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Isolation and Characterization of Strains from a New Group of Acidophilic Actinobacteria

Bachelor's Thesis

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Sustainable Use of Natural Resources

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Declaration

I hereby declare that I have authored this bachelor's thesis carrying the name „Isolation and Characterization of Strains from a New Group of Acidophilic Actinobacteria" independently under the guidance of my supervisor. Furthermore, I confirm that I have used only professional literature and other information sources that have been indicated in the thesis and listed in the bibliography at the end of the thesis. As the author of the bachelor's thesis, I further state that I have not infringed the copyrights of third parties in connection with its creation.

In Prague on 22nd April 2022 _____

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Isolation and Characterization of Strains from a New Group of Acidophilic Actinobacteria

Summary:

Previously done research on acidophilic bacteria from waterlogged acidic forest soils in Třeboň region found a new deeply branched cluster of *Actinobacteria*. The Trebon Clade has had one species described yet, *Trebonia kvetii,* within a family Treboniaceae. Due to this discovery, another research needed was to support this observation by isolation of relatives of this new family.

Actinobacteria are an interesting group of bacteria for their importance in the cycles of elements, decomposition of organic matter and xenobiotics, and the ability to create secondary metabolites. They are so far the main source of currently used antibiotics. They can live under extreme conditions, which may be limiting for other organisms.

Two sampling points were chosen for isolation of noval strains from soil. Locations near the towns of Zakopaná and Třeboň had similar soil properties, thus the samples of soil for isolation were collected to obtain strains with wider adaptations and physiology. Locations are characteristic mostly by their low soil pH. At both sites, the presence of Treboniaceae family was proved by 16S rRNA gene sequencing of the whole soil community. In this study, after plating, transferring, DNA extraction, and PCR preparation, 17 strains of Actinobacteria belonging to genera Actinospica, Nocardia....were found to be good enough for the sequencing and further creation of phylogenetic trees to get an idea of their genetic origin.

Keywords:

Actinobacteria, acidic soil, isolation of bacteria

Content

1 Introduction

Bacteria are all around us and they are significant for the ecosystem and for humankind. They are playing important roles in global ecosystems. The cycles of nutrients such as nitrogen, carbon, and sulfur are completed by them. They are able to decay and release carbon back into the ecosystem or bind atmospheric nitrogen. Then beneficial bacteria which help us digest. Not all of them have always positive influence. Pathogenic bacteria are also playing their role in the ecosystem and cause problems also to humans, crops, and animals.

Actinobacteria is a phylum of the *Bacteria* domain. They are known for their ability to live under conditions that could be limiting for other organisms. Due to these conditions, they have become the target of researchers investigating their properties. After years of studies, they have been a great source of natural antibiotics, and around 70 % of the world's used antibiotics are obtained from *Actinobacteria*. They are gram-positive bacteria common as representatives of soil microbial life. They play a significant role in the decomposition of organic compounds. Some Actinobacteria can cause health diseases, but some as mentioned before are producers of antibiotics.

A novel actinobacterial strain was isolated from the acidic waterlogged forest soils nearby the town of Třeboň in the Southern Bohemia in the Czech Republic. After sequencing based on 16sRNA new clade, Trebonia Clade was proposed based on numerous strains occurring in the same cluster, with one representative described as *Trebonia kvetii*. Therefore, the intention to study the site and to find possible new strains has risen. In total of 17 strains were good enough to present and classify.

2 Aims of the thesis

Actinobacteria are known for their specific properties which make them unique. We can find them living under extreme conditions. These conditions make them so interesting for further studies mostly for their production of secondary metabolites. Since we are facing the ineffectiveness of antibiotics in pharmacological use due to the growing resistance of common bacteria causing health problems to humans, seeking new possible antibiotics is one great way for fighting such resistance. Already Actinobacteria were described for their production of secondary metabolites which are in use (around 80 % of antibiotics are from Actinobacteria) or are undergoing trials.

Pharmacological use is not the only field of interest. In this study, the observed Actinobacteria are from acidic waterlogged soils of deciduous forest and from acidic soils of coniferous to mixed forest. Actinobacteria are also needed to be understood for their ecological function in their place of origin where they live under specific conditions. They may influence the decay and soils cycle processes and based on observing new strains and their DNA, the possible influence can be observed for the production of specific metabolites. The genomic sequences of new strains can be compared to already described strains so the possible metabolite production can be estimated.

Described novel species by Rapoport *et al.* (Rapoport et al. 2020), *Trebonia kvetii* 15TR583^{T}, was found in one of the studied locations in this work. It was one strain that currently belongs to the novel Trebon Clade (TC), proposed by Kopecký *et al.* (Kopecky et al. 2011), which dominated a clone library of waterlogged acidic soil. Due to these previous discoveries, further studies of such localities are needed to support novel knowledge and to understand the decomposing of the litter which can seasonally differ. Actinobacteria are also not cosmopolitan distributed (Wawrik et al. 2007) but can be distributed not only over large distances (Wawrik et al. 2007) but also just within a single sampling location (Bredholdt et al. 2007). Therefore, the presence of Actinobacteria is hard to determine since they can be unevenly distributed within one location. Because of this, it is important to continue searching for new species which can appear to have the ability to create desired secondary metabolites.

Based on the sequencing of 16s rRNA of plated bacteria that were isolated from the two sampling sites new strains could be found and may create separated clades or have interesting physiological activities.

3 Actinobacteria

Actinobacteria are a group of Gram-positive bacteria that can be terrestrial or aquatic. Their DNA has a high content of guanine and cytosine. They are unicellular-like bacteria and produce a nonseptate and slenderer mycelium. This bacterial group includes some of the most common soil, freshwater, and marine types, which play an important role in the decomposition of organic matter. Thereby they are playing an important vital part in organic matter turnover, carbon cycle, refilling of the supply of nutrients in the soil, and formation of humus (Ranjani et al., 2016).

The mycelia which they form are an aerobic mycelium known as substrate and aerial. They reproduce by producing spores or conidia, by binary fission, they sporulate through fragmentation, and segmentation or conidia formation. The appearance of Actinobacteria is compact, often matte with a dry surface on culture media, and usually forms aerial mycelium. Often embedded in agar and hard to extract from the surface except for spore collecting from aerial mycelium.

A variety of secondary metabolites with high pharmacological and commercial interest are produced by actinobacteria. For its ability to decay biological material they may play important role in the future possible production of biofuels. If in such a process were wisely used specific species, the possible production would be of high efficiency (Lewin et al. 2016).

The phylum *Actinobacteria* has six classes: *Acidimicrobiia, Actinomycetia, Coriobacteriia, Nitriliruptoria, Rubrobacteria,* and *Thermoleophilia.*

Class *Acidimicrobiia* is a deeply rooted lineage and is comprised of few cultivable representatives, which were isolated mostly from extremely acidic environments (Zhi et al. 2009, Gao et al. 2006, Ludwig and Euzéby 2012). Four extremely acidophilic species namely, *Acidimicrobium ferroxidans, Acidithrix ferrooxidans, Ferrimicrobium acidiphilum,* and *Ferrithriy thermotolerans* have optimal pH at around 2.0 for their growth. They are also able to oxidize ferrous iron (Cleaver et al. 2007, Ludwig and Euzéby 2012). These species were primarily isolated from geothermal sites or acidic mine drainage. They were responsible for regenerating ferric iron in the acidic ecosystem (Clark & Norris 1996, Johnson et al. 2009, Jones & Barrie Johnson 2015). But in contrast, other class members are not necessarily acidophiles and inhabit more diverse aquatic environments. For example, filamentous *Candidatus* Microthrix parvicella (also called *M. Parvicella*) from wastewater sludge, neutrophilic *Iamia majanohamensis* isolated from the abdominal epidermis of sea cucumber, and members of the genus *Ilumatobacter* from estuary sediment of seashore sand (Kurahashi et al. 2009, McIlroy et al. 2013).

Actinomycetia class comprises 34 orders with 60 families, while the other 5 classes contain only 20 families (Salam et al. 2020). They are widely distributed in natural habitats and participate in different biological and metabolic processes, for example, the production of extracellular enzymes (Ghorbani-Nasrabadi et al. 2013). Actinomycetes can produce pigments that can be red, green, yellow, brown, or black in color (Narain et al. 2014). Members of the genus *Streptomyces* are leading producers of antibiotics, followed by *Nocardia* and *Micromonospora*. Based on many studies most of the novel antibiotic-producing actinomycetes were isolated from soil (Baniya et al. 2018). Beneficial influence can be also observed (Chouvenc et al. 2018) in termite colonies where *Streptomyces* can opportunistically colonize

the fecal material and may result in defensive mutualism with termites of genus *Coptotermes*. Hosted *Streptomyces* can provide a level of protection to the termite population against soil fungal pathogens. It is done by producing an array of antifungal compounds which are distributed throughout the fecal nest, which is created by the termites out of fecal material (Chouvenc et al. 2013, Mevers et al. 2017).

Coriobacteriia are spore-forming, non-motile bacteria with the ability of anaerobic growth (Haas & König 1988), Wurdemann et al. 2009, Saunders et al. 2009, Clum et al. 2009). *Coriobacteriia* is one of the deepest branching lineages of the phylum *Actinobacteria*, branching closely with phylum *Firmicutes* (Stackebrandt & Schumann 2006, Yarza et al. 2008, Ludwig Wolfgang and Euzéby 2012). Nowadays the class *Coriobacteriia* contains 29 species grouped into 13 genera of a single order called *Coriobacteriales*, and of a single family, *Coriobacteriaceae* (Collins & Wallbanks 1992, Haas & König 1988, Stackebrandt & Schumann 2006, Euzéby 1997). Some genera, such as *Atopobium,* Olsenella, and *Cryptobacterium* have species responsible for infection of periodontal/endodontic kind, whilst species of genus *Eggerthella* have been found in severe blood bacteriaemia cases (Copeland et al. 2009; Mavrommatis et al. 2009; Göker et al. 2010).

Class *Nitriliruptoria* has five members, which are halophilic or halotolerant *Actinobacteria*. These microorganisms survive in various high-salt environments, like saline lakes, saline soil, and marine (Mohamad & Li 2018; Jiang & Huang 2018). Antibiotics, other bioactive compounds or enzymes produced by these microorganisms can be used in industrial and medical applications (Mohamad & Li 2018). Studies on mechanism of osmoregulation suggested that two strategies are adopted as a reaction to saline shock (Roeßler M & Müller V 2001, Roberts MF 2004, Vaidya S *et al.* 2018). The first is the salt-in strategy, the so-called accumulation of inorganic ions, mostly K^+ and Cl^-.

Thermoleophilia and *Rubrobacteria* classes belonged under one *Rubrobacteridae* subclass within the phylum *Actinobacteria* (Stackebrandt et al. 1997) and have been assigned into two newly established classes (Ludwig Wolfgang & Euzéby 2012).

The class of *Rubrobacteria* includes the established *Rubrobacterales* order with the family *Rubrobacteraceae* (Stackebrandt et al. 1997) and its genus *Rubrobacter* (Suzuki et al. 1988). *Thermoleophilia* class consists of two orders *Thermoleophilales* and *Solirubrobacterales* (Reddy & Garcia-Pichel 2009). Order *Thermoleophilales* has one single family *Thermoleophilaceae* (Stackebrandt 2004; Zhi et al. 2009). The order *Solirubrobacterales* has three mesophilic, mostly soil-derived bacteria families *Solirubrobacteraceae, Conexibacteraceae,* and *Patulibacteraceae* (Stackebrandt 2004; Zhi *et al.*, 2009). *Solirubrobacteraceae* includes the genus *Solirubrobacter* (Singleton et al. 2003), which includes five species so far, *Solirubrobacter pauli* (Singleton *et al.*, 2003), *S. soli* (Kim et al. 2007), *S. ginsenosidimutans* (An et al. 2011), *S. phytolaccae* (Wei et al. 2014), and *S. taibaiensis* (Zhang et al. 2014). The second family *Conexibacteraceae* contains the genus *Conexibacter* (Monciardini et al. 2003), which consists of two species *Conexibacter woesei* (Monciardini *et al.*, 2003), and *C. arvalis* (Seki et al. 2012). The last family *Patulibacteraceae* has one genus *Patulibacter* (Takahashi et al. 2006), which includes four species *Patulibacter minatonensis* (Takahashi *et al.*, 2006), *Patulibacter americanus* (Reddy & Garcia-Pichel 2009), *Patulibacter ginsengiterrae* (Kim et al. 2012), and *Patulibacter medicamentivorans* (Almeida et al. 2013).

3.1 Habitat of Actinobacteria

3.1.1 Terrestrial

Streptomycetes are considered to be the most isolated samples. Isolated *Actinobacteria* from anoxic mangrove rhizosphere produced novel antibiotics with antibacterial activity. Genera such as *Streptomyces*, *Nocardioform*, and *Micromonospora* were abundant in this specific area of tidal influence (Oskay et al. 2004). *Nocardia*, also isolated from the mangrove soil, produces cytotoxic metabolites and is shown to be an inhibitor of human cell lines, such as gastric adenocarcinoma (Kathrin Schneider et al. 2006). Another extreme terrestrial environment is desert soil. Several reports show that Actinobacteria are distributed in various locations. Sandy soil (Cario, Egypt; Falmouth, MA), sandy loam soil (Keffi Metropolis, Nigeria; Presque Isle, PA), black alkaline soil (Karnataka, India), alkaline dessert soil (Wadi El Natrun, Egypt; Wadi Araba, Egypt), and subtropical desert soil (Thar, Rajasthan). The mostly isolated were *Streptomyces sp*., followed by *Nocardia*, *Nocardiopsis* (Cundell & Piechoski 2016). *Actinobacteria* play an important part in the rhizosphere microbial community for the turnover of hardly decomposable plant organic matter. Thus, the rhizosphere region is considered as one of the best habitats for isolating such microorganisms of this group.

3.1.2 Freshwater

Based on a study (Priyadharsini & Dhanasekaran 2015) *Actinobacteria* can be easily isolated from freshwater sites. The major genera include *Actinoplanes, Rhodococcus, Micromonospora, Streptomyces,* and the endospore-forming *Thermoactinomyces*. *Actinoplanes* can be found in soils, lakes, and rivers. They have the ability to survive long-term desiccation, but once they are rehydrated, the motile spores are released (Cross T 1981). In numerous freshwater habitats a large fraction of total bacteria (30-70 %) is represented by *Actinobacteria* (Glöckner et al. 2000; Sekar et al. 2003; Warnecke et al. 2005; Allgaier & Grossart 2006) and a growing number of records suggests that these bacteria are important players in energy and nutrient cycling in freshwater and estuarine waters (Sharma et al. 2008). The composition of aquatic *Actinobacteria* is affected by pH value (Stepanauskas et al. 2003; Newton et al. 2007), phytoplankton-derived dissolved organic matter (Stepanauskas *et al.* 2003), UV-irradiation (Warnecke et al. 2005) and other factors (Allgaier et al. 2007).

3.1.3 Marine

Ocean and sea environments provide novel species of *Actinobacteria* and their possibly useful metabolites. The marine environment offers various extreme conditions for life. Deep waters with high pressure and anaerobic cold environment around 0 – 8°C at the seafloor on one side, and highly acidic conditions with temperatures 8 – 100°C nearby hydrothermal vents on the second. The first of characterized isolates was *Rhodococcus marinonascene*, *Actinomycete* species. As indigenous marine *Actinobacteria* genera are considered *Rhodococcus, Streptomyces, Dietzia, Salinispora, Salinibacterium, Marinophilus, Williamsia maris, Verrucosispora,* and *Aeromicrobium marinum* (Stach et al. 2004; Magarvey et al. 2004; Bull et al. 2005; Jensen et al. 2005).

3.2 Mycelium

3.2.1 Aerial Mycelium

Aerial mycelium is formed of hyphae developed from the substrate mycelium which grows out of agar (medium) into the air. In some cases, the aerial hyphae and substrate mycelia are hard to distinguish. It is also one of the most important criteria for Streptomyces genus classification into species, including structure (powdery, velvety, or cottony), formation of concentric zones or rings, and pigmentation (Li et al. 2016).

3.2.2 Substrate Mycelium

Substrate mycelium is known as vegetative or primary. Mycelium grows into or right on the surface of the medium. This mycelium has a wide variety in size, shape, and mass thickness. The color of mycelium ranges from white to light yellow, red, brown, orange, green, grey, or black (Li et al. 2016). Some hyphae can also produce water or fat-soluble pigment. The water-soluble pigment can change the color of the culture medium. The fat-soluble pigment affects the colony color. It has the function of absorbing nutrients from the substrate for its growth. With a close-up view with a microscope, the mycelium is more slender, transparent, and more branched than the upper aerial one. The hyphae do not usually create diaphragms and fractures, but they can develop some branches. A few groups (such as *Nocardia*) are branched like the roots, hyphae in substrate often fragment into coccoid to rod-shaped, nonmotile elements once they grow into a certain stage. The hyphae can form sclerotium in some genera.

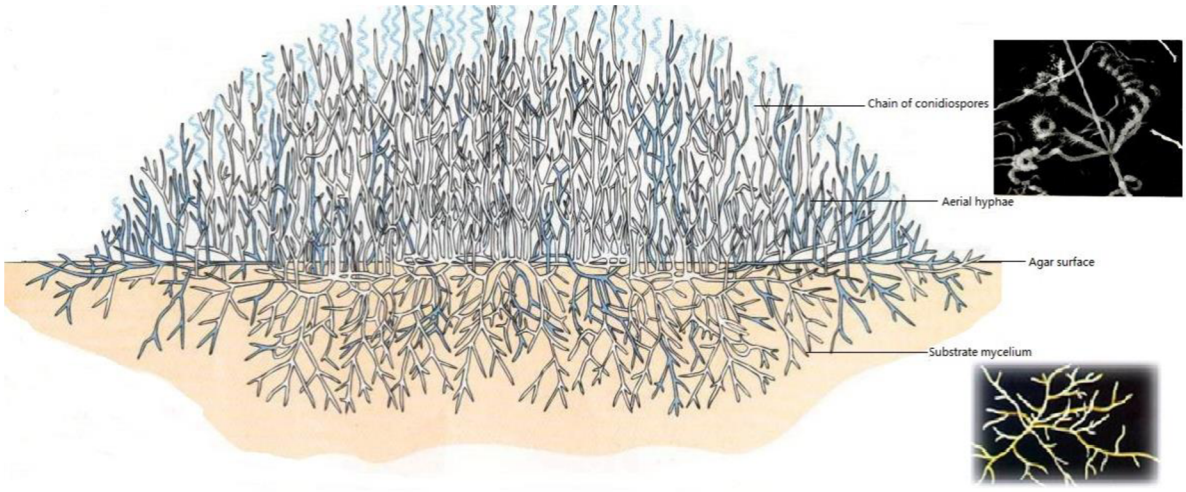

Figure 1 - example of the common morphology of actinomycetes with the crossection of a colony showing the substrate mycelium and aerial mycelium with the chains of conidiospores (Li et al. 2016)

3.3 Spores

Hyphae division and production of spores start with the formation of a cross-wall. The sporulation may occur by three different processes: (I.) When substrate hyphae are fragmented, the septum known as a split septum may occur and create a spore, e.g. in the genus *Micromonospora*. (II.) Pre-existing hyphal elements with a thin fibrous sheath form spores by its septation and disarticulation. The spore wall is at least partly formed from the wall layers of

the parent hypha. This is so-called *holothallic development* (Locci & Sharples 1984) and is known in many other actinomycetes, e.g. the genus *Streptomyces*. (III.) Some strains of *Thermoactinomyces* have globose spores which are formed in both aerial and substrate mycelium and produce spore walls. Such spores are classical bacterial endospores with all the properties, relative to the process of formation, ultrastructure, and its physiology. Spore formation is the most important morphological recognition criterion of actinomycetes. Widely, the formation of spores is limited to the morphological group of sporoactinomycetes, where well-defined parts of mycelium undergo sporulation. Several different genes are involved in the formation of spores and different cultivation conditions can have an influence on their formation (Chater & Chandra 2006). The characterization of spores played a key role in describing species for many years. The spores which are produced individually or in shorter chains are in most cases thicker than the hyphae. While those developed in long chains are usually of the same diameter as the hyphae. Spores are about 1 – 2 μm thick and differ in terms of shape and surface characteristics. Common morphology shapes are globose, coliform, ovoid, rod-shaped, reniform, and allantoid. The motile spores have flagella which provide active movement. Non-motile spores can be smooth or might present special ornamentation on their surface. Surface ornamentation has also been recognized as a taxonomic character. Some genera are well studied for different types of ultrastructures. They are grouped into several forms: rugose, warty, knobby, smooth, verrucose, spiny, or irregular.

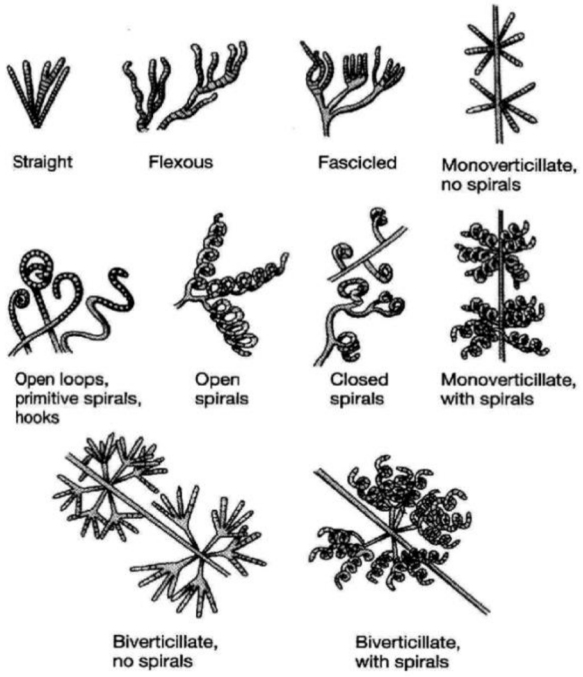

Figure 2 - spore baring body types (Barka et al. 2016)

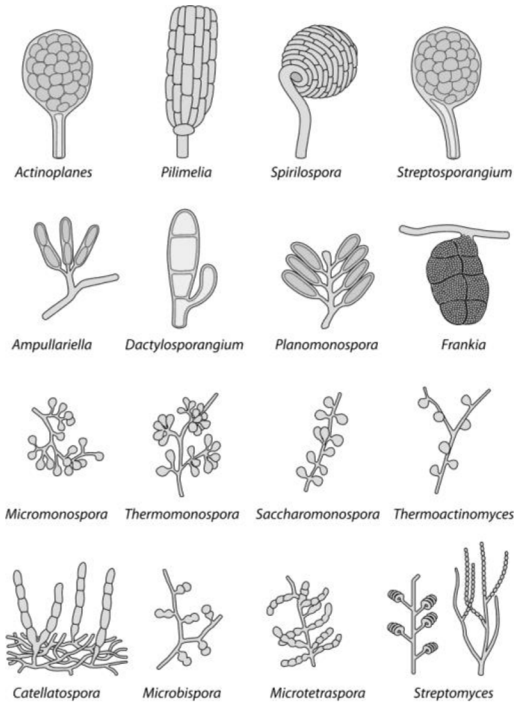

Figure 3 - example of actinobacterial species and their spore baring bodies (Barka et al. 2016)

3.4 Types of Actinobacteria

3.4.1 Thermophilic *Actinobacteria*

Based on several studies, the researchers confirm the existence of extremophilic and extreme tolerant soil *Actinobacteria* (acid and alkali tolerant, thermotolerant, and

psychrotolerant, haloalkaliltolerant and halotolerant or xerophilic). Mesophilic *Actinobacteria* prosper at an optional temperature between 20°C and 42°C, among which the thermotolerant species exist and can survive even at 50°C. Moderately thermophilic ones have their optimum growth at 45°C-55°C (Jensen et al. 2005). Strictly thermophilic Actinobacteria grow in the range between 37°C-65°C with the optimum between 55°C-60°C (Jiang & Xu 1993). The set incubation temperatures are 28°C, 37°C, and 45°C for representatives of mesophilic, thermotolerant, and moderately thermophilic *Actinobacteria*.

3.4.2 Halophilic *Actinobacteria*

Halophilic *Actinobacteria* are, based on their growth in media of different concentrations of salt, categorized into different types. Halophiles prosper and grow in media containing 2.5-5.2 M NaCl, extreme halophiles do best in media with 1.5-4.0 M NaCl, moderate halophiles in media containing 0.5-3.5 M salt, and at last halotolerant that show no requirements to salt for growing, yet mostly grow well up to very high salt concentrations and can tolerate at least 100 g/l salt (like 1.7 M salt). Seawater, salt lakes, saline soils, brines, and alkaline saline habitats are one of the most efficient habitats for halophilic Actinobacteria isolating. Broadly, most of the halophilic Actinobacteria have been isolated from saline soils. Isolated halophilic Actinobacteria from the marine environments are defined as a few genera, including *Rhodococcus, Micromonospora,* and *Streptomyces* (Maldonado et al. 2005). Another group includes *Salinispora, Solwaraspora, Dietzia, Marinophilus, Aeromicrobium, Microbacterium, Salinibacterium, Grofonia, Mycobacterium, Nocardiopsis, Pseudonocardia, Saccharopolyspora, Actinomadura, Streptosporangium, Qilliamsia, Nonomuraea,* and *Verrucosispora* (Maldonado et al. 2005; Jensen et al. 2005; Magarvey et al. 2004; Riedlinger et al. 2004).

3.4.3 Endophytic *Actinobacteria*

Endophytic A*ctinobacteria* are defined as inhabitants of internal parts of plants, which do not cause any visible damage or changes to the hosts. These *Actinobacteria* have specific roles for plant life, like protecting the host against insects and diseases. Endophytic *Actinobacteria* represent a large part of the rhizosphere, they are also found inside the plants and are extensively studied species from the genus *Frankia*, which is nitrogen-fixing bacteria of nonleguminous plants (Berry et al. 1993). And a few species of the genus *Streptomyces* are phytopathogens. Endophytic Actinobacteria generally include *Streptomyces*, but the genera *Nocardia, Micromonospora, Actinopolyspora, Streptoverticilu, Kitasatospora, Microbispora, Glycomyces Pseudonocardia, Kibdelosporangium, Promicromonospora,* and *Streptosporangium* can be also found in plants like *Calycophyllum acreanum, Croton lechleri, Palicourea longifolia, Monstera spruceana, Cantua buxifolia, Eucharis cyaneosperma,* and *Siparuna crassifolia*. Different methods have been used for the isolation of this type of *Actinobacteria*. The diversity of obtained actinobacteria depends on the methods of isolation (Takahashi & Omura 2003). The most applied method for the detection and calculation involves isolation from the sterilized surface of the host plant tissue. Isolation depends on various factors, such as host plant species, sampling season, age and type of tissue, geographical and habitat distribution, selective media, and conditions of culture (Hallmann 2001; Gaiero et al. 2013).

Medical plants are a very important source of precious bioactive compounds. As a result of a long-term correlation of endophytes with these plants might be the participation of Actinobacteria in metabolic pathways. They may enhance their natural bioactivity or could gain some useful genetical information for the production of specific biologically active compounds such as those produced by the host plant. So genetic exchange of information may take a place

(Stierle et al. 1993; Eyberger et al. 2006; Mitchell et al. 2010; Kumar et al. 2013; Chithra et al. 2014).

3.4.4 Endosymbiontic *Actinobacteria*

The endosymbionts are any organisms that live within the bodies or cells of other organisms. Endosymbiosis can be sometimes obligate, where the endosymbiont or the host cannot survive without the other organism. *Actinobacteria* phylum has been identified with the abundance of its members of sponge-associated microbial communities. *Mycobacterium* along with *Micromonospora, Microbacterium, Micrococcus, Brevibacterium, Rhodococcus, Kocuria, Corynebacterium, Dietzia, Streptomyces, Rubrobacter, Brachybacterium, Actinokineospora, Arthobacter, Rhodonia, Nocardiopsis,* and *Rothia* species observed for their life as endosymbionts of marine sponges *Callyspongia aff. Implexa, Aplysina aerophoba, Hemimycale columella, Dysidea tupha, Spheciospongia vagabunda, Callyspongia* sp., *Dysidea avara, Negombata magnifica,* and *Amphimedon* sp. Nevertheless, endosymbionts of *Actinobacteria* have been also discovered in other animal groups, such as *Rhinopithecus roxallanae, Rhinopithecus bieti, Hylobates hoolock, Panthera tigris altaica, Panthera tigris tigris, Panthera tigris amoyensis, Ursus thibetanus, Ailurus fulgens, Cavnlvara zlrsidae, Cervus elaphus, Vicugna pacos,* and *Elaphurus davidianus*.

In research by R. Gandhimathi *et al.* (2008), there was observed actimicrobial activity against the growth of some human pathogens in the endosymbiotic marine actinomycetes. Within the primary screening, 76.9 % showed antimicrobial activity. The only potential producers were considered just actinomycetes and amongst the bacterial and fungal isolated was perceived no producer. 27 % of actinomycetes were found associated with F. cavernosa and C. diffusa to be the potential producers. Sponge isolates of *Streptomyces* spp., *Saccharomonospora* spp., and *Micromonospora* spp. showed the highest antimicrobial activity. All the potential producers showed antimicrobial activity against Gram-positive bacteria and just 60 % against Gram-negative bacteria. This percentage trend has been already reported (Kokare et al. 2004), and Gram-positive strains, especially *M. luteus* were used as a testing strain for standard antibiotic trials.

As observed in the study of Gandhimathi *et al.* (2008), actinomycetes associated with sponges, showed high antimicrobial activity against Gram-negative bacteria like *K. pneumoniae* and *Pseudomonas aeruginosa.*

3.4.5 Symbiotic *Actinobacteria*

Around 15 % of the fixed nitrogen in the world is a result of symbiotic relationships between various species of the *Frankia* which belongs to the phylum of *Actinobacteria*. Actinorhizal plants are those forming symbiotic relationships with *Frankia*. In many research has been found that over 160 plants have *Actinobacteria* hosts. Such plants include sweet fern, bitterbrush, cliffrose, Russian olive, bayberry, and alders. The *Frankia* can provide the most or all of the nitrogen needed by the plant. Numbers of representatives of this genus including *Casuarina* isolates form nitrogen-reducing vesicles *in vitro* and in planta (Thajuddin et al. 2015). These bacteria which fixate nitrogen, and their host plants are often pioneer species on mostly young, nitrogen-deficient soils such as volcanic flows, moraines, and sand dunes.

3.4.6 Acidophilic *Actinobacteria*

Acidophilic *Actinobacteria* are common in terrestrial habitats, such as acidic forests, mine drainage soil, waterlogged acid soils (Kopecky et al. 2011), acidic mine sediments (Lu et al. 2010), pine forest soils (Hui et al. 2012), paddy fields (Liu et al. 2014), and extremely acidic

rivers (García-Moyano et al. 2007). They grow in the pH range between 3.5 and 6.5, but the optimum rate at pH 4.5 to 5.5 (Khan & Williams 1975; Hagedorn 1976). The major factor of abundance and diversity of prevailing lingeages of soil bacteria are temperature, pH value, land use, and the local vegetation (Youssef & Elshahed 2009). *Actinobacteria* represent up to 50 % of total soil bacterial communities through many different ecosystems.

In the case of isolates of novel extremely acidophilic, iron-oxidizing actinobacteria were found. Isolate $T23^T$ from a mine site located in North Wales in the UK, and second, $Y005^T$, from a geothermal site in Yellowstone National Park in Wyoming, USA. The isolate $T23^T$ had its optimum at pH 2.0, minimum at pH 1.4, and grew optimally at 35 °C, but not at 45 °C. Second isolate $Y005^T$ had its optimum at pH 1.8 and pH 1.6 minimum. The second isolate was able to grow at 50 °C of its maximum (Johnson et al. 2009).

Another research, done on paddy field soil in Osmancık found 3 possible new species which exhibited antifungal activity. Isolates were tested against two main fungi which cause significant damage to the production of rice. Isolates *Streptomyces* sp. PT513, *Streptomyces* sp. PT579, and *Streptomyces* sp. PT597 performed their possible use in biological control of phytopathogenic fungi *Fusarium moniliforme*., more beneficial than currently, although effective, chemicals which pose a certain risk to the environment and a human (Veyisoglu & Tatar 2021).

Acidophilic *Actinobactera* can also synthesize silver in form of bioactive silver nanoparticles. *Streptacidiphilus durhamensis* HGG16n synthesized bio(AgNPs) of a spherical shape and size range of 8-48 nm which showed antimicrobial activity. This isolate can be used for its efficient synthesis of bioactive AgNPs by an inexpensive, non-toxic, and eco-friendly method. The obtained bio(AgNPs) showed unique biochemical and physicochemical properties. New development of antimicrobial agents with synergistic enhancement could be done against clinical bacteria. The highest antimicrobial activity was performed against *Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, Escherichia coli, Bacillus subtilis,* and *Klebsiella pneumonia* (Buszewski et al. 2018).

3.4.7 Gut *Actinobacteria*

Some *Actinobacteria* can be found in intimate association with invertebrates and vertebrates. Symbiotic interactions are crucial mostly for survival and reproduction because they take a part in nutrition, detoxification of certain compounds, protection against pathogenic bacteria, and growth performance. Based on many studies, some symbiotic *Actinobacteria* species, which are probiotics, do control bacterial diseases in livestock, aquaculture, and poultry. They may be also beneficial by converting the feed into microbial biomass and fermentation end-product then can be utilized by the animal host.

Streptomyces, Nocardiopsis, and Oerskovia were isolated from the healthy goat feces (Tan et al. 2009).

3.5 Use of Actinobacteria

As mentioned in the beginning, *Actinobacteria* are well recognized for their production of both primary and secondary metabolites, which have a big potential throughout many fields. They are also a promising source of wide range of important enzymes, which could be produced on an industrial scale. The vast majority of antibiotic in the market is obtained thanks to *Actinobacteria*. They produce inhibiting enzymes useful for the treatment of cancer and immunomodifiers that enhance the immune response. Other great abilities are to degrade a wide

range of hydrocarbons, pesticides, aromatic and aliphatic compounds, and to perform microbial transformations of organic compounds (a field of great commercial value). Representatives of many genera can be potentially used in the bioconversion of underutilized urban and agricultural wastes into chemical products.

3.5.1 Antibiotics (Antimicrobials)

The importance of *Actinobacteria* for their possible novel secondary metabolites is significant after a recent decrease in the effectiveness of current pharmaceutical drugs. There is a struggle with multidrug-resistant pathogens that are harder and harder to treat with current medicaments. Products of natural origin have been observed for their useful activities (Dancer 2004). Since some certain antibiotics are toxic, their usage led to be limited although thousands of antibiotics have been discovered. The majority of antibiotics are derived from microorganisms, where most of them are Actinobacteria which take a part in almost 80% of the world's antibiotics. Most are from two genera, *Streptomyces* and *Micromonospora* (Jensen et al. 1991; Hassan et al. 2011). Streptomyces are producers of around 7600 compounds, where most of them are secondary metabolites that are strong antibiotics. Thanks to that, Streptomyces are the primary producers of antibiotics and are exploited by the pharmaceutical industry (Jensen et al. 2007; Ramesh & Mathivanan 2009). The ability of the genus S*treptomyces* to produce such significant compounds is hidden in its large DNA complement (Kurtböke 2012). One of the first antibiotics called streptomycin is produced by *Streptomyces griseus* (Schatz et al. 2005). It has been assessed that during the last 50 years has been discovered over 12,000 antibiotics. Out of this amount, for 70% were responsible *Actinobacteria*, and the remaining 30% were products of filamentous fungi and other bacteria. Actinobacterial antibiotics are divided into several major structural classes, starting with aminoglycosides (e.g., streptomycin and kanamycin), anthracyclines (e.g., doxorubicin), beta-lactam (cephalosporins), macrolides (e.g., erythromycin), and tetracycline. If different conditions for culturing are applied, different abilities for antibiotic production of *Streptomyces* occur. So, if proper ways are applied, the production could be enhanced, thus the biosynthesis would be much more effective. They have a different chemical nature, toxicity to animals, antimicrobial action, and potential in chemotherapy. Some of the so far isolated antibiotics are crude preparations, whilst others have been crystallized, and great information has been obtained concerning their chemical nature. Some Actinobacteria can produce more than one substance of antibiotics (e.g., *S. griseus*), and the same antibiotics can be produced by different species of Actinobacteria (e.g., actinomycin, streptothricin). Thus, certain antibiotics may be identical even if they were produced by different species.

3.5.2 Enzymes

Both, marine and terrestrial *Actinobacteria* produce a wide variety of biologically active enzymes. For example, some secrete amylases into the outside, which helps with carrying out extracellular digestion. Amylase is significant for biotechnological applications in food, textile, fermentation, and paper industries where its ability to degrade starch is desired (Pandey et al. 2000). Since many *Actinobacteria* have been isolated from various habitats, the biological functions mainly depend exactly on their origin. Actinobacteria, especially *Streptomyces*

secrete multiple proteases in the culture media (Sharmin et al. 2005). Species like *Streptomyces griseus*, *S. albidoflavus*, *S. karnatakensis*, and *Nocardia sp*. have been found as a great resource of L-asparaginase. (DeJong 1972; Narayana et al. 2008).

3.5.3 Vitamins

A natural vitamin B12 may be produced by bacteria or *Actinobacteria* (Robbins WJ *et al.* 1950). The possible production of vitamin B12 has its interest-based on actionbacterial fermentation (Rickes et al. 1948; Lichtman et al. 1949). If cobalt salt is added to the media, it acts as a precursor to producing vitamins, which applies to all *Actinobacteria*. As long as the cobalt is added carefully, it does not antibacterial effect. The fermentations producing antibiotics aureomycin, grisein, neomycin, and streptomycin will also produce some vitamin B12 without any affection of yield of antibiotic substances while the cobalt is supplied in the medium. Based on some studies, Actinobacteria not producing antibiotics are preferable for their higher production of this vitamin than antibiotic-producing Actinobacteria. Other water-soluble vitamins were found to be produced too.

3.5.4 Probiotics

Probiotics are microbial supplement which has a beneficial effect on the host. Probiotics can modify the associated or ambient microbial community of the host. It is done by improving the use of feed or enhancing the nutritional value, improving its nutrition, enhancing the response of the host against diseases, or by improving the surrounding environment.

Regardless of several other important uses, marine *Actinobacteria* got attention for their use as probiotics. The potential use against shrimp pathogenic *Vibrio spp*. made marine *Actinobacteria* a possible probiotic strain for their ability to degrade the macromolecules (e.g., protein and starch), the formation of spores resistant to heat and desiccation, and the production of antimicrobial agents (You et al. 2007). In another case, *Streptomyces* sp. showed again its use, where an antibiotic product extracted from the S*treptomyces* sp. was added to the feed of black tiger shrimp to observe the *in vivo* effect on the white spot syndrome virus (Das et al. 2006).

3.5.5 Pigments

Actinobacterial pigment production is a great substitute for synthetic pigments which have some limitations. The limiting aspects are the hazardous chemicals used in the production, the worker's safety, and in the end the hazardous waste. Some *Actinobacteria* are characterized by the production of pigments of various colors on both, natural and synthetic media. These pigments usually have colors of shades of blue, red, rose, violet, yellow, brown, green, or black. The pigments can be dissolved into the medium, exopigments, or may remain within the mycelium, endopigments. Some antibiotics are considered pigments since the pigmental formation is influenced by the pH of the medium, temperature, aeration of the growth, and sources of carbon and nitrogen. So far just a little is known about the exact chemical nature of these pigments.

3.5.6 Plant diseases control

The tendency to search for natural products for biocontrol and protection of plants in the market has raised significantly during past years. Streptomyces again as representative of *Actinobacteria* appeared to be a good candidate for research of finding new ways in controlling plant diseases. A great number of novel insecticides and herbicides originate from *Streptomyces* within the last years (Tanaka & Omura 1993). *Streptomyces kasugaensis* produces kasugamycin, a bactericidal and fungicidal metabolite, which has inhibiting properties in protein biosynthesis in microorganisms but means no harm to mammals and has excellent toxicological properties (Umezawa et al. 1965). Polyoxin B and D were isolated metabolites of *Streptomyces cacaoi* var. *asoensis* as a new natural fungicidal class in 1965 (Isono et al. 1967). Regarding environmental considerations, polyoxins are acceptable for their ability to interfere with the fungal cell wall synthesis thanks to especially inhibiting chitin synthase (Endo & Misato 1969). Polyoxin B found its use against many fungal pathogens in vegetables, fruits, and ornamentals. Polyoxin D is more precisely marked for the control of rice sheath blight caused by *Rhizoctonia solani*.

3.5.7 Enhance of plant's growth

Even though the history of *Streptomyces* is well documented for their use in biocontrol and preliminary evidence of their ability to enhance the growth of plants (Aldesuquy et al. 1998), Streptomyces have been not examined for their potential as PGPR (plant growth-promoting rhizobacteria). Whereas the beneficial effect is certain for some PGPR strains on particular plants, its own mechanism is unclear (Glick 1995). The effect of PGPR on plants can be direct or indirect. In case of indirect effect, the PGPR lessens or prevent the effects of one or more harmful microorganisms. This is achieved via biocontrol or the antagonism of soil-plant pathogens. Colonization or the biosynthesis of antibiotics or other secondary metabolites can prevent the invasion and establishment of pathogens. Direct enhancement of the plant growth happens when the compound synthesized by the bacteria is supplied to the plant, or when PGPR enhances plant uptake of the nutrients from the soil. *Streptomyces griseus* was reported (Merriman et al. 1974) for its use in seed treatment of barley, carrot, oat, and wheat to increase their growth. The increase in the average grain yield, tiller number, foliage weight, and advanced head emergence of both oat and wheat over controls was not significant. But in contrast, the use of *Streptomyces griseus* for seeds of carrot, the treatment was more successful. Purchasable yields increased over controls by 17% and in two separate fields by 15%. Both experiments even indicated an increase in the yield of large to very large carrots over controls (Merriman et al. 1974).

4 Methodology

Two study sites were observed for their similar conditions and properties of soil. The first site was a coniferous forest located in the region of Český ráj, next to Zakopaná village. This location was chosen for its conditions. The novel proposed actinobacterial family Treboniaceae might be supported if new strains were found to be related to this family. The second location, more known, is near Třeboň in the southern part of the Czech Republic. The study site is a deciduous forest dominated by *Populus tremula*. After the collection, the soil samples were

diluted, and the diluted water-soil suspension was plated on agar media. The media were of three types, differing in their pH value. After some time, which differed for each bacteria type, the most promising colonies were picked and transferred to fresh media. The purification was repeated 3 times before transferring to liquid media for the final preparation of biomass. After proper cleaning of the biomass, the DNA extraction was performed using a DNA kit. Some changes in the process had to be done to achieve higher efficiency. Using an optimized method of PCR amplification, the specific 16S rRNA gene amplicons were obtained in sufficient yield. The PCR products were further sent to be sequenced.

4.1 Soil samples

Soil samples were collected with some deviation at previously known sites. The first samples from Zakopaná were collected on the 2nd of February while from Třeboň on the 7th of June 2021. At both sampling points, the litter was removed carefully before collecting the soil. In both cases, the soil samples were collected from two horizons, the upper (Ah horizon) and the lower horizon (Go horizon). Disinfected equipment was used and cleaned before collecting from each soil horizon. Samples were collected and transported in plastic bags. The samples were stored in the fridge before the dilution.

4.1.1 Zakopaná site

In Zakopaná, the sampling point was on arenick podzol based on FAO classification (The Food and Agriculture Organization of the United Nations). The forest is dominated by *Pinus sylvestris*. The location is a part of Hruboskalské sandstones. Thus, the scenery is typical of sandstone cliffs. The litter was mostly composed of fallen needles and partly covered by small shrubby plants, such as *Vaccinium myrtillus* and *Cytisus scopariu*. Komárovský pond, with an area of 38 ha maximal depth of 2.5 m, is around 600 m from the sampling location with no or slight influence since the location is at higher altitudes.

4.1.2 Třeboň site

In the case of the Třeboň location, the soil is classified as a gleysol (stagnogley) according to the FAO classification. Iron reduction is a typical characteristic of this soil due to its waterlogging. The Ah horizon (the “upper horizon”) was a 10 cm thick, loamy sand horizon with moderate humus, and the Go horizon (the “lower horizon”) was loamy sand soil with mottling between 15 and 50 cm. The subsoil water table’s fluctuation depended on the nearby fishpond’s water level of an area of 1.6 km^2 and an average depth of around 2 m. The water table could reach up to 10 cm below the soil surface, which meant penetration of the whole lower horizon. Thus, the upper layer receives the water only in form of precipitations or capillary forces, so it can completely dry out during hot summer days.

4.2 Plating the samples

Obtained soil samples were 10^3, 10^4, and 10^5 –fold diluted in the distilled water. 100 µl of diluted soil suspension was added onto each medium and was spread over the whole surface of the plate. A proper description of the samples was done for more accurate further steps without loss of data. The both sets of samples from both locations were incubated at 20 °C to assure the growth of slow-growing strains just like *Actinobacteria*. This step took a long time. Approximately after a month or two, colonies most similar to Actinobacteria were selected. Colonies that were tough, matte, inlaid in agar, and created just smaller colonies in comparison to other bacteria from the soil, were desired for further transfer. Transferring of colonies was made just by a tip of a toothpick again onto the same medium. The streak plate method was applied for obtaining just individual bacterial colonies for further isolation. These secondly transferred samples were labelled by numbers and information matching the properties of the plating media and the type of horizon. The secondly transferred samples were again incubated at 20 °C and after 14 days to one month were transferred again onto a VL medium of matching pH but incubated at a higher temperature of 28 °C. If no contaminating fungal colonies occurred, the final transfer took a place finally onto the R2A medium of the matching pH. All transfers were done after 1 - 3 weeks in case of incubating at 28 °C. When the strains have grown on the R2A, colonies were transferred into the liquid R2A medium and incubated in the shaking incubator Ecotron (INFORS HT, Switzerland) at 28 °C. After a week the biomass was collected.

4.2.1 VL medium

A selective medium was used in the first plating and the following two cleaning transfers. The media were supplemented with cycloheximide (CX), that is a naturally occurring fungicide produced by *Streptomyces griseus*, to avoid the spread of unwanted molds to obtain clean strains. Prepared VL solution and agar were autoclaved at 120 °C for 20 min. Before mixing the VL solution and agar were incubated at 50 °C. Then the vitamins and CX were added to the VL solution, mixed with agar, and poured in the desired amount onto the Petris dishes.

Solution 1 – selenite/tungstate solution (mg(ml)/l): 0.4 g – NaOH pellets, 2 mg – Na_2SeO_3, 4 mg - $Na_2WO_4 * 1.5H_2O$.

Solution 2 – trace element solution (mg(ml)/l): 7.7 ml – conc. HCl, 190 mg – $CoCl_2*6H_2O$, 2 mg - $CuCl_2*2H_2O$, 4.5 g – $FeCl_2*2H_2O$, 45 mg – $NaBO_3$ (or H_3BO_3), 100 mg – $MnCl*4H_2O$, 36 mg - $Na_2MoO_4*2H_2O$, 24 mg – $NiCl*6H_2O$, 140 mg - $ZnCl_2$.

Vitamins 1 (mg/l): 40 mg – 4-aminobenzonate, 10 mg – (1)-biotin, 100 mg – Nicotin acid, 50 mg – hemicalcium D-(1)-pantothenate, 150 mg – pyridoxamine hydrochloride (vitamin B6), 100 mg – thiamine chloride hydrochloride, 50 mg – cyanocobalamine.

Vitamins 2 (mg/l): 10 mg – DL-6,8-thiotic acid (or Lipoic), 10 mg – riboflavin, 4 mg – folic acid.

VL 55 medium – 0.5 l	
MES	1.95 g
$MgSO_4$	0.025 g
$CaCl_2$	0.035 g
$(NH_4)_2HPO_4$	0.03 g

Solution 1	0.5 ml
Solution 2	0.5 ml
Vitamins 1	1 ml
Vitamins 2	3 ml
Cyclohexamide (CX)	12 ml (10 mg/ml)
Agar	7.5 g / 250 ml $_{d}H_2O$ + 250 ml VL solution
$_{d}H_2O$	250 ml + 250 ml

Table 1 - VL 55 medium

4.2.2 R2A medium

For the last transferring of strains onto the clean Petris dishes R2A medium was used without any added CX to see if the cultures contained just the desired strains. In the first place the transfer was onto the solid R2A medium and for the last step of producing the biomass, the liquid one. The prepared R2A solution and agar were autoclaved at 120 °C for 20 min. When ready, the solution with agar was incubated at 50 °C, mixed in the flow box, and poured onto the Petri dishes. In the case of the liquid media for the incubation in the shaker incubator, the solution was after mixing the ingredients filled into 100 ml Erlenmayer flasks and autoclaved at 120 °C for 20 min.

R2A medium – 1 l	
Bacto protease peptone	1 g
Yeast extract	1 g
Casamino acid	1 g
Glucose	1 g
Soluble starch	1 g
Na – pyruvate	0.6 g
K_2HPO_4	0.6 g
$MgSO_4$ x $7H_2O$	0.05. g
MES (4 – Morpholineethanesulfonic acid)	3.9 g
Agar *	30 g / 1 l $_{d}H_2O$ + R2A solution

Table 2 - R2A medium

4.3 Collection of the biomass

After the biomass was grown in the liquid medium, it was collected after a week. The whole amount was poured into 50-ml centrifuge tubes. Samples were centrifuged for 10 min at 16 000 x g at 5 °C. Supernatant was discarded to obtain just the sedimented biomass from the bottom of the tubes. The next step was washing the samples with $_{dd}H_2O$ to get rid of the remaining medium in the collecting 2 ml tubes, using a small centrifuge. Again, the excess liquid was discarded. Part of the biomass was conserved in the TRIS-glycerol solution in the cryotubes at -70 °C. The rest was used for the DNA extraction using the DNA isolation kit.

4.3.1 Conservation of strains

A solution of 200 mM TRIS and 50 % glycerol was used at a rate of 1:1 for the storage of strains for possible further work. The prepared solution was added into 2.5 ml cryotubes, 1.5 ml per tube, and autoclaved at 120 °C for 20 min. After cooling down to room temperature, under the sterile condition in the flow box, an adequate amount of biomass was added into the tubes. The samples were stored in the -70 °C freezer in a signed box.

4.4 DNA extraction

DNeasy PowerLyzer Microbial Kit (QIAGEN, Hilden, Germany) was used for the isolation of high-quality DNA from microbial cultures with the use of a bead-based homogenizer. The protocol was modified for better results since the original procedure was not efficient and showed low or no DNA yield. The procedure was modified at the 3rd, 4th, and the 15th step. In the 3rd were used different Bead Tubes than those provided by the kit, because the bacteria were hard to crush with only 0.1 mm glass beads. Before homogenizing in the 4th step, the Bead Tubes were heated up to 80 °C, and once this temperature was reached the samples were disintegrated in the Mini BeadBeater-16 (Biospec Products, Bartlesville, OK, USA) for 3 min at speed of 3400 rpm. In the 15th step, the use of 50 µl elution buffer was divided into two steps where the first 25 µl were added and left for 2 min and then centrifuged and it was repeated with the remaining 25 µl. The DNA preparations were checked for yield and fragmentation by electrophoresis and stored in a -20 °C freezer before further procedures.

The modified procedure was as followed:

1. Add 1.8 ml of microbial culture to a 2 ml Collection Tube and centrifuge at 10,000 x g for 30 s at room temperature. Decant the supernatant and spin the tubes at 10,000 x g for 30 s at room temperature and completely remove the media supernatant with a pipet tip.
2. Resuspend the cell pellet in 300 µl of PowerBeand Solution and gently vortex to mix. Transfer resuspended cells to a PowerBead Tube Glass, 0.1 & 0.5 mm (1:1).
3. Add 50 µl of Solution SL to the PowerBead Tube. And heat up to 80 °C and then move to the 4th step.
4. Homogenize using Mini BeadBeater-16 for 3 min at 3400 rpm.
5. Make sure the PowerBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge the tubes at a maximum of 10,000 x g for 30 s at room temperature.
6. Transfer the supernatant to a clean 2 ml Collection Tube and expect 300 to 350 µl of supernatant.
7. Add 100 µl of Solution IRS and vortex for 5 s. Incubate at 4 °C for 5 min.
8. Centrifuge at 10,000 x g for 1 min at room temperature.
9. Avoiding the pellet, transfer all the supernatant to a 2 ml Collection Tube. Expect approximately 450 µl of supernatant and a small carryover of glass beads is possible without affecting the results.
10. Add 900 µl of Solution SB to supernatant and vortex for 5 sec.
11. Load about 799 µl into an MB Spin Column and centrifuge at 10,000 x g for 30 s at room temperature. Discard the flow-through, add the remaining supernatant to the MB Spin Column, and centrifuge at 10,000 x g for 30 sec at room temperature.
12. Add 300 µl of Solution CB and centrifuge at 10,000 x g for 30 s at room temperature.

13. Discard the flow-through and centrifuge at 1é,000 x g for 1 min at room temperature.
14. Being careful not to splash liquid on the spin filter basket, place MB Spin Column in a new 2 ml Collection Tube.
15. Add 25 µl of Solution EB to the center of the white filter membrane and still for 2 min.
16. Centrifuge at 10,000 x g for 30 s at room temperature.
17. Repeat the 15th step followed by the 16th step.
18. Discard the MB Spin Column. The DNA is now ready for downstream application.

4.4.1 Electrophoresis

For checking the quality of the DNA templates, electrophoresis was used. 50 ml of a 1% agarose gel was prepared with the use of 50 ml of TAE buffer with dissolved 0.5 g of agarose. After cooling down, 5 µl SYBR GREEN in DMSO (1:9 ratio) was added to the solution, mixed, and poured into the casting tray. After solidifying, the gel was put into the TAE buffer in the electrophoresis bath. DNA samples were prepared in the microtiter plate and mixed with the loading buffer, 2 µl of the sample, and 3 µl of the loading buffer. 5 µl of marker (1 Kb Plus DNA ladder) was loaded into the first lane and followed by the samples mixed with the loading buffer. Each loading took 5 µl. The electrophoresis was run from minus to plus at 95 V, about 95 mA. The run was done once the orange color reached approximately 2/3 of the way down. Then the gel was visualised under the UV light and snaps were taken.

4.4.2 Use of spectrophotometer

After the DNA preparations were done the concentration and purity were measured with a micro-volume spectrometer Nanophotometer (Implen, München, Germany). For most of the measurements, lid No. 50 was used with 2 µl of the sample. If the concentration was too low the lid was changed for the No. 10 with the use of 4 µl of the sample.

4.5 PCR amplification of 16S rRNA genes

Amplification was done with the GoTaq polymerase (Promega) using forward primer 27F 5'- AGA GTT TGA TCM TGGC TC AG-3' and backward primer 1492·R 5'-TAC GGY TAC CTT GTT ACG AC TT-3'.

Amplification with GoTaq polymerase – 1 sample of 25 µl	
$_dH_2O$	13.125 µl
Colorless GoTaq Reacting Buffer	5 µl
DMSO	1.25 µl
$_d$NTP (10mM)	0.5 µl
Forward primer – 27F	1 µl
Reverse primer – 1492·R	1 µl
GoTaq DNA Polymerase G2 HOT START	0.125 µl
DNA	1 µl

Table 3 - Amplification with GoTaq polymerase

The polymerase was prepared firstly without the DNA templates to avoid contamination with the DNA of the workplace, where only polymerases were prepared after sterilization of the workplace with UV light. Then the polymerase was moved to the UV-sterilized box again where firstly the DNA templates were diluted based on the concentration of the samples if needed. If the concentration was higher than 20.0 ng/ µl then the dilution ratio increased with each additional 15 – 20 units. For example, a concentration of 75 was diluted in the ratio of 1:3. In the case of lower concentrations than 10 units, the amount of distilled water in the PCR mixture use was decreased as a result of the needed increase of the DNA templates. Thus, when the concentration was 7 units, the amount of DNA template was doubled to 2 µl, and the amount of $_dH_2O$ decreased to 11.125 µl. PCR mixture was pipetted into the PCR stripes of 8 samples each in an amount of 24 µl or modified, then the DNA templates were added in the desired amount and mixed with the tips. If needed the centrifuge was used to assure that the solutions were at the bottom of the stripes for further steps of the reaction in the Thermal Cycler.

4.5.1 PCR

For the polymerase chain reaction was used C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). A specific procedure was modified for better results of the reaction. The protocol consisted of 7 steps where after reaching the 5th step, steps 2, 3, and 4 were repeated 34 times, and after the repetition, the procedure terminated with the 6th and 7th steps. In the 1st step, the samples were heated to 95.0 °C for 5:00 min, followed by the 2nd step of the same temperature but only for 0:30 min, 3rd at 56.0 °C for 0:50 min, 4th at 72.0 °C for 1:30 min. As mentioned before the 5th step was a repetition of 3 previous steps 34 times. Then the 6th step was for 5:00 min at 72.0 °C and the final step cooled down to 10 °C and was kept “forever” for storing before another manipulation of the PCR product.

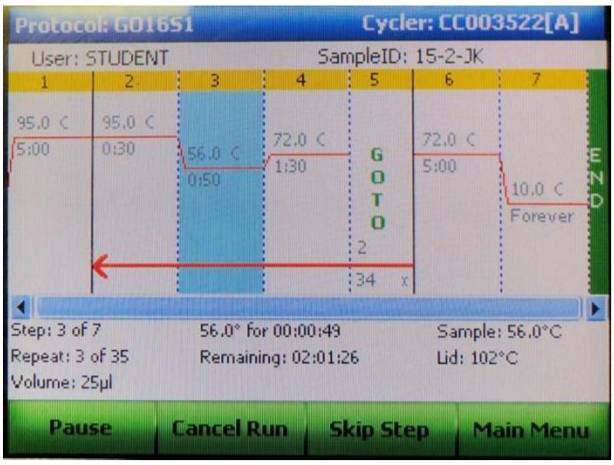

Figure 4 - GO16S1 Protocol

4.5.2 Electrophoresis

For checking the quality of the PCR products, electrophoresis was used again. 50 ml of a 1% agarose gel was prepared with the use of 50 ml of TAE buffer with dissolved 0.5 g of agarose. After cooling down, 5 µl SYBR GREEN in DMSO (1:9 ratio) was added to the solution, mixed, and poured into the casting tray. After solidifying, the gel was put into the TAE buffer in the electrophoresis bath. PCR products were prepared in the microtiter plate and mixed with the loading buffer, 1 µl of the PCR product, and 2 µl of the loading buffer. 5 µl of marker (1 Kb Plus DNA ladder) was loaded into the first lane and followed by the PCR products mixed with the loading buffer. Each loading took 3 µl. The gel ran from minus to plus – 95 V, about 95 mA. The run was done once the orange color reached approximately 2/3 of the way down. Then the gel was visualized under the UV light and snaps were taken.

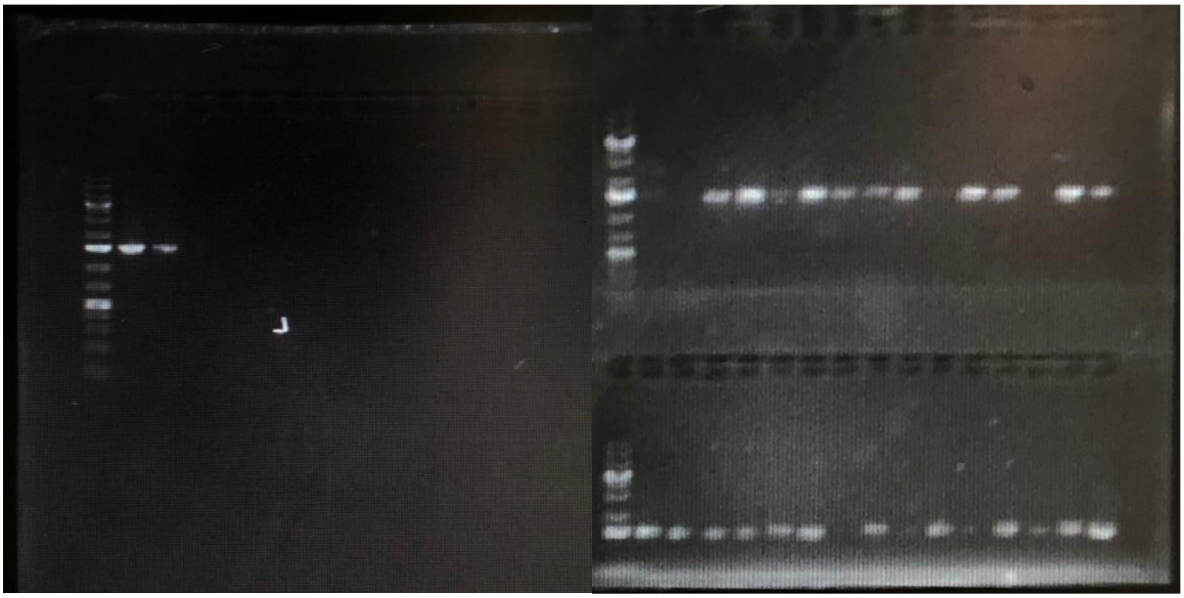
Figure 5 - PCR products after electrophoresis under UV light, left one with the failed procedure and the right one with modified amount of DNA sample used for the PCR

4.6 Phylogram construction

After receiving sequences, the editing was done using Chromas (Technelysium – DNA Sequencing Software, South Brisbane, AU), if some genes overlayed each other, the codes were used instead. After choosing the useable part of the sequence, the sequence was saved as the FASTA file. The sequences with the names were put into a single FASTA file and were uploaded to Silva's database (The SILVA ribosomal RNA database project, Max Planck Institute for Marine Microbiology and Jacobs University, Bremen, DE) of 16s sequences. The classification of strains was then downloaded as a .csv file. The classification was ensured using Nucleotide Blast (National Library of Medicine, National Center for Biotechnology Information, Bethesda, USA), to find the most related species, if the sequences had a low identity percentage, the sequences were compared to see if the edited sequences were right and really occurred in the same classification. If needed, some corrections were done. Based on the classification, using the browser in Silva, reference sequences of the six genera were downloaded as FASTA files. Also, representatives (one for each genus) of related genera were exported to outgroup the rest in the creation of the phylogenetic trees. To the reference sequences, the observed strain and outgrouping species (roots) were added and aligned, using Sina 1.2.11 – ACT section in Silva. Aligned files were opened by SeaView software (Gouy et al. 2010) and gaps were deleted using the edit options. The sequences were checked in case of the observed strains and their length was noted for the next step. Files were then saved and opened by ClustalX where the cutting was done to just have the part of the sequence where the observed strains appeared. These works were saved as FASTA and PHYL files. Using FastTree (Price et al. 2010) the approximately-maximum-likelihood phylogenetic trees were inferred. Before the visual creation of the phylogenetic trees. The genera were put into the Excel files each and the lists of the names and codes were created, in the second list the inferred trees from FastTree were imported, and by using macra the codes in the trees were replaced by the species names. These renamed tree texts were then used in FigTree (available from http://tree.bio.ed.ac.uk/software/figtree/) for the visualization of phylogenetic trees. Firstly, the species which were added to outgroup the genera we re-rooted. Then the visual editing was done for better readability. Phylogenetic trees were then exported as pdf. documents and used in this work. Phylogenetic trees can be found in the appendices.

5 Results

5.1 Description

Based on the information about the locations and previous samplings, strains from Zakopaná were more isolated from Go horizon (B) and mostly grew the best in the medium of pH 3.5 or 5.5. The dilution rate was mostly represented by 10^4 or 10^5.

On the contrary, the majority of strains from Třeboň were isolated from A horizon (A). The dilution probably did not play an important role, and the samples were evenly distributed, but the strains grew mostly at pH 4.5 and 5.5.

Zakopaná			
Sample N°	Location+horizon	Dilution 10^x	pH * 0,1
11-Z	B	4	55

3-Z	B	4	55
14.B-Z	B	5	55
23.1-Z	B	5	55
53-Z	B	4	35
54-Z	B	4	35
57-Z	B	4	35
100-Z	B	3	55
1031-Z	B	3	45
R-203-Z	B	5	35
205-Z	A	4	55
Třeboň			
31-T	A	4	45
31a-T	B	3	45
32-T	A	4	45
35-T	A	3	55
R-46-T	A	3	45
70-T	A	5	55

After constructing the phylogenetic trees, the isolated strains could be compared to the described species occurring within the genus. All isolated strains were from 6 genera: *Actinospica, Nocardia, Pseudonocardia, Rhodococcus, Streptacidiphilus*, and *Streptomyces*. After all the previous procedures for obtaining the results, 17 strains were good enough to present and classify in the trees. All the phylogenetic trees can be found in the Appendices section.

5.2 Phylogeny

Obtained sequence information for each strain and their further classification made it clear, which samples were representing the phylum of *Actinobacteria*. After classification from the Silva database, 8 samples appeared to be unclassified within Bacteria. Four samples (5-T, 6-T, R-4-T, R-44-T – all from the Třeboň site) were classified as strains of the family *Burkholderiaceae* of the *Burkholderiales* order. Three samples (R-33-T, R-332-T, R-333-T – from the Třeboň site), were classified in the Pseudomonas genus of the *Pseudomonadales* order. Both orders *Pseudomonodales* and *Burkholderiales* are within the *Gammaproteobacteria* class of Proteobacteria phylum.

5.2.1 *Actinospica*

A total of 3 strains were identified within the genus *Actinospica*. All of them, R-2031-Z, 53-Z, 57-Z., are from the first sampling location, Zakopaná. and created distinct branches in the phylogenetic tree, where 53-Z and 57-Z seemed to create their own cluster. The closest to

53-Z and 57-Z were undescribed actinobacterium Aac-40 AB180781 and actinobacterium_Aac-40_AB180762. Other closest strains are undescribed in the same cluster with both AB180762 and AB180781. The closest described species, but still genetically distinct, is *Actinocrinis puniiceicyclus* (Kim et al. 2017), and undescribed bacterium_Ellin5022_AY234439. In the case of R-2031-Z, it is quite distinct from the other species within the cluster. The closest described species are *Actinospica durhamensis* KJ445732, KC554451 (Golinska et al. 2015), and *A. robiniae* AJ865863 (Cavaletti et al. 2006) which create their own cluster.

5.2.2 *Nocardia*

Three strains were found in the genus of *Nocardia*. Here again, two strains, 54-Z and 3-Z, both from the Zakopaná site, created a separate cluster. *Nocardia sungurluensis* JN989289 (Camas et al. 2014) and *N. halotolerans* KM577163 (Moshtaghi Nikou et al. 2015) were genetically the closest ones. Then strain 31a-T, from Třeboň site, was quite distinct and also created a different branch of the cluster containing *N. crassostreae* AF430049, Z37989.1, *N. mexicana* GQ376178 (Moser et al. 2011), *N. miuyunensis* GQ376179 (Moser et al. 2011), AY639901 (Cui et al. 2005), *N. carnea* AF430035 (Roth et al. 2003), NBRC 14403 GQ376165 (Moser et al. 2011), NBRC 14403 BAFV0100, X80602.2, Z36929.1 (Chun & Goodfellow 1995), *N. testacea* JF797319 (Conville et al. 2010), GQ853070 (Moser et al. 2011), AB121769 (Kageyama et al. 2004), AB192415, BAGD0100, *N. sienata* JF797316 (Conville et al. 2010), AB121770 (Kageyama et al. 2014), GQ853066 (Moser et al. 2011), AB516654.

5.2.3 *Pseudonocardia*

Only one strain was obtained from *Pseudonocardia*, 205-Z from Zakopaná site. It obviously created a separate branch of the tree. The only two closest species were *Pseudonocardia acacia* EU921261 (Duangmal et al. 2009) and *Pseaudonocardia eucalypti* FJ805426 (Kaewkla & Franco 2011) from the same cluster.

5.2.4 *Rhodococcus*

Only one representative from Zakopaná site, 14B-Z, occurred in the genus *Rhodococcus*. Strain 14B-Z clustered with strains of *R. rhodnii* X80621.1 (Rainey et al. 1995), X81935.1 (Ruimy et al. 1995), and KF410352. Thus, it was still quite distinct.

5.2.5 *Streptacidiphilus*

Two representatives from the Zakopaná site and two from the Třeboň site occurred to be strains of *Streptacidiphilus*. They were evenly separated and not related to each other. Just samples from Zakopaná, 11-Z and 231-Z, seemed to be from the same cluster with *Streptacidiphilus sp.* Aac-5 AB180783, *S. albus* AF074415, and representatives of S. durhamensis, *S. albus*, and *S. sp*. Then 31-T strain from the Třeboň site was clustered with *S. specus* JX401485, *S. albus* MH241008, and *S. carbonis* MH241010. At last, the R-46-T strain, also from the Třeboň site, was distinct and clustered with *S. sp.* AM-06 DQ904536, AM-08

DQ904538, AM-10 DQ904545, AM-18 DQ904548, AM-24 DQ904550, and *S. anmyonensis* DQ904546, all described by Cho *et al.* (2006).

5.2.6 *Streptomyces*

Five isolated strains were from the *Streptomyces* genus. Two from the Zakopaná site and three from the Třeboň site. The both strains from Zakopaná, 1031-Z and 100-Z were clustering together with a strain from Třeboň, the 32-T. The most related was *S. sparsus* AJ849545 (Jiang et al. 2011). The last two representatives from Třeboň occurred distinct from each other. 35-T clustered with *S. siamensis* AB773848 (Sripreechasak et al. 2013) whilst 70-T with *S. kaempferi* HE591382 (Santhanam et al. 2013).

6 Discussion

The novel group of bacteria was found based on the sequencing of 16S rRNA but just one representative was isolated and described as *Trebonia kvetii.* This work was done to support this Trebon clade with possible findings of other new species within this phylogenetic cluster. The work aimed at obtaining novel strains of Actinobacteria because they are an important source of antibiotics but also a source of enzymes for organic matter decomposition, degradation of xenobiotics, and nutrient turnover (Ranjani et al., 2016).

6.1 Highlights

The strains closely related to *Trebonia kvetii* were not found as in the work of Kopecký *et al.* (2011). The reason might be wrong dilution rates, thus high concurrency avoided the isolation, or a colder incubation temperature should have been applied to avoid spoiling by other bacteria. Yet, several interesting new strains were isolated and seemed to make separated clusters. These strains were from 6 genera. Three from *Actinospica*, three from *Nocardia*, one from *Pseudonocardia*, one from Rhodococcus, four from *Streptacidiphilus*, and seven from *Streptomyces*.

6.2 Findings

More than half of the strains from Zakopaná grew on very acidic media of pH 3.5, whereas in the case of Třeboň only one representative grew under the same pH conditions. Based on the number of strains from different soil dilution rates, there is no significant result to make a conclusion about which dilution rate was the best for plating the samples. But that did not apply to the horizon type of origin. The vast majority was isolated from the Go horizon in Zakopaná and from the A horizon in Třeboň. That may be influenced by the season when the samples were collected because the first samples were collected in the winter and the second at the end of spring.

6.2.1 *Actinospica*

Strains found within this genus were hard to estimate their physiological properties or metabolic activities. The strains 53-Z and 57-Z together were related to yet not described strains that were just isolated, but no further work has been written, describing their properties. The distinct strain R-2031-Z which had the genetically closest strains *Actinospica durhamensis* KJ445732, KC554451 (Golinska et al. 2015), and *A. robiniae* AJ865863 (Cavaletti et al. 2006) which created their own cluster, seemed to be also separated. R-203-Z creates yellow endopigments in the aerial mycelium while the substrate mycelium is more brownish. This strain had the ability of spore formation.

6.2.2 *Nocardia*

The assumption of properties of the isolated strain is more reliable because of extensive previously done research on this genus. The strain 31-T clustered with many described *Nocardia* species which were found to be pathogens causing so-called nocardiosis. Some were found to cause infection of European Pacific oysters (*N. crassostreae*), *N. mexicana* causing pulmonary nocardiosis harming human lungs (Rodríguez-Nava et al. 2004) with *N. testacea* (Taj-Aldeen et al. 2013), *N. carnea* causing pacemaker pocket infection (Guedez López et al. 2022). From this information, we can suggest potentially pathogenic properties of this strain.

6.2.3 *Pseudonocardia*

Only one strain was isolated within this genus. The two related species *Pseudonocardia acacia* EU921261 (Duangmal et al. 2009) and *Pseaudonocardia eucalypti* FJ805426 (Kaewkla & Franco 2011) had no significant properties, thus there cannot be any suggestions on the 205-Z strain.

6.2.4 *Rhodococcus*

One strain isolated, 14B-Z, was related quite close to *R. rhodnii* X80621.1 (Rainey et al. 1995), *R. rhodnii* can biotransform cortisone steroid creating two novel steroids. Cortisone is a steroid used as an anti-inflammatory drug, that can suppress the immune system (Zappaterra et al. 2021).

6.2.5 *Streptacidiphilus*

Strains found in this genus were evenly distinct and did not form any cluster. Just 11-Z with 231-Z seemed to be close. Close species to these strains were not known for any significant properties or metabolites production.

6.2.6 *Streptomyces*

A total of five trains were isolated from *Streptomyces*. Visible clustering was for 1031-Z, 100-Z, and 32-T with closely related *S. sparsus* AJ849545 (Jiang et al. 2011). Then separately, 35-T clustered with *S. siamensis* AB773848 (Sripreechasak et al. 2013) whilst 70-T with *S.*

kaempferi HE591382 (Santhanam et al. 2013). None of the close species were found to be producers of any interesting metabolites or have been deeply observed.

6.3 Limitations

After a year of working on the samples in the laboratory, many improvements can be done in my possible future research. The lack of knowledge from the practical part was significant but that was also the reason for choosing this topic, to gain it. In the possible future work, the labeling and order in the samples collected would be crucial for having a more complete data set and to be able to easily find a sample in a large amount of Petri dishes.

6.4 Recommendations

Acidophilic bacteria, namely *Actinobacteria* are far from being completely described despite their importance in both, natural processes and practical applications. It is important to seek new antibiotics to fight the resistance to prescribed drugs, to describe new species and their metabolic processes to understand the ecosystem and all the processes. This work is focused on the phylum of *Actinobacteria*, of which the species are proving their ability to survive and live under extreme conditions. Extreme conditions mean a specific adaptation to it, so it is a great source of metabolites and so on. Strains found in this thesis might be further studied to assure the accuracy of the genetic classification and their possible metabolic properties. Processes for obtaining the DNA templates and PCR products were modified for a better yield in this thesis. These processes were repeated many times in many variations, but these written in the Methodology were those which worked well and secured the results. Therefore, they may be applied in further research on *Actinobacteria* collected from the acidic (forest) soils.

7 Conclusion

- Samples were collected from two soil horizons at two locations, Zakopaná and Třeboň.
- After growing on solid media genomic DNA extraction and PCR procedures were modified to enhance the yield of the PCR amplicon.
- Based on the 16S rRNA gene sequences, the isolated strains were classified into six genera, *Actinospica* (3 strains), *Nocardia* (3 strains), *Pseudonocardia* (1 strain), *Rhodococcus* (1 strain), *Streptacidiphilus* (4 strains), and *Streptomyces* (5 strains).
- The phylogeny of isolated strains was analyzed using phylogenetic trees of related strains from the database. Strain physiology was estimated based on the position of individual isolates within the phylogenetic tree.

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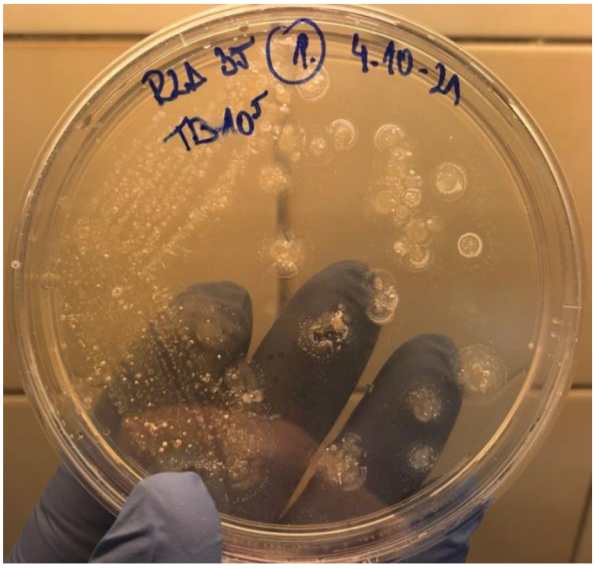
9 List of abbreviations and symbols

TC	Trebonia Clade
CX	Cyclohexamide
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
FAO	The Food and Agriculture Organization of the United Nations

10 Appendices



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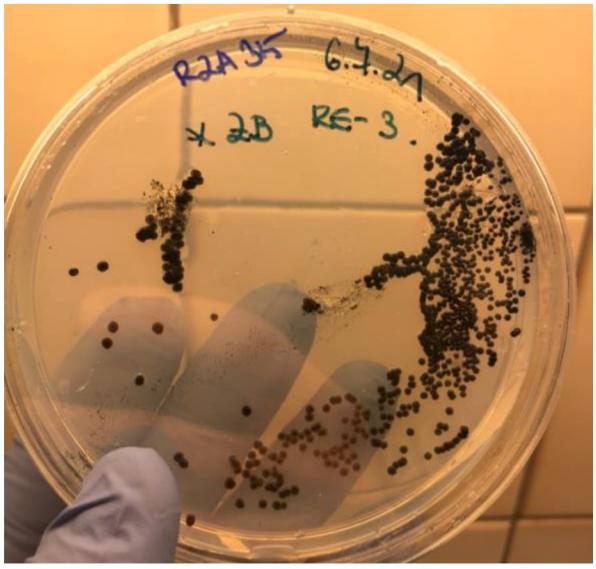

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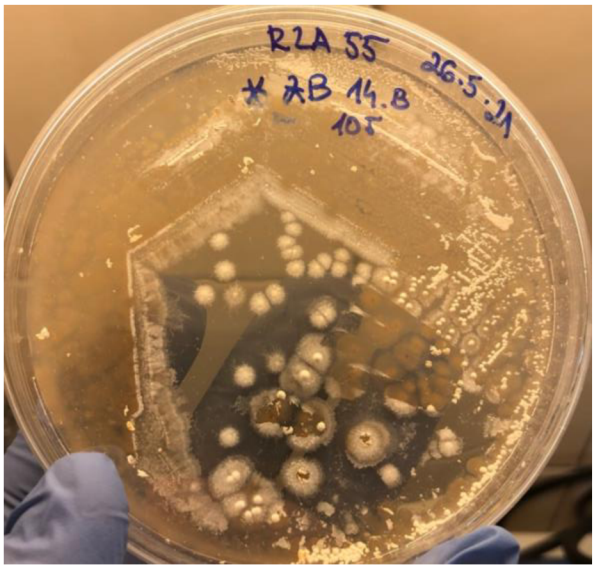

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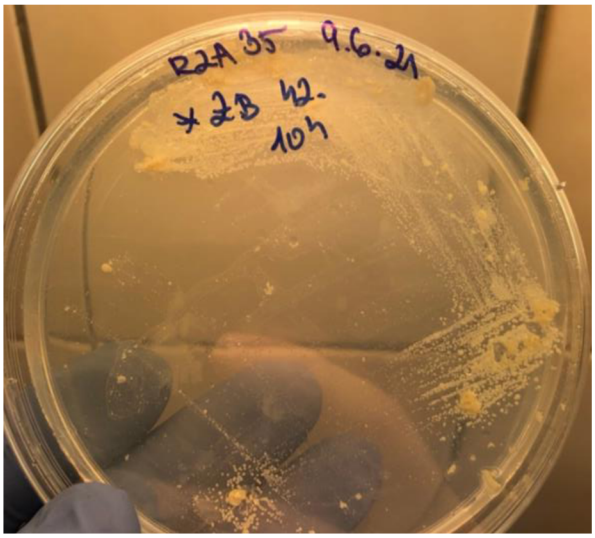
Strain 31-T

Strain 35-T



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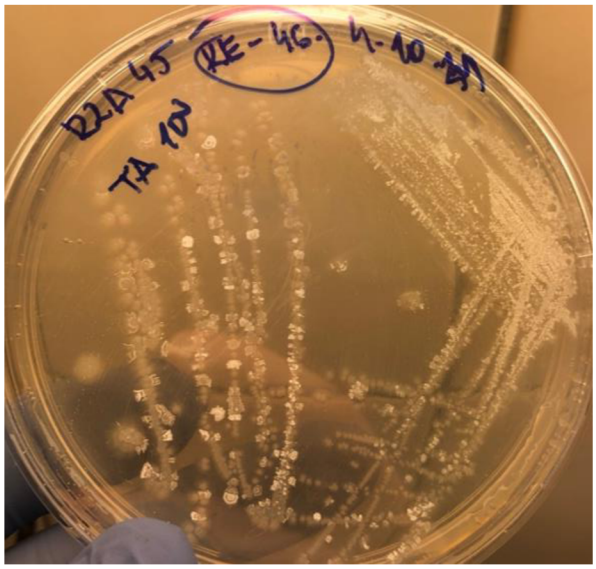

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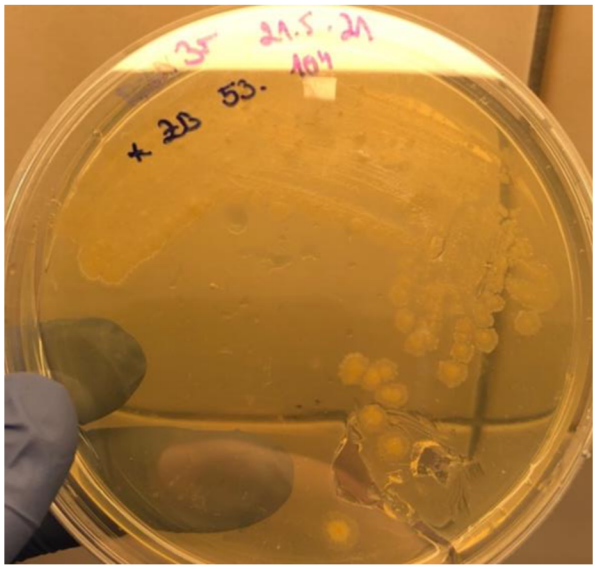

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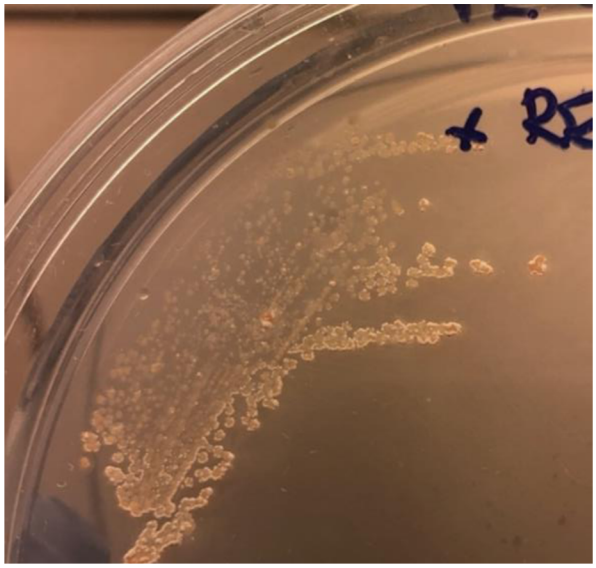

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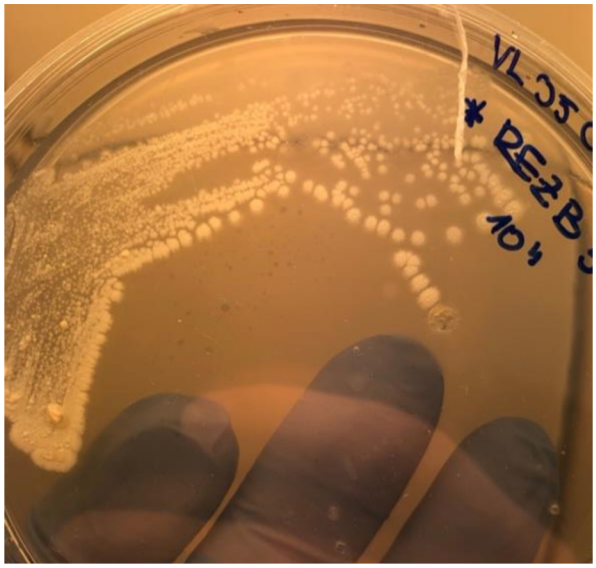
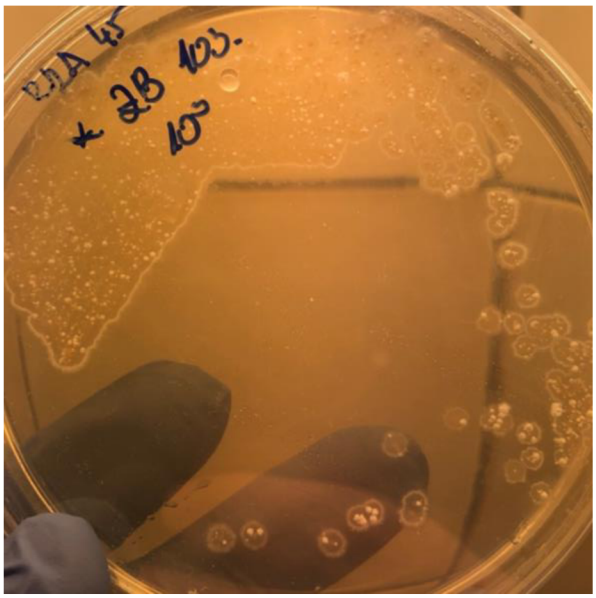


Strain 57-Z

Strain 70-T



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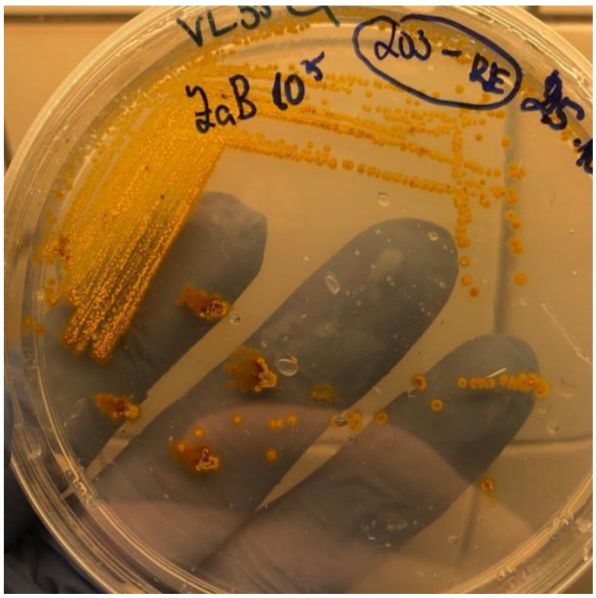
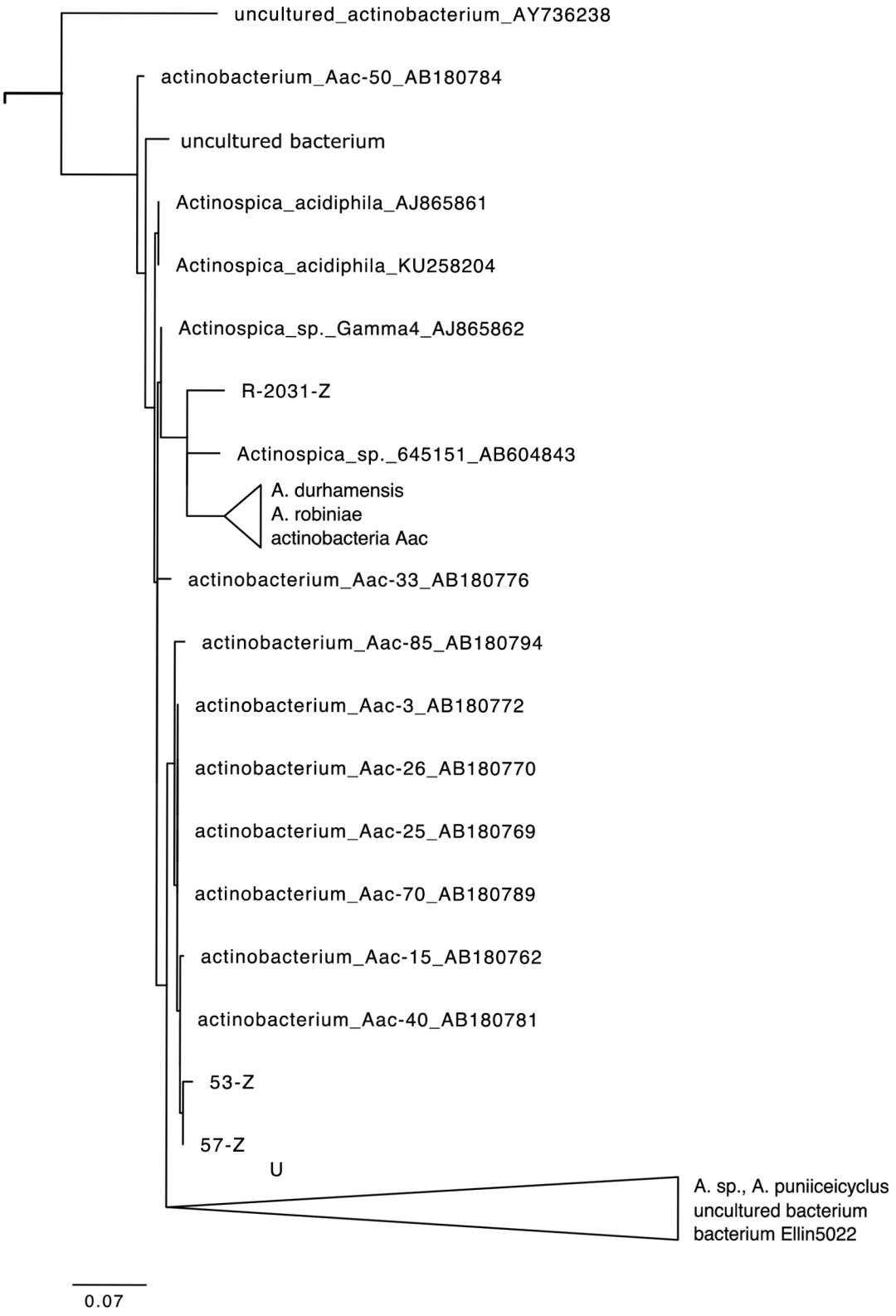
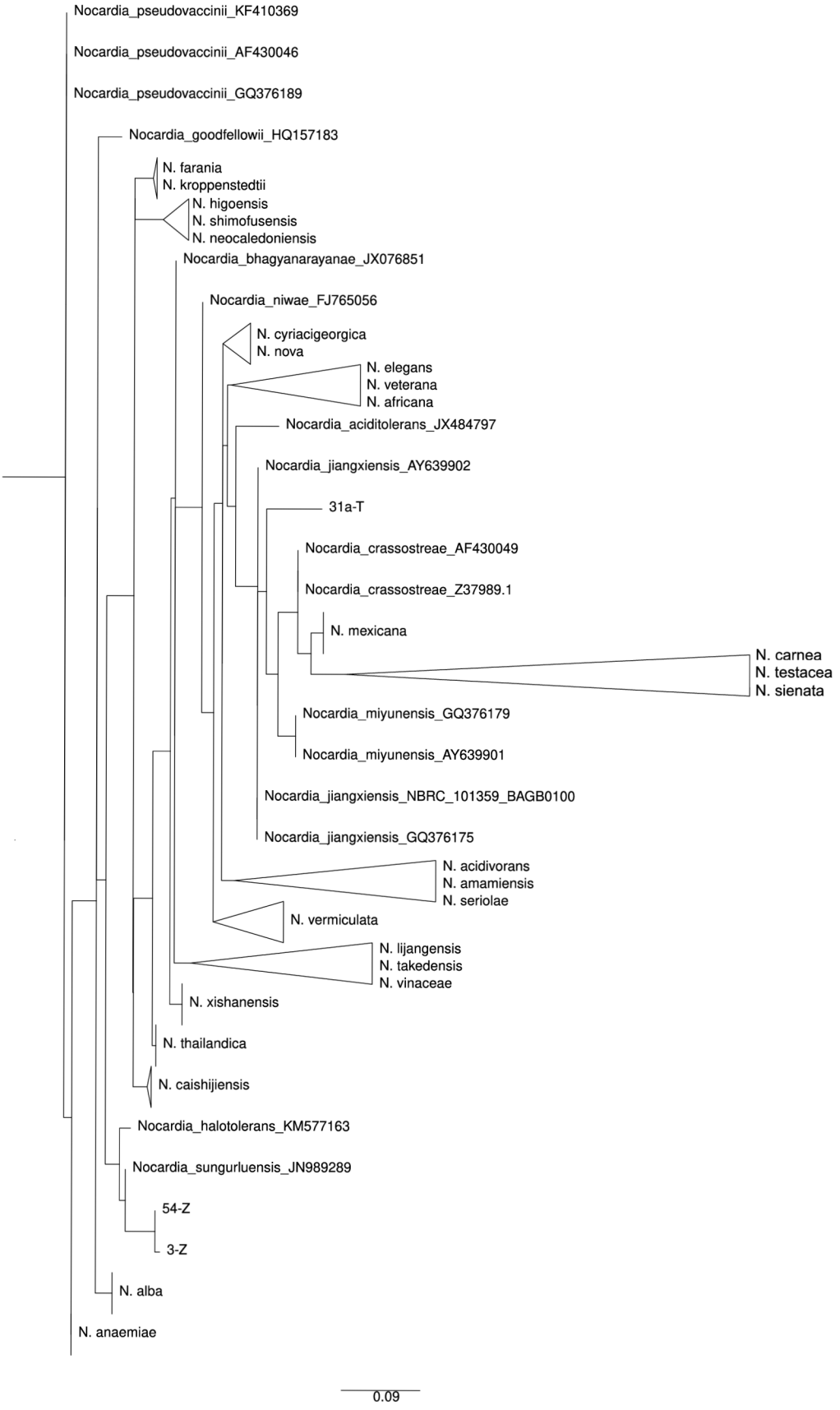


Strain 2031-Z

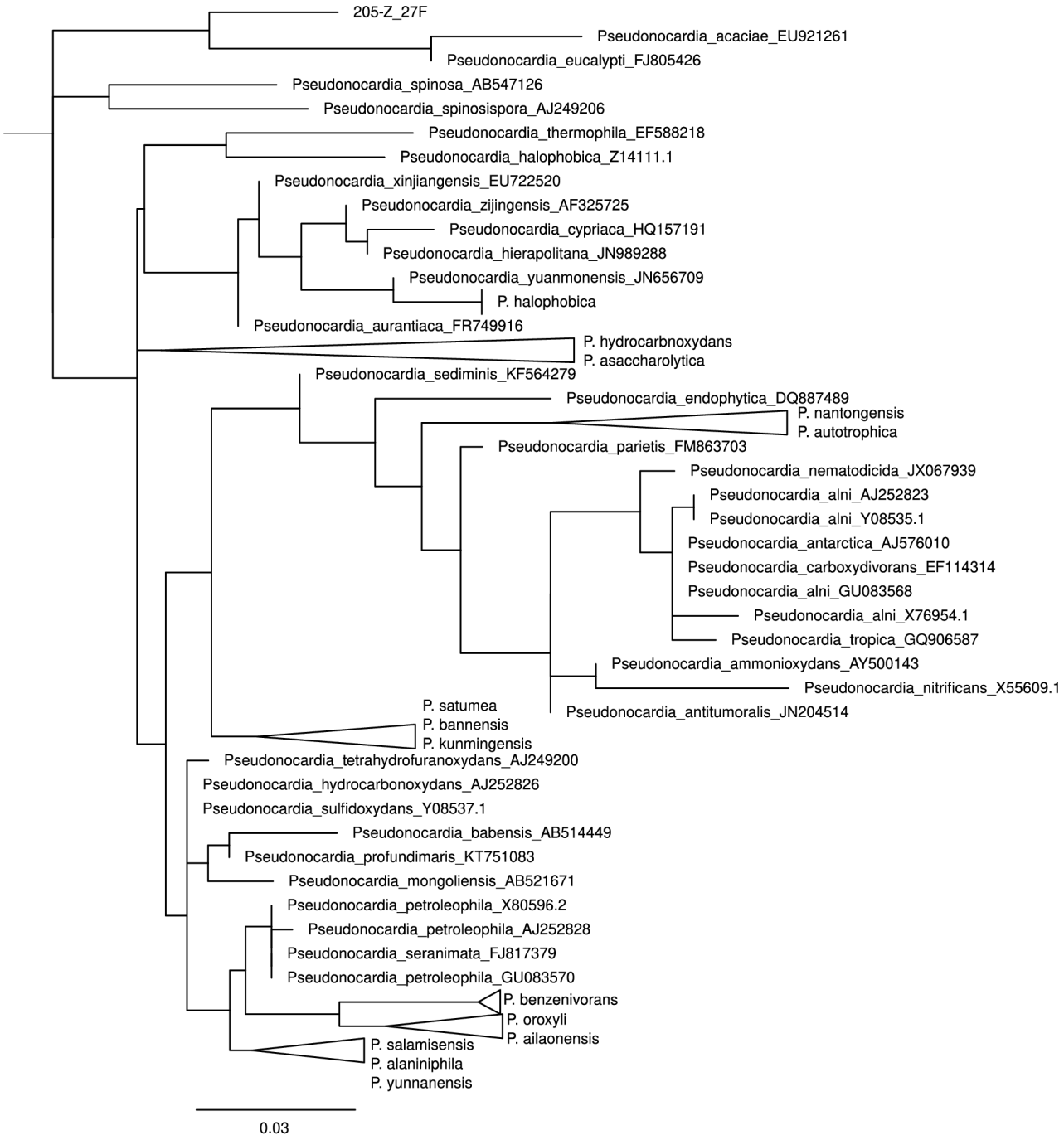
Appendix 1 - Observed strain



Appendix 2 - The phylogeny of the *Actinospica* genus was inferred by maximum-likelihood analysis of 3 sequences of the 16S rRNA gene from the isolated strains and 64 reference sequences out of 277, in total, of genus Actinospica from the Silva database. Branch lengths correspond to evolutionary distances (scale bar, 0.07). The phylogram was outgrouped using the *Catenulispora fulva* sequence FJ796419.

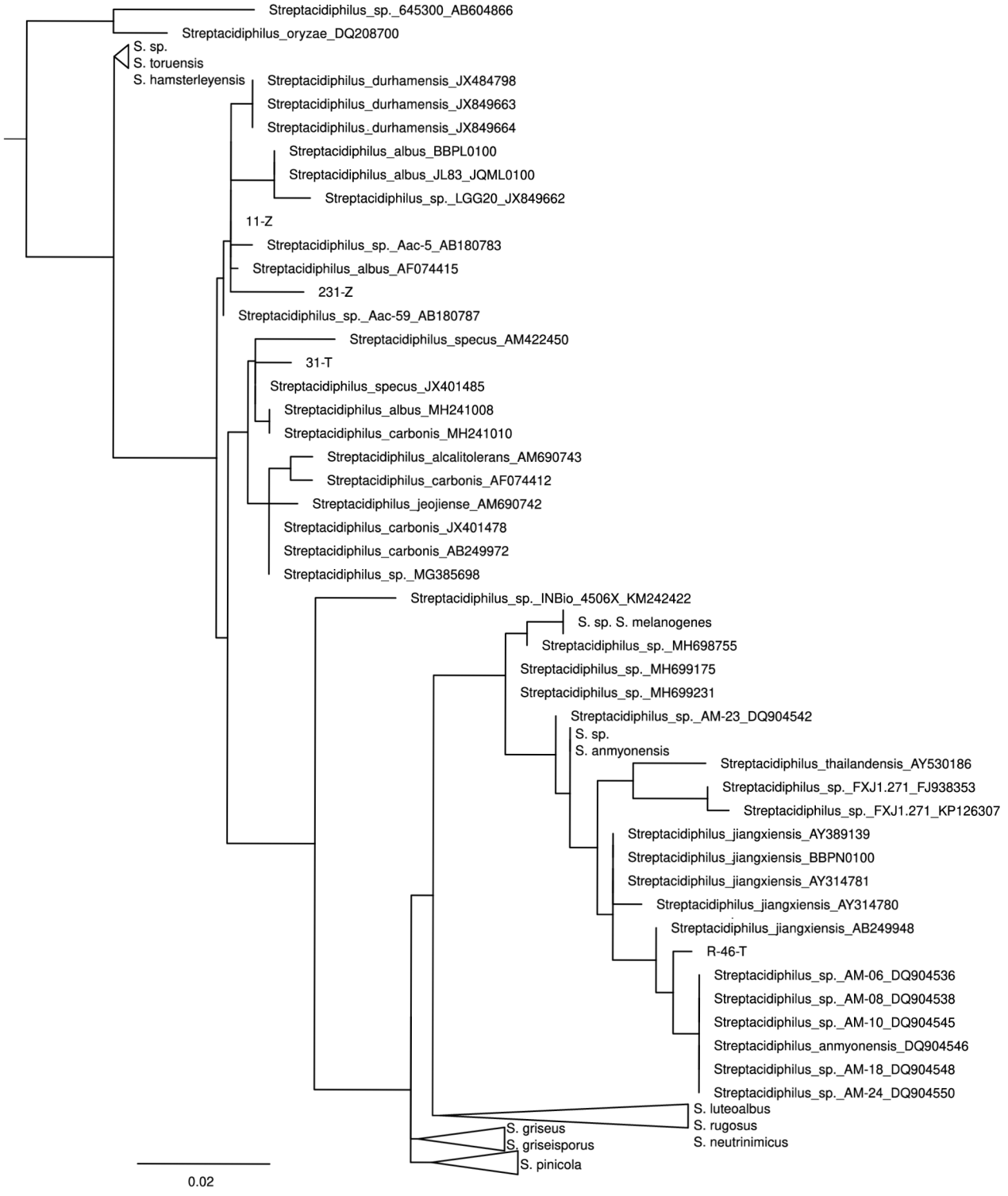


Appendix 3 - The phylogeny of the *Nocardia* genus was inferred by maximum-likelihood analysis of 3 sequences of the 16S rRNA gene from the isolated strains and 277 reference sequences out of 4158, in total, of the genus Nocardia from the Silva database. Branch lengths correspond to evolutionary distances (scale bar, 0.09). The phylogram was outgrouped using the *Gordonia effusa* sequence AB162799.1.

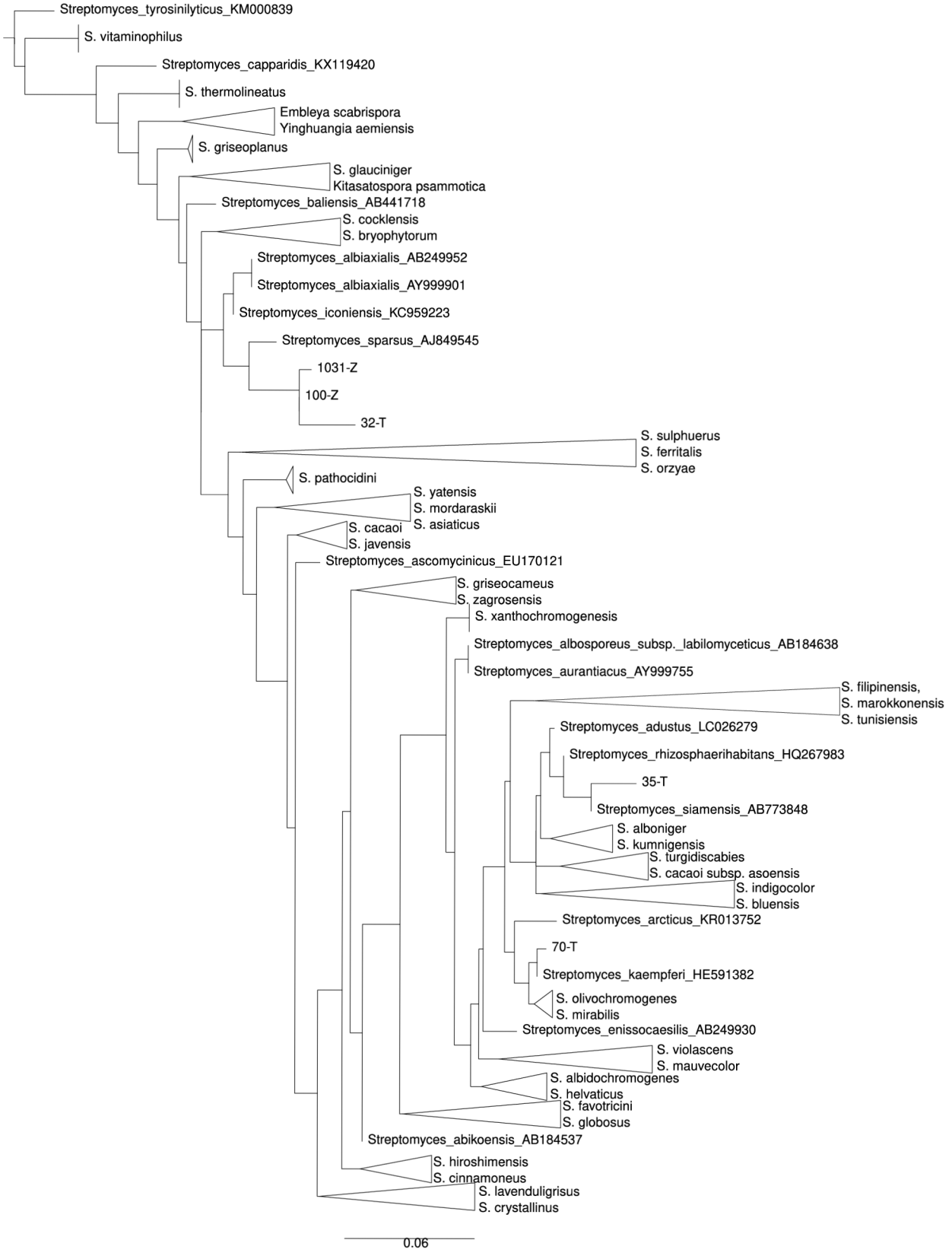


Appendix 4 - The phylogeny of the genus *Pseduonocardia* was inferred by maximum-likelihood analysis of 1 sequence of the 16S rRNA gene from the isolated strains and 73 reference sequences out of 4377, in total, of the genus *Pseudonocardia* from the Silva database. Branch lengths correspond to evolutionary distances (scale bar, 0.03). The phylogram was outgrouped using the *Actinopolyspora erythraea* sequence CP022752.

Appendix 5 - The phylogeny of the *Rhodococcus* genus was inferred by maximum-likelihood analysis of 1 sequence of the 16S rRNA gene from the isolated strains and 94 reference sequences of 10663, in total, of the genus *Rhodococcus* from the Silva database. Branch lengths correspond to evolutionary distances (scale bar, 0.07). The phylogram was outgrouped using the *Gordonia effusa* sequence AB162799.1.



Appendix 6 - The phylogeny of the *Streptacidiphilus* genus was inferred by maximum-likelihood analysis of 4 sequences of the 16S rRNA gene from the isolated strains and 113 reference sequences of 322, in total, of the *Streptacidiphilus* genus from the Silva database. Branch lengths correspond to evolutionary distances (scale bar, 0.02). The phylogram was outgrouped using the *Allostreptomyces indica* sequence GQ357647.1.



Appendix 7 - The phylogeny of the *Streptomyces* genus was inferred by maximum-likelihood analysis of 4 sequences of the 16S rRNA gene from the isolated strains and 1520, in total, of the *Streptomyces* genus reference sequences of 46594 from the Silva database. Branch lengths correspond to evolutionary distances (scale bar, 0.06). The phylogram was outgrouped using the *Allostreptomyces indica* sequence GQ357647.1.