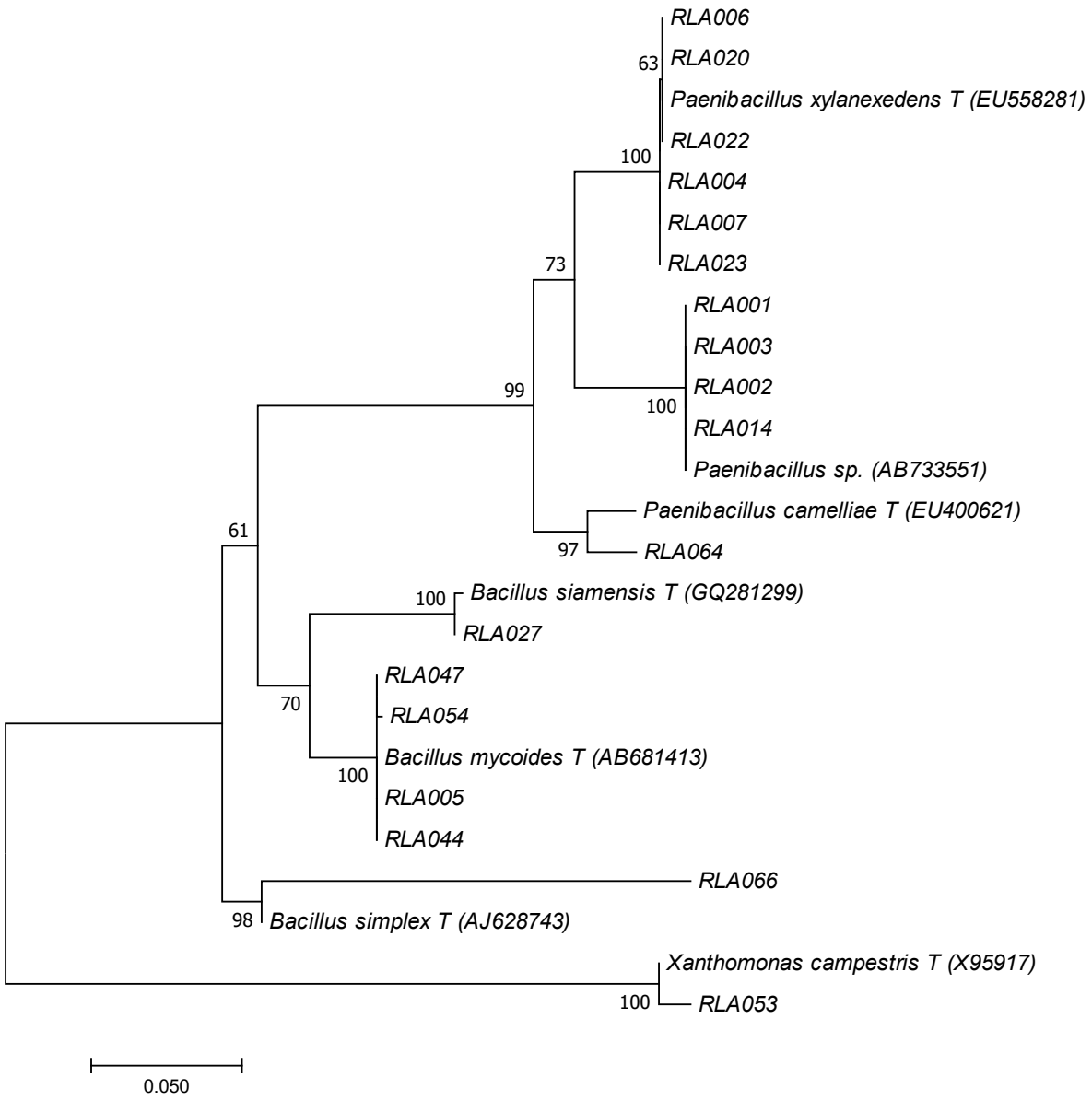


Fig.16: Neighbor-joining phylogenetic tree of various Bacteria species was compiled using maximum likelihood method and K2+G model (-lnL = 4746,348; no. of sites = 1504). Numbers at branch notes are bootstrap percentages based on 1000 resamplings.

Final table

Isolate ID	Medium / Treatment	Sequence length [nt]	16S rRNA based Phylogeny			Notes
			BLAST		RDP	
			Closest Match	ident		
RLA001	GAC / P NC	1440	*Paenibacillus* sp. MBEE99	99%	*Paenibacillus* sp.	bioactive
RLA002	GAC / P NC	1445	*Paenibacillus* sp. MBEE99	99%	*Paenibacillus* sp.	bioactive
RLA003	GAC / M NC	1437	*Paenibacillus* sp. MBEE99	99%	*Paenibacillus* sp.	bioactive
RLA004	GAC / M NC	1434	*Paenibacillus* sp. S8	99%	*Paenibacillus* sp.	bioactive
RLA005	GAC / M NC	1409	*Bacillus cereus* 262AG4	100%	*Bacillus* sp.	bioactive
RLA006	GAC / M NC	1437	*Paenibacillus xylanexedens* IARI-KP4	99%	*Paenibacillus* sp.	bioactive
RLA007	GAC / M NC	1434	Bacterium UKR A17	99%	*Paenibacillus* sp.	bioactive
RLA008	GAC / K NC	1403	*Streptomyces nojiriensis* T29	99%	*Streptomyces* sp.	-
RLA009	GAC / K NC	930	*Paenibacillus* sp. IHB B 3310	99%	*Paenibacillus* sp.	-
RLA010	GAC / K NC	1004	*Streptomyces lavendulae* M3-3	99%	*Streptomyces* sp.	-
RLA011	GAC / K NC	1399	*Streptomyces goshikiensis* HBUM173750	99%	*Streptomyces* sp.	-
RLA012	GAC / K NC	1396	*Streptomyces* sp. ESM205	99%	*Streptomyces* sp.	-
RLA013	GAC / K NC	1398	*Streptomyces* sp. WW4-2c	99%	*Streptomyces* sp.	-
RLA014	GAC / M NC	1444	*Paenibacillus* sp. MBEE99	99%	*Paenibacillus* sp.	bioactive
RLA015	GAC / K NC	941	*Paenibacillus amylolyticus* I-7	99%	*Paenibacillus* sp.	-
RLA016	GAC / K NC	991	*Streptomyces* sp. 27-35	99%	*Streptomyces* sp.	-
RLA017	GAC / K NC	956	*Streptomyces cirratus* IHB 10402	99%	*Streptomyces* sp.	-

Tbl.10: Bacterial isolates from the rhizosphere of *Leontopodium nivale* ssp. *alpinum*. K = control pre-treatment, H = heat pre-treatment, M = microwave pre-treatment, P = phenol pre-treatment. 0 = no antibiotics, NC = Nystatin and Cycloheximide, NCN = Nystatin, Cycloheximide and Novobiocin.

Isolate ID	Medium / Treatment	Sequence length [nt]	16S rRNA based Phylogeny			Notes
			BLAST		RDP	
			Closest Match	ident		
RLA019	GAC / K NCN	908	*Micromonospora* sp. ZSGR11	100%	*Micromonospora* sp.	-
RLA020	GAC / P 0	1437	*Paenibacillus* sp. IHB B 3310	99%	*Paenibacillus* sp.	bioactive
RLA021	GAC / M 0	1400	*Streptomyces* sp. ISE_29	99%	*Streptomyces* sp.	-
RLA022	GAC / M 0	1432	*Paenibacillus xylanexedens* IARI-KP4	99%	*Paenibacillus* sp.	bioactive
RLA023	GAC / M 0	1439	Bacterium UKR A17	99%	*Paenibacillus* sp.	bioactive
RLA024	GAC / K 0	847	*Variovorax* sp. URHB0020	99%	*Variovorax* sp.	-
RLA025	GAC / K 0	883	*Bacillus* sp. BA-110-09	99%	*Bacillus* sp.	-
RLA026	GAC / K 0	951	*Paenibacillus* sp. JG-TB16	99%	*Paenibacillus* sp.	-
RLA027	GAC / H 0	1435	*Bacillus vallismortis*	100%	*Bacillus* sp.	-
RLA029	GAC / K 0	1395	*Streptomyces* sp. QLS92	99%	*Streptomyces* sp.	-
RLA030	GAC / K 0	1398	*Streptomyces goshikiensis* HBUM173360	99%	*Streptomyces* sp.	-
RLA034	MC / K 0	1401	*Streptomyces avidinii*	99%	*Streptomyces* sp.	-
RLA035	MC / K 0	1403	*Streptomyces* sp. PL21	99%	*Streptomyces* sp.	-
RLA036	MC / K 0	1398	*Streptomyces atratus* T11	99%	*Streptomyces* sp.	-
RLA037	MC / K 0	1403	*Streptomyces herbaricolor* P3	99%	*Kitasatospora* sp.	-
RLA038	MC / K 0	1394	*Streptomyces atratus* HBUM175124	99%	*Streptomyces* sp.	-
RLA039	MC / K 0	1393	*Streptomyces* sp. PL21	99%	*Streptomyces* sp.	-
RLA040	MC / K 0	1398	*Streptomyces* sp. GS16	99%	*Streptomyces* sp.	-

Tbl.10: Bacterial isolates from the rhizosphere of *Leontopodium nivale* ssp. *alpinum*. K = control pre-treatment, H = heat pre-treatment, M = microwave pre-treatment, P = phenol pre-treatment. 0 = no antibiotics, NC = Nystatin and Cycloheximide, NCN = Nystatin, Cycloheximide and Novobiocin.

Isolate ID	Medium / Treatment	Sequence length [nt]	16S rRNA based Phylogeny			Notes
			BLAST		RDP	
			Closest Match	ident		
RLA041	MC / K NC	593	*Streptomyces* sp.	93%	Unclass. Actinomycetales	-
RLA042a	MC / K NC	1400	*Streptomyces scabrisporus* 173877	99%	*Streptomyces* sp.	-
RLA042c	MC / K NC	1395	*Streptomyces scabrisporus* Cac02	99%	*Streptomyces* sp.	-
RLA043	MC / K NC	1403	*Streptomyces avidinii*	99%	*Streptomyces* sp.	-
RLA044	MC / K NC	1432	*Bacillus* sp.	99%	*Bacillus* sp.	bioactive
RLA046	MC / K NC	1399	*Streptomyces atratus* T11	99%	*Streptomyces* sp.	-
RLA051	MC / K NC	1409	*Streptomyces brevispora* BK160	99%	*Streptomyces* sp.	-
RLA052	MC / K NC	1358	*Streptomyces* sp. RM 27-35	99%	*Streptomyces* sp.	-
RLA053	MC / K NC	1426	*Xanthomonas* sp. 33DCP	99%	*Xanthomonas* sp.	-
RLA054	MC / K NC	894	*Bacillus mycoides* 0511ARD10J4	99%	*Bacillus* sp.	-
RLA056	GAC / K 0	1392	Uncultured bacterium clone ncd560g05c1	99%	*Mycobacterium* sp.	-
RLA057	GAC / K 0	1401	Uncultured bacterium clone FCPT705	99%	*Leifsonia* sp.	-
RLA058	GAC / K 0	902	*Mycobacterium neglectum* GMC128	99%	*Mycobacterium* sp.	-
RLA059	GAC / M 0	907	*Micromonospora* sp. Ume45	100%	*Micromonospora* sp.	-
RLA060	GAC / K 0	862	*Xanthomonas translucens* RFP2	99%	*Xanthomonas* sp.	-
RLA063	GAC / K 0	1405	*Streptomyces nojiriensis* T29	99%	*Streptomyces* sp.	-
RLA064	MC / M 0	1443	*Paenibacillus* sp. HA10	98%	*Paenibacillus* sp.	-

Tbl.10: Bacterial isolates from the rhizosphere of *Leontopodium nivale* ssp. *alpinum*. K = control pre-treatment, H = heat pre-treatment, M = microwave pre-treatment, P = phenol pre-treatment. 0 = no antibiotics, NC = Nystatin and Cycloheximide, NCN = Nystatin, Cycloheximide and Novobiocin. Unclass. = unclassified.

Isolate ID	Medium / Treatment	Sequence length [nt]	16S rRNA based Phylogeny			Notes
			BLAST		RDP	
			Closest Match	ident		
RLA065	MC / K 0	1180	*Bacillus* sp. PG-2010-18	80%	Unclass. Bacteria	-
RLA066	MC / K 0	1486	*Bacillus* sp. PG-2010-18	89%	Unclass. Bacillales	-
RLA067	MC / K 0	1393	*Actinokineospora diospyrosa* 173276	99%	*Actinokineospora* sp.	-
RLA068	MC / K 0	743	*Actinokineospora* sp.	99%	Unclass. Pseudo-nocardiaceae	-
RLA069	MC / K 0	1391	*Actinokineospora diospyrosa* 173276	99%	*Actinokineospora* sp.	-
RLA070	MC / K 0	821	*Kitasatospora* sp. SCAUEIIIA1r-1	99%	*Kitasatospora* sp.	-
RLA072	MC / K NC	1388	*Actinokineospora diospyrosa* 173276	99%	*Actinokineospora* sp.	-
RLA073	MC / K NC	844	*Microbacterium* sp. Zs19	99%	*Microbacterium* sp.	-
RLA075	GAC / K NC	1366	*Streptomyces* sp.	99%	*Streptomyces* sp.	-
RLA076	GAC / P NC	1387	*Micromonospora* sp. GUI14	99%	*Micromonospora* sp.	-
RLA085	MC / H 0	1398	*Micrococcus luteus* I-A-R-2	99%	*Micrococcus* sp.	-
RLA086	MC / H 0	908	*Micrococcus yunnanensis* 0911MAR18R4	99%	*Micrococcus* sp.	-
RLA087	GAC / K 0	1287	*Nocardia soli* KSI1234	99%	*Nocardia* sp.	-
RLA098	MGA / K 0	1402	*Streptomyces* sp. A48	98%	*Streptomyces* sp.	-
RLA099	MGA / K 0	948	*Streptomyces* sp. ACTY	99%	Unclass. *Streptomycetaceae*	-
RLA103	MGA / K NC	882	*Streptomyces* sp. F4475	99%	*Streptomyces* sp.	-
RLA108	MGA / K NC	766	*Nocardia* sp. 4HB-9	99%	*Nocardia* sp.	-

Tbl.10: Bacterial isolates from the rhizosphere of *Leontopodium nivale* ssp. *alpinum*. K = control pre-treatment, H = heat pre-treatment, M = microwave pre-treatment, P = phenol pre-treatment. 0 = no antibiotics, NC = Nystatin and Cycloheximide, NCN = Nystatin, Cycloheximide and Novobiocin. Unclass. = unclassified.

Isolate ID	Medium / Treatment	Sequence length [nt]	16S rRNA based Phylogeny			Notes
			BLAST		RDP	
			Closest Match	ident		
RLA112	AV / H 0	932	Uncultured bacterium clone nbt124g10	99%	*Brevibacillus* sp.	-
RLA115	AV / K 0	818	*Streptomyces* sp. MJM9110	98%	*Kitasatospora* sp.	-
RLA120	AV / K 0	883	*Streptomyces* sp. 746G1	99%	*Streptomyces* sp.	-
RLA121	AV / K 0	903	*Streptomyces* sp. A120	99%	*Streptomyces* sp.	-
RLA129	AV / H NC	915	*Bacillus cereus* RGN1.3	99%	*Bacillus* sp.	-
RLA131	AV / M NC	1353	*Streptomyces drozdowiczii* IHBA 11040	99%	*Streptomyces* sp.	-
RLA132	AV / M NC	891	*Streptomyces vinaceus* AS-65	100%	*Streptomyces* sp.	-
RLA137	MGA / K 0	909	*Mycobacterium* sp. Lor30	99%	*Mycobacterium* sp.	-
RLA139	MGA / K 0	942	*Streptomyces* sp. S1B	98%	*Streptomyces* sp.	-
RLA148	MC / H 0	869	*Micrococcus yunnanensis* G1-7-20	96%	*Micrococcus* sp.	-
RLA152	AV / K 0	1072	*Streptomyces* sp. RM-27-35	99%	*Streptomyces* sp.	-
RLA156	AV / K 0	1400	*Streptomyces* sp.SIIB_Cd_R4	99%	*Streptomyces* sp.	-
RLA164	MGA / M NCN	875	*Micromonospora* sp. HBUC10207	99%	*Micromonospora* sp.	-
RLA165	MGA / M NCN	922	*Micromonospora* sp. HBUC10207	99%	*Micromonospora* sp.	-
RLA166	MGA / M NCN	898	*Micromonospora* sp. YIM 65395	99%	*Micromonospora* sp.	-
RLA167	MGA / M NCN	915	*Micromonospora* sp. HBUC10207	99%	Unclass. *Micromonosporaceae*	-
RLA175	MGA / M NCN	943	*Micromonospora maoerensis* NEAU-MES19	99%	*Micromonospora* sp.	-

Tbl.10: Bacterial isolates from the rhizosphere of *Leontopodium nivale* ssp. *alpinum*. K = control pre-treatment, H = heat pre-treatment, M = microwave pre-treatment, P = phenol pre-treatment. 0 = no antibiotics, NC = Nystatin and Cycloheximide, NCN = Nystatin, Cycloheximide and Novobiocin. Unclass. = unclassified.

Isolate ID	Medium / Treatment	Sequence length [nt]	16S rRNA based Phylogeny			Notes
			BLAST		RDP	
			Closest Match	ident		
RLA177	MGA / M NCN	945	*Micromonospora maoerensis* NEAU-MES19	99%	*Micromonospora* sp.	-
RLA180	MGA / M NCN	799	*Micromonospora* sp. HBUC10207	99%	*Micromonospora* sp.	-
RLA183	MGA / M NCN	875	*Micromonospora* sp. HBUC10207	99%	*Micromonospora* sp.	-
RLA188	AV / K NC	800	*Stenotrophomonas maltophilia* JYD-4	99%	*Stenotrophomonas* sp.	-
RLA191	AV / M NC	953	*Streptomyces exfoliatus* 0911TES26Z4	99%	*Streptomyces* sp.	-
RLA195	AV / K 0	539	*Paenibacillus* sp. UA-VH0139	94%	Unclass. Bacillales	-
RLA198	MC / P 0	865	*Paenibacillus* sp. PhyCEm-108	99%	*Paenibacillus* sp.	-
RLA199	MC / P 0	843	*Asanoa ferruginea* 6257-C	99%	*Asanoa* sp.	-
RLA201	MGA / P NCN	1389	*Micromonospora* sp. ZSGR11	99%	*Micromonospora* sp.	-
RLA204	MGA / P NCN	582	*Micromonospora saelicesensis* YIM130886	93%	Unclass. *Micromonosporaceae*	-
RLA205	MGA / P NCN	1377	*Micromonospora* sp. HBUB10301	99%	Unclass. *Micromonosporaceae*	-
RLA208	HV / K NCN	935	Bacterium XD-H1	99%	*Stenotrophomonas* sp.	-
RLA209	HV / K NCN	965	*Stenotrophomonas maltophilia*	99%	*Stenotrophomonas* sp.	-
RLA210	HV / K NCN	895	*Stenotrophomonas rhizophilia*	100%	*Stenotrophomonas* sp.	-
RLA215	MGA / P NCN	933	*Micromonospora maoerensis* NEAU-MES19	99%	*Micromonospora* sp.	-
RLA223	LSV / M 0	918	*Bacillus* sp. DU164(2010)	99%	*Paenibacillus* sp.	-
RLA232	HV / K NCN	932	*Pseudomonas migulae* MS2	99%	*Pseudomonas* sp.	-

Tbl.10: Bacterial isolates from the rhizosphere of *Leontopodium nivale* ssp. *alpinum*. K = control pre-treatment, H = heat pre-treatment, M = microwave pre-treatment, P = phenol pre-treatment. 0 = no antibiotics, NC = Nystatin and Cycloheximide, NCN = Nystatin, Cycloheximide and Novobiocin. Unclass. = unclassified.

Isolate ID	Medium / Treatment	Sequence length [nt]	16S rRNA based Phylogeny			Notes
			BLAST		RDP	
			Closest Match	ident		
RLA251	HV / K NC	795	*Methylobacterium* sp.	99%	*Methylobacterium* sp.	-
RLA252	HV / P NC	891	*Paenibacillus* sp. DSL09-3	99%	*Paenibacillus* sp.	-
RLA259	LSV / M 0	903	*Bacillus* sp. 262XG4	99%	*Bacillus* sp.	-
RLA278	HV / H 0	541	*Micrococcus aloeverae* PP-06	99%	*Micrococcus* sp.	-
RLA280	HV / K 0	866	*Microbacterium* sp.	99%	*Microbacterium* sp.	-

Tbl.10: Bacterial isolates from the rhizosphere of *Leontopodium nivale* ssp. *alpinum*. K = control pre-treatment, H = heat pre-treatment, M = microwave pre-treatment, P = phenol pre-treatment. 0 = no antibiotics, NC = Nystatin and Cycloheximide, NCN = Nystatin, Cycloheximide and Novobiocin.

6. Discussion

In this project we obtained 287 bacterial isolates by applying various selective treatments. The genera some of these bacteria belong to that were identified are discussed in this section. Furthermore, the experimental design will be discussed in detail.

Selective media & pre-treatments

Most of the isolates were obtained from MGA agar, closely followed by GAC agar. *Micromonospora* spp. were exclusively obtained on MGA and GAC agar. Phenol pre-treatment is known to eliminate the majority of present bacteria, including *Streptomyces* species. *Micromonospora* spp., however, were emerging after phenol pre-treatment (Hayakawa et al, 1991). For MGA medium, phenol pre-treatment resulted in four *Micromonospora* spp. and microwave exposure resulted in eight *Micromonospora* spp. There is evidence, that microwave exposure elicits growth of rare actinomycetes, especially *Micromonospora* sp. (Bulina et al., 1997). Therefore, MGA medium combined with phenol pre-treatment or microwave exposure is suitable for the isolation of *Micromonospora* spp.

Isolates with observed bioactivity in contact with other bacteria sourced mainly from GAC agar and were predominantly assigned to the genus *Paenibacillus*. For GAC medium, no pre-treatment as a control resulted in eleven *Streptomyces* spp. *Streptomyces* spp. were especially well growing on GAC and MC media, therefore those media are best for the isolation of *Streptomyces* species.

The highest variety of genera was obtained from MC agar, which could perhaps be associated with the ingredient $NaNO_3$, which is unique to this medium compared to others. For MC medium, phenol pre-treatment resulted in one *Asanoa* sp. and no pre-treatment as a control resulted in four *Actinokineospora* spp., one *Kitasatospora* sp. and one *Microbacterium* sp. Therefore, MC medium is also suitable for the isolation of rare actinomycetes, especially those belonging to the abovementioned genera.

Heat pre-treatment facilitates the selection of rare actinomycetes, according to Jiang et al. (2006). We experienced that dry heat pre-treatment diminished the number of cultivable microorganisms compared to control and other pre-treatments significantly. This reduction could give slowly growing rare actinomycetes an advantage over faster growing bacteria, e. g. *Streptomycetes*. This claim is supported by report from Hayakawa et al. (1991). The mentioned survey also reports, that *Streptomyces* and *Micromonospora* are susceptible to heat and were not isolated after heat pre-treatment, which is consistent with our observation. Additionally, the number of *Streptomyces* isolates after microwave exposure is strikingly low, which could have been caused by the heating effect of the microwave pre-treatment. Vela and Wu (1979) claimed, that the lethal effect of microwave exposure to microorganisms is based on thermal heating.

Antibiotics

Three different antibiotics were added to agar plates to reduce the number of common and abundant bacteria and fungi. Isolated genera from agar plates without antibiotics added compared to isolated genera on media with Nystatin and Cycloheximide (NC) treatment do not differ significantly. *Streptomyces* spp. occur on media without antibiotics added and media with NC treatment, but are absent on media with Nystatin, Cycloheximide and Novobiocin (NCN) treatment. However, *Micromonospora* is the most frequently occurring genus in NCN containing agar plates. Hayakawa et al. (1991) report, that Novobiocin effectively selects *Micromonospora* spp., which is consistent with our observations. Consequently, hardly any *Micromonospora* spp. were observed on selective media without antibiotics added.

Compiling of phylogenetic trees

Phylogenetic analysis depicts evolutionary relationships between organisms (Nei and Kumar, 2000) and allows us to taxonomically characterize the bacteria isolated in this project. Comparison of 16S-rDNA sequences from isolated bacteria with known type strains facilitates the description of obtained bacteria.

Phylogenetic trees: rare actinobacteria

Micromonospora spp. are frequently occurring among the studied isolates. *Micromonospora* is, just as *Streptomyces*, a soil dwelling bacterial genus and a prolific producer of antibiotics (Qiu et al., 2008). Furthermore, *Micromonospora* species are commonly widespread in nature. They inhabit terrestrial and aqueous environments, as well as plant tissues (Ruixia et al., 2014).

Isolates RLA201 and RLA076 are distantly related to the type strain of *Micromonospora saelicesensis* (AJ783993) which was described for the first time by Trujillo et al. (2007). According to available literature there is nothing known about substances with antibiotic properties produced by *Micromonospora saelicesensis*.

Isolate RLA205 is distantly related to type strain *Micromonospora pisi* (AM944497) which was described for the first time by Garcia et al. (2010). According to available literature there is nothing known about substances with antibiotic properties produced by *Micromonospora pisi*.

Isolate RLA199 is distantly related to type strain *Asanoa ferruginea* (AF152108). Little is known about the rare actinomycetes genus *Asanoa.* As mentioned by Lee (2002) *Asanoa* spp. colonies are looking orange, hardly sporulate, and *Asanoa* spp. can be isolated from various soils. *Asanoa* spp. are also reported as inhabitants of the rhizosphere (Niemhom et al., 2016). According to the available literature, nothing is known about substances with antibiotic properties produced by *Asanoa* spp. and further research in this matter would be desirable.

Three seemingly identical isolates (RLA072, RLA069 and RLA067) are distantly related to type strain *Actinokineospora diospyrosa* (AF114797). Tamura et al (1995) reported, that *Actinokineospora* spp. can be found in soil and decomposing leaves. *Actinokineospora* belong to the rare actinomycetes genera and have motile spores (Hayakawa, 2008). *Actinokineospora spheciospongiae* was recently shown to produce a compound with antibiotic properties, named actinokineosin (Takasaka et al., 2017). Furthermore rhizosphere-associated *Actinokineospora bangkokensis* was shown to produce two compounds with antifungal properties, namely thailandin A and B (Intra et al., 2016). Additional studies concerning *Actinokineospora* species are necessary.

Isolate RLA056 is distantly related to type strain *Mycobacterium neglectum* (AB741467) and was first described by Nouioui et al. (2018). The genus *Mycobacterium* hosts among others prominent pathogenic representatives like

Mycobacterium tuberculosis and *Mycobacterium leprae* (Lagier et al., 2015). According to similarities in the 16S rDNA of *Mycobacterium* spp., several *Mycobacterium* species are not reliably distinguishable by 16S rDNA barcoding. Further comparative measures are necessary, for example determination of 16S-23S internal transcribed spacer (ITS) sequences (Roth et al., 1998).

Isolate RLA087 is ident with the type strains of *Nocardia soli* (AF430051) and *Nocardia cummidelens* (AF430052). Both type strains were described for the first time by Maldonado et al. (2000). Many *Nocardia* spp. cause infections in immunocompetent and immunocompromised humans. *Nocardia* spp. differ little in their 16S rDNA sequences and results produced by 16S rDNA sequencing can be misleading (Conville et al., 2004).

Isolate RLA085 is related to type strain *Micrococcus luteus* (AJ536198). *Micrococcus luteus* is known to produce an antibiotic named neoberninamycin (Biskupiak et al., 1988).

Isolate RLA057 is distantly related to type strain *Leifsonia kafnienesis*. Members of the genus *Leifsonia* are inhabiting plants, soil and watery ecological niches (Barka et al, 2016). *Leifsonia kafnienesis* was described for the first time by Pindi et al. (2009). According to available literature there is nothing known about substances with antibiotic properties produced by *Leifsonia kafnienesis*.

Phylogenetic tree: *Streptomyces* spp.

According to Tiwari and Gupta (2012) actinomycetes are ubiquitous in natural environments. *Streptomyces* spp. are abundantly occurring in soil, compost and decomposing leaves and prefer dry conditions and neutral to alkaline pH in soil (Waksman, 1959; Alexander, 1961; Ceylan et al., 2008). Consistent with these reports, *Streptomyces* is the most frequently appearing genus among the isolates from the rhizosphere of *Leontopodium alpinum* ssp. *nivale* originating from the Rax Mountain.

The genus *Streptomyces* is the most extensively studied producer of antibiotics among microbes (Watve et al., 2001). *Streptomyces* species produce 80 % of the antibiotics originating from Actinobacteria. Further research is expected to yield more promising antimicrobial agents (Barka et al., 2016). Isolates of the genus *Streptomyces* from this study included isolate RLA021, which is closest related to type strain *Streptomyces*

avidinii (AB184395). This species is known to produce streptavidin (Stapley et al., 1963; Chaiet and Wolf, 1964). The isolates RLA156 and RLA075 are related to type strain *Streptomyces subrutilus* (X80825), producer of antibiotics hydroxystreptomycin (Arai et al., 1964), deoxynojirimycin and deoxymannonojirimycin (Hardick et al., 1991).

Isolate RLA012 is distantly related to type strain *Streptomyces mirabilis* (AB184412). *S. mirabilis* produces miramycin (Ruschmann, 1952) and the bioactive compound di- (2-ethylhexyl) phthalate (DEHP, El-Sayed, 2013).

Isolate RLA040 is very similar to type strain *Streptomyces anulatus* (AB184875). *S. anulatus* produces telomestatin (Shin-ya et al., 2001) and was recently shown to also produce compounds with antibacterial and antifungal properties. The ethyl acetate extract from a strain of *Streptomyces anulatus* was observed to be effective against Gram positive and Gram negative bacteria as well as against fungus *Candida albicans*. Furthermore, the extract inhibited multidrug-resistant *Staphylococcus aureus*, among others. Substances produced by *Streptomyces anulatus* are of current interest in drug discovery (El-Naggar et al., 2017).

Isolates RLA036, RLA038 and RLA046 are related to type strain *Streptomyces atratus* (DQ026638). *S. atratus* is known to produce compounds with antibiotic activity. Higashide et al. (1962) report, that rufomycin A and rufomycin B active against *Mycobacterium tuberculosis* are produced by *Streptomyces atratus*. Furthermore, *S. atratus* produces cytotoxic hydrazidomycins (Ueberschaar et al., 2011).

Isolates RLA042a and RLA042c are distantly related to type strain *Streptomyces scabrisporus* (AB030585). RLA042 was morphologically non-homogeneous, and therefore suspected to consist of more than one isolate. However, according to the sequencing results both isolates are identical. *Streptomyces scabrisporus* produces the substance hitachimycin, which has antibiotic properties (Ping et al., 2004). The clade containing *Streptomyces scabrisporus* forms an outgroup, which could be explained by the distant relatedness of *Streptomyces scabrisporus* to other *Streptomyces* species (Ping et al., 2004).

Isolate RLA037 is seemingly identical to *Kitasatospora* sp. (KY788226). *Kitasatospora* sp. has several features common for *Streptomyces* species (Zhang et al., 1997) and was subsequently grouped into the *Streptomyces* phylogenetic tree. Strains of the genus *Kitasatospora* were shown to produce the antibiotic compounds setamycin

(Omura et al., 1982), terpentecin (Tamamura et al., 1985) and hazimycins (Koyama et al., 2015). Additionally *Kitasatospora setae* produces bafilomycins (Nara et al., 2017).

Phylogenetic tree: other Bacteria

The genus *Paenibacillus* is frequently occurring in natural habitats, especially in soil and rhizosphere. Just like closely related *Bacillus* spp., *Paenibacillus* spp. are spore forming organisms. This feature allows them to survive in unfavorable conditions. Some of *paenibacilli* are plant pathogens and endophytes as well. Additionally, *Paenibacillus* spp. are well known for producing bioactive substances, particularly *Paenibacillus polymyxa.* (Rybakova et al., 2015). *Bacillus* spp. and predominantly *Paenibacillus* spp. produce lipopeptide antibiotics, for example polymyxins, octapeptins, polypeptins, iturins, surfactins, fengycins, fusaricidins, tridecaptins and kurstakins (Cochrane and Vederas, 2016).

Isolates RLA006, RLA020 and RLA022 are similar to type strain *Paenibacillus xylanexedens* (EU558281). Furthermore, closely related to *Paenibacillus xylanexedens* are RLA004, RLA007 and RLA023. *P. xylanexedens* was described for the first time by Nelson et al., (2009). *P. xylanexedens* inhabits the rhizosphere, tolerates alkaline pH and forms creamy white colonies (Verma et al., 2016). According to available literature, nothing is known about substances with antibiotic activity produced by *P. xylanexedens*. All isolates from the rhizosphere of *Leontopodium alpinum* ssp. *nivale* which are related to *P. xylanexedens* showed bioactivity in form of visible inhibition zones on original plates. Therefore, further research in this matter appears promising.

Isolate RLA064 is related to type strain *Paenibacillus camelliae* (EU400621) and was described for the first time by Oh et al. (2008). According to available literature, no antibiotics are known to be produced by *Paenibacillus camelliae*.

RLA027 is closely related to type strain *Bacillus siamensis* (GQ281299). *Bacillus siamensis* was described for the first time by Siribaed (1935) and is similar to *Bacillus vallismortis* and *Bacillus subtilis* among others, according to the 16S rRNA gene sequencing results (Sumpavapol et al., 2010). A strain of *Bacillus siamensis* was shown to produce antifungal lipopeptides (Chen et al., 2016).

Isolates RLA047, RLA005, RLA044 and RLA054 are similar to type strain *Bacillus mycoides* (AB681413). *B. mycoides* is associated with the *Bacillus cereus sensu lato* group, however it is not pathogenic. *B. mycoides* forms curly chain patterns on cultivation plates, hence its morphology is rather distinguishing. *B. mycoides* is widespread and abundantly occurring in soil and the rhizosphere. Furthermore, some of the strains are endophytes and produce antimicrobial substances, e. g. bacillomycin D, fengycin, zwittermicin A and volatiles (Yi et al., 2018).

Isolate RLA066 is distantly related to type strain *Bacillus simplex* (AJ628743), and it was impossible to find a more closely related type strain. *B. simplex* was shown to produce a heat stable toxin, which is similar to the emetic toxin cereulide, produced by *B. cereus* (Taylor et al., 2005).

Isolate RLA053 is related to type strain *Xanthomonas campestris* (X95917) and builds an outgroup in the bacteria – tree. The genus *Xanthomonas* comprises 20 species, mostly prominent for causing plant diseases. *Xanthomonas* spp. afflict variable plants and plant parts from root to fruit. Members of this genus appear only in tight contact with plants and not independently (Hayward,1993).

Further genera, identified by BLAST search

Three of the isolates were assigned to the genus *Stenotrophomonas,* two of them are similar *to S. maltophilia* (RLA188, RLA209) and one is similar to *S. rhizophilia* (RLA210). *Stenotrophomonas maltophilia* inhabits the rhizosphere as well as aqueous environments. *S. maltophilia* plays a role by protecting plants from pathogenic fungi, promoting plant growth and furthermore *S. maltophilia* can be used for decontamination of soil (Wolf et al., (2002). *S. rhizophilia* was described for the first time by Wolf et al., who observed antifungal activity against plant-pathogenic fungi (2002).

Isolate RLA232 was assigned to the genus *Pseudomonas*. *Pseudomonas* spp. are Proteobacteria and widespread in soil and water. Additionally they can be found on the surface of or inside plants. *Pseudomonas* species are well known producers of antibiotics (Bérdy, 2005).

One isolate was assigned to the genus *Microbacterium* (RLA073). *Microbacterium* spp. inhabit various natural environments, e. g. the rhizosphere. An endophytic strain is *Microbacterium zeae* (Gao et al., 2017).

Another isolate was assigned to the genus *Variovorax* (RLA024). *Variovorax* is a genus of typical rhizosphere bacteria (Li et al., 2014). Glick (2010) claimed, that *Variovorax paradoxus* is beneficial for root growth in presence of toxic Cadmium ions.

Successful isolation and taxonomical characterization of bacteria led to various meaningful results. The rhizosphere of *Leontopodium alpinum* ssp. *nivale* is inhabited by a plethora of microorganisms which produce bioactive compounds. Many of the isolated bacteria, rhizosphere inhabitants as well as endophytes, are promising potential sources of new antibiotic substances. Testing for bioactivity is an ensuing step, especially isolates which showed bioactivity in contact with other bacteria are promising candidates. Pure cultures can be cultivated in liquid culture and pellets are produced by centrifugation. Extracts gained by solvents, e. g. ethyl acetate, can be directly used for bioassays.

7. Materials and Methods

Rhizosphere soil samples from *Leontopodium nivale* subsp. *alpinum* were collected by Franz Tod (Core Facility Botanischer Garten, Rennweg 14/2, 1030 Vienna) on September 26 2015 at the Rax plateau at 1624 m above sea level for the isolation of bacteria. The precise location has the common name “Praterstern” and is located 569 m NE of the “Otto-Schutzhaus”; coordinates: N47-42-58.34, E15-45-42.63, L624. The following maps (Fig.17, Fig.18) illustrate the location of the source.

Fig.17: Map of Austria: Location of the Rax Mountain SSW of the capital Vienna marked with a red rectangle.

http://www.nationsonline.org/oneworld/map/austria-political-map.htm, accessed 2017-11-10

Fig.18: Close-up of the Austrian Hiking map: depicting the locations of the Otto-Schutzhaus and the Praterstern at the Rax Mountain.

In the described area three plants of *Leontopodium nivale* subsp. *alpinum* were collected, which grew in a distance of approx. 20 m to each other. Three soil samples of 1 g each were collected per plant, nine soil samples in total. All soil samples were collected in sterile 50 ml tubes.

In the lab, 9 ml of sterile deionized water were added to the tubes and sonicated for 3 min (35 kHz, 85 W) in a sonic water bath (Transsonic T460, Elma Schmidbauer GmbH, Singen, Germany) on the same day. 10 ml of 40 % glycerol were added per sample and vortexed. All samples were stored at -80 °C until processing.

Selective media

The isolation of bacteria was accomplished using generally nutrition-poor media. Rare actinomycetes can be easily outcompeted by abundant microbes in nutrient-rich media, but were expected to persist in harsh conditions (Cross, 1982; Goodfellow and Williams, 1986). Six different nutrient media were chosen, which have been described before to be advantageous for the isolation of rare actinomycetes (Nonomura and Ohara, 1969; 1971; Hayakawa and Nonomura 1987; Hayakawa et al. 1996a; Hayakawa 2008).

Tbl.11: Ingredients of selective media per 1 L of nutrient medium used for isolating microorganisms from rhizosphere soil from *Leontopodium nivale* subsp. *alpinum*.

Ingredients (g / l)	AV agar[1]	MC agar[2]	MGA-SE agar[2]	GAC agar[2]	HV agar[3]	LSV-SE agar[4]
Glucose	1,0	2,0	2,0	2,0	-	-
Glycerol	1,0	-	-	-	-	-
Humic acid	-	-	-	-	1,0	-
Lignin	-	-	-	-	-	1,0
Soil extract	-	-	200 ml	-	-	100 ml
L-Arginine	0,3	-	-	-	-	-
L-Asparagine	-	-	1,0	1,0	-	-
Soy bean flour	-	-	-	-	-	0,2
$NaNO_3$	-	0,5	-	-	-	-
$CaCO_3$	-	-	-	-	0,02	0,02
K_2HPO_4	0,3	0,3	0,5	0,3	-	-
NaH_2PO_4	-	-	-	-	0,5	0,5
KCl	-	0,3	-	0,3	1,7	1,7
NaCl	0,3	-	-	-	-	-
$MgSO_4*7H_2O$	0,2	0,3	0,5	0,3	0,5	0,5
$Fe_2(SO_4)_3$	0,01	-	-	-	-	-
$FeSO_4*7H_2O$	-	0,01	0,01	0,01	0,01	0,01
$CuSO_4*5\ H_2O$	0,001	0,001	0,001	0,001	-	-
$ZnSO_4*7H_2O$	0,001	0,001	0,001	0,001	-	-
$MnSO_4*7H_2O$	0,001	0,001	0,001	0,001	-	-
B-vitamins[5]	+	-	-	-	+	+
Agar	15	20	20	20	18	18
pH	6,4	7,4	7,4	7,4	7,2	7,2

[1] Nonomura and Ohara (1969)

[2] Nonomura and Ohara (1971)

[3] Hayakawa and Nonomura (1987)

[4] Hayakawa et al. (1996a)

[5] Composed of 0,5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxin-HCl, inositol, Ca-pantothenate, p-aminobenzoic acid and 0,25 mg of biotin.

Nutrient media were prepared using ingredients listed in Table 1. Components were dissolved in distilled water with a magnetic stir bar and gently warmed. The B-Vitamins mixture and the soil extract were prepared separately beforehand.

B-Vitamins

Powdered B-Vitamins were weighted in and mixed consecutively in a mortar, starting with biotin and thiamine-HCl. Next riboflavin was added to the mixture. Niacin and pyridoxin-HCl were mixed in a second mortar and were added to the mixture. All B-Vitamins were blended thoroughly for the stock and weighted into 1,5 ml Eppendorf tubes at dosages for 1 l of media, taped with parafilm and stored at -20 °C until use.

Soil extract

Soil extract was produced using surface garden soil from the medicinal plants garden (Fig.19; Faculty of Pharmaceutical Sciences, Althanstraße 14, 1090 Vienna).

Fig.19: Surface garden soil for production of soil extract

Common garden soil was filled in a beaker and dried using a heating oven at 120 °C for 90 minutes. Big clumps of soil were grounded with a mortar after cooling down and were granulated with a broad-meshed sieve. 400 g of dried and finely granulated soil were placed into a 2 l Schott Duran bottle and mixed with 960 ml of deionized and distilled H_2O, and autoclaved 121 °C, 100,000 Pa, for 60 min. The soil extract was cooled to room temperature and stored overnight at 4 °C.

The following day, the soil extract was decanted into 50 ml Falcon tubes and centrifuged at 4000 rpm for 10 min. A pleated filter was used for filtration of the soil extract. This soil extract was poured into a Schott bottle and kept at 4 °C, if used within several days. For longer storage times, it was poured into 50 ml tubes and stored at -20 °C.

Antibiotics

Six different isolation media were combined with three different antibiotics:

1. Nutrient media without antibiotics (0)
2. Nutrient media with Nystatin and Cycloheximide (NC)
3. Nutrient media with Nystatin, Cycloheximide and Novobiocin (NCN)

Stock solutions of antibiotics were prepared, sterile-filtered (except DMSO-based Nystatin-solution) and poured into 1,5 ml Eppendorf tubes for storage at -20 °C.

Nystatin is a pore-forming polyene antifungal active against a wide range of fungi species (Dos Santos et al., 2017). Therefore it was used to suppress fungi growth. Nystatin was dissolved in Dimethyl sulfoxide (DMSO) and a stock solution contained 10 mg/ml Nystatin (Sigma Aldrich®, St. Louis, Missouri, USA; N3503-5MU ≥4,400 USP units/mg). Thus 2 ml stock solution was used to achieve a final concentration of 20 µg/ml of Nystatin in isolation media.

Cycloheximide suppresses protein biosynthesis by inhibiting translation in eukaryotes, especially in yeast and filamentous fungi (Schneider-Poetsch et al., 2010). Cycloheximide was dissolved in Ethanol and a stock solution contained 40 mg/ml Cycloheximide (Sigma Aldrich®, St. Louis, Missouri, USA; 01810-1G). Thus 0,5 ml stock solution was used to achieve a final concentration of 20 µg/ml of Cycloheximide in isolation media.

Novobiocin is an aminocoumarin antibiotic that blocks bacterial synthesis of nucleic acids by inhibiting gyrase (Collin F. et al., 2011). It is frequently used for selecting rare actinomycetes, such as *Micromonospora* strains for instance (Qiu et al., 2008). Novobiocin was dissolved in deionized water and a stock solution contained 50 mg/ml Novobiocin (Sigma Aldrich®, St. Louis, Missouri, USA; Novobiocin sodium salt, N1628-1G). Thus 2 ml stock solution was used to achieve a final concentration of 100 µg/ml of Novobiocin in isolation medium.

Frozen antibiotic solutions were thawed slowly if required. Antibiotics were added near the solidification point of the agar. Afterwards, media were poured into Petri dishes with vents and a diameter of 10 cm. Agar plates were stored at 4 °C in the dark until use.

Pre-treatments, dilutions and inoculation of isolation plates

Four pre-treatment methods were used for this experiment: No pre-treatment as a control, dry heat, phenol treatment and exposure to microwaves.

Petri dishes were left open in the sterile bench for approx. 20 min before inoculation to evaporate condensed water. Soil samples were thawed slowly at 4 °C and centrifuged at 4000 rpm for 10 min. Supernatant was decanted under sterile conditions.

Plates were inoculated after pre-treating and diluting the soil samples. Per plate, 0,1 ml of each soil dilution were pipetted and spread thoroughly using a sterile glass Drigalski spatula. The workflow of the soil sample preparation is shown on Fig.20.

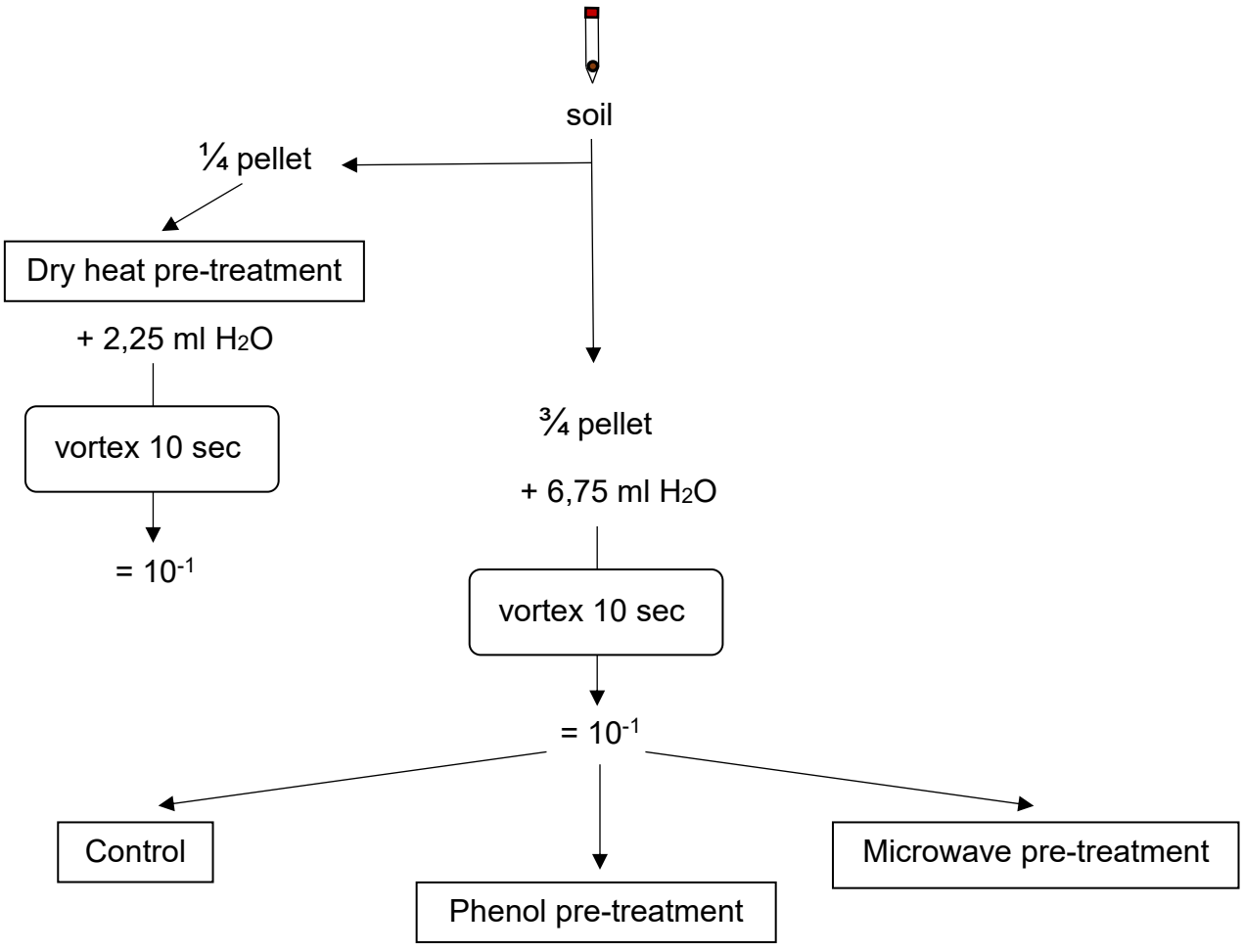


Fig.20: Allocation and preparation of a soil sample for the four different pre-treatments

Dry heat pre-treatment

A quarter of the soil pellet was placed in a sterile glass culture dish and heated using a heating oven at 100 °C for 60 min. 2,25 ml distilled and sterile H_2O were added after cooling down to room temperature and vortexed for 10 sec. 1 ml of 10^{-1} dilution was added to 9 ml sterile H_2O, vortexed shortly and the resulting 10^{-2} dilution was applied to agar plates. Further dilutions of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were made and spread on agar plates.

Control

The 10^{-1} dilution was used without further pre-treatment for serial dilutions and inoculating agar plates.

Phenol pre-treatment

0,5 ml of 10^{-1} dilution were mixed with 4,5 ml of sodium phosphate buffer, which contained 1,5 % phenol, and vortexed for 10 sec. Thirty minutes after treatment, the resulting 10^{-2} dilution was spread on agar plates. Further dilutions were made and also plated. Inoculated agar dishes were wrapped into aluminium foil and kept in a fume hood at room temperature for three days to ensure that phenol fumes were able to evaporate safely. Afterwards, plates were moved to an incubator set at 28°C.

Microwave exposure

2 ml of 10^{-1} dilution were exposed to microwaves for 30 sec. (Panasonic combination device, type number NN-D801/NN-D851; 2460 MHz frequency, 100 W power), diluted and spread onto agar plates like explained above.

Cultivation

Isolation plates were incubated at 28 °C and visually inspected, at least once a week. Colonies with morphologies similar to those described for actinomycete bacteria (Labeda, 1987b; Barka et al., 2016; Li et al., 2016) or colonies with mycelial appearance (Srinivasan et al., 1991) were chosen primarily. In addition, colonies which

produced visible zones of inhibition in contact with other bacteria were chosen. Quickly growing colonies or mixed colonies were removed and excluded from picking.

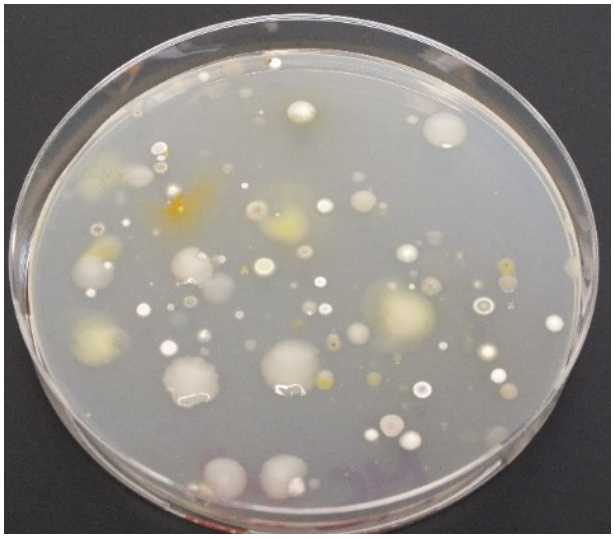


Fig.21: Isolation plate with morphologically diverse colonies

Picking of colonies

Selected colonies were picked with a sterile tooth pick, inoculated onto a new agar plate of the same nutrient and antibiotic scheme and incubated at 28 °C. If bacteria didn't grow or were growing slowly, the same colony was picked a second time and cultivated on antibiotic-free International *Streptomyces* Project Medium 2 (ISP2 medium, Shirling and Gottlieb, 1966). Resulting cultures were used for production of liquid cultures and spore isolation plates.

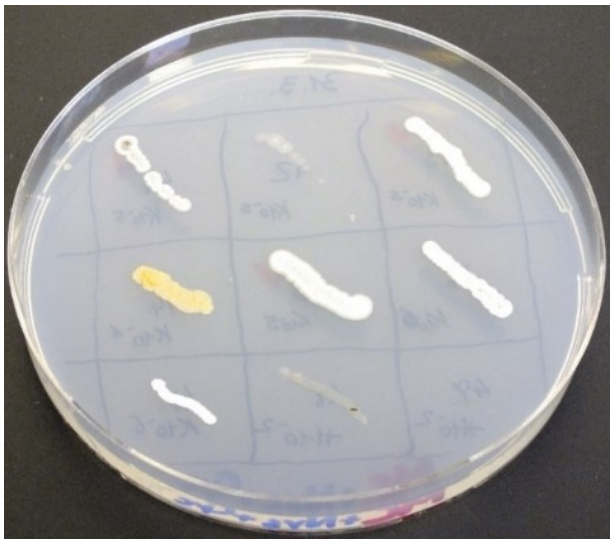


Fig.22: Cultivation plate with picked colonies

Preparation of liquid cultures

Liquid bacterial cultures were prepared using sterile tubes with 3 ml of tryptic soy broth (TSB). Cultures were transferred with a sterile loop for inoculation. If bacterial growth in the TSB medium failed, 2x Yeast Extract/Tryptone (2xYT) liquid medium was used instead. Liquid cultures were incubated at 28 °C at 200 rpm until sufficient growth (judged by culture turbidity) was observed.

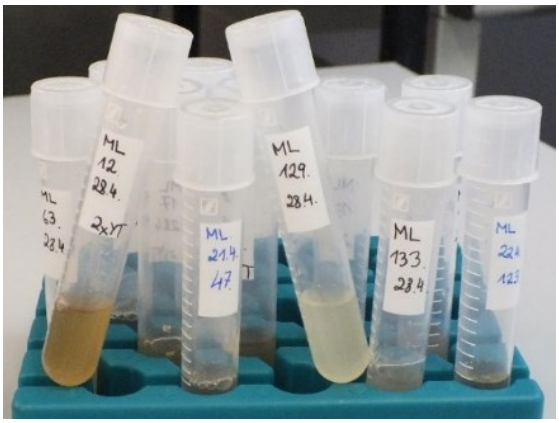


Fig.23: 2xYT or TSB liquid nutrient medium were used for liquid cultures

1 ml of liquid culture was poured into a sterile Eppendorf tube, centrifuged at 13.000 rpm for 4 min and supernatant was removed. Pellets for DNA-extraction were kept at -20 °C until processing. Pellets for the back-up collection were resuspended in 1 ml of 20 % glycerol, transferred to cryotubes and stored at -80 °C.

Preparation of sporulating plates

Cultures of isolates that were able to produce spores on isolation plates were streaked out onto new agar dishes of the same nutrient and antibiotic scheme in a high density. After incubation at 28 °C, sporulating plates were used for harvesting spore suspensions. Time of incubation varied from three to eight weeks, depending on how fast bacteria grew. Bacterial growth failed in some cases, then a second type of spore isolation plate was produced using ISP2 medium. However, some strains didn’t grow on such plates at all.

Fig.24: Densely grown spore isolation plate

Densely grown cultures displayed spore formation on the surface of agar plates (Fig.24) after some weeks of inoculation. Plates were inundated with 5,5 ml of 20 % Glycerol. Spores and mycelium were detached from the aerial mycelium with a pipette tip. Spore suspensions were resuspended repeatedly and filtered using sterile 5 ml syringes stowed with sterile cotton plug. Filtrated spore suspensions were transferred into cryotubes in amounts of 0,5 ml to 1,0 ml per cryotube and stored at -80 °C.

Non-sporulating isolates were scraped off from the media with an inoculation loop and resuspended in 0,5 ml of 20 % glycerol and also stored at -80 °C.

DNA extraction

DNA extraction was performed with Wizard® SV Genomic DNA Purification System (Promega Corporation, Madison, Wisconsin, USA). The Quick protocol provided by manufacturers was used and adapted for the requirements of this project: Microbial pellets obtained from liquid cultures (see preparation of liquid cultures) were thawed and resuspended in 0,8 ml of sterile deionized water. Half of the mixture was transferred to a new 1,5 ml tube, both tubes were centrifuged and supernatant was removed. One pellet was used for DNA extraction, the second one was frozen and kept as backup sample in case of process failure.

Next, microbial pellets were re-suspended in Digestion Solution Master Mix for DNA extraction, which was prepared in accordance with user's manual. Lysis was performed in a heat block for one hour at 55 °C. After incubation, Wizard® SV Lysis Buffer was added and the mixture was vortexed.

Genomic DNA from lysate was purified with Wizard® SV Minicolumn Assembly according to manual instructions. Genomic DNA (gDNA) was eluted with nuclease-free water using a volume of 0,2ml

Purified gDNA was kept frozen at -20 °C.

Gel electrophoresis I

Successful gDNA extraction was checked by Gel electrophoresis (Fig.25).

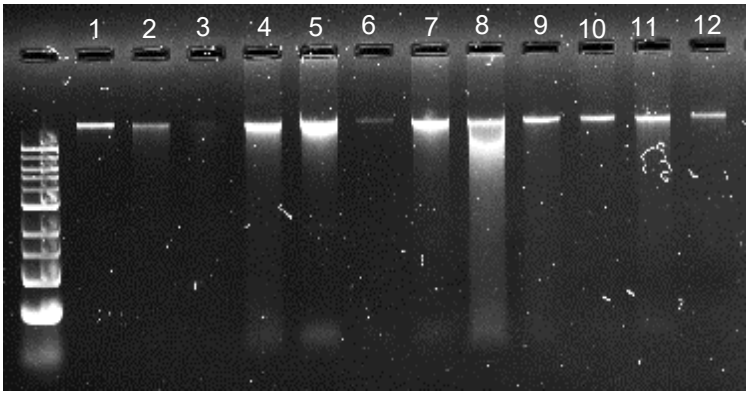


Fig.25: Digital image of an agarose gel for checking genomic DNA extraction. Bands show low but sufficient amounts of gDNA considering sample 3 and 6, as well as considerable gDNA amounts of remaining samples.

Gel electrophoresis was performed using 0,8 % agarose gel with GelGreen intercalating nucleic acid stain (50 µl/l), Tris/Borate/EDTA (TBE) buffer and 110 V electric current. Applied DNA size marker was 2 µl of 1 kbp ladder mixed with 8 µl of sterile deionized water. 2 µl of each gDNA sample were mixed with 1 µl blue loading dye and 7 µl of sterile deionized water before adding to gel well.

Small DNA fragments are migrating faster during electrophoresis than big DNA fragments (Graw, 2015). Genomic DNA samples bands in Fig.25 show relatively large gDNA fragments.

PCR

Polymerase chain reaction (PCR) for amplification of 16S-rRNA gene fragments was done using Taq PCR Kit (New England BioLabs®, Canada). 1 µl of purified gDNA was added to 39 µl of PCR master mix, which was prepared following instructions of the manufacturer's manual.

The master mix contained 4 µl of Taq buffer, 0,5 µl of deoxy nucleotide triphosphates (dNTPs), 1 µl of FW primer 10 mM 27F, 1 µl of RV primer 10 mM 1492, 0,5 µl of Taq DNA polymerase and 32 µl of sterile deionised water. The mixture was transferred to micro-tubes. Amplification was performed with Mastercycler® nexus X2 (Eppendorf AG, Hamburg, Germany).

PCR conditions (Fig.26) were defined as: (1) initial denaturation at 95 °C for 2 min, followed by (2) 30 cycles of denaturation at 95 °C for 30 sec, (3) annealing at 62 °C for 30 sec and (4) extension at 72 °C for 3min. Final Extension took place at 72 °C for 5min (5). After the completion of the cycler program a temperature of 4 °C was maintained (6) until micro-tubes were removed.

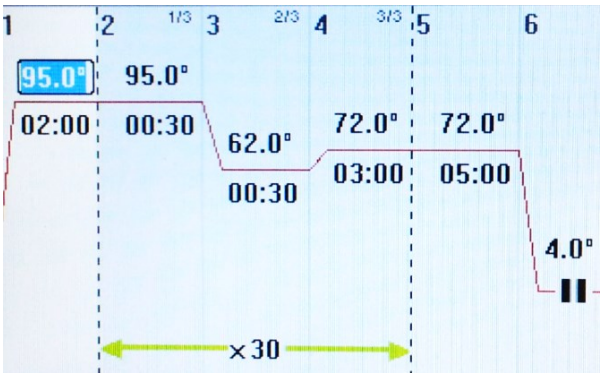


Fig.26: Digital image of chosen PCR conditions for amplification of DNA.

Gel electrophoresis II

Successful PCR was checked by Gel electrophoresis (fig.27). 1 µl of the PCR reaction, 1 µl loading dye and 8 µl of sterile deionised water were mixed. The slot in the first row of the gel was loaded with a mixture of 2 µl 1 kbp ladder and 8 µl sterile deionised water.

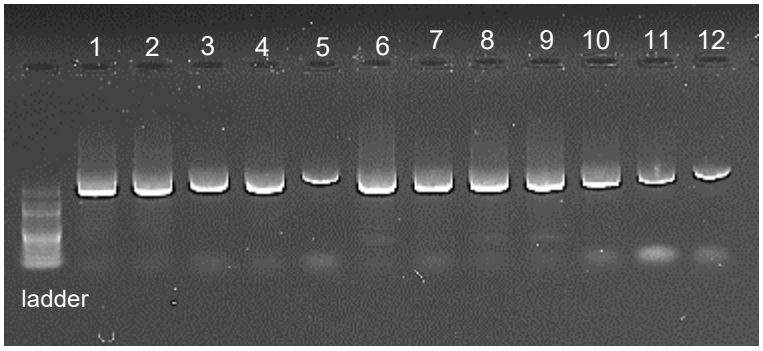


Fig.27: Digital image of an agarose gel for checking PCR amplification of DNA. Bands show sufficient amounts of DNA.

All twelve samples were amplified successfully and show one sufficient band of DNA fragments of expected size, ca. 1,4 kb.

Nanodrop measurement

With the Thermo Scientific™ NanoDrop Lite Spectrophotometer the concentration of PCR products was measured. Nanodrop results were used to calculate the necessary sample volume for a concentration of at least 250 ng/µl DNA needed for subsequent DNA sequencing.

Sequencing of PCR products

Appropriate amounts of PCR products were mixed with forward or reverse primers and transferred to coded tubes of the Mix2SeqKit (Eurofins Genomics AT GmbH, Austria) in accordance with requirements of the provider. Samples were submitted to Eurofins overnight sequencing facility, which uses Sanger sequencing method.

Raw sequences data were manually edited using the software application Geneious. In addition, Molecular Evolutionary Genetics Analysis program (MEGA) 7 was used. Edited sequences were aligned and consensus sequences were blasted against gene bank database between June and October 2016. The most likely match with the highest score was used to identify the isolate and the respective strain's sequence was downloaded for downstream phylogenetic analyses. Furthermore sequences were fed to the Ribosomal Database Project's Classifier (RDP, Wang et al., 2007). Results from RDP Classifier were compared to those from Basic Local Alignment Search Tool (BLAST).

Compiling of phylogenetic trees

Homologous sequences to 16S rDNA sequences of the isolates from the rhizosphere of *Leontopodium nivale* subsp. *alpinum* were detected by BLAST search. Ascertaining of Type strains was conducted with straininfo.net and GenBank. If no appropriate Type strain was available, sequences of the closest matches from BLAST search were downloaded. Sequences were aligned by algorithm ClustalW in MEGA 7 and the resulting alignmentblock was edited manually. Best DNA model was detected by model test. Phylogenetic trees were constructed with neighbour-joining method, combined with maximum likelihood method. Reliability of phylogenetic trees was estimated by defining bootstrap values.

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