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Bacterial interactions and secondary metabolites in plant and soil environment
Bakteriální interakce a sekundární metabolity v rostlinném a půdním prostředí

Doctoral thesis

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract

Phylum Actinobacteriota represents one of the major phyla in plant and soil environments. Known for the ability to produce numerous secondary metabolites, Actinobacteriota may affect other bacteria and plants in various ways. The thesis aimed at assessing the connection between the production of secondary metabolites and interactions of actinobacteria in plant and soil environments.

In the first part, we assessed how rare actinobacteria from acid soil affect cultivable soil bacteria. We isolated a collection of actinobacteria from acid soil dominated by previously uncultivable lineage and isolated a representative described as a new family *Treboniaceae* and multiple other potentially novel species of known genera. The genome and metabolome analysis of *Trebonia kvetii, sp.nov. gen.nov*., demonstrated its ability to produce unusual and potentially novel bioactive metabolites. We designed an experiment where a single strain was co-cultivated with the total soil bacterial community of the same soil in a set up, where a free exchange of diffusible metabolites was allowed between them. Three actinobacteria strains coming from distinct lineages were selected for this interaction. Each strain significantly and specifically affected cultivable bacteria as well as the metabolite pool, part of which was induced by co-cultivation.

In the second part of the thesis, we explored phytopathogenic and biocontrol actinobacteria connected to the common scab of potato. The pathogenicity islands (PAI) bearing virulence genes determining the ability to cause scab disease have been studied in relation to the geographical and phylogenetic origin of the phytopathogenic strains. Both factors influenced PAI type, although in some cases evidence for horizontal gene transfer of similar PAI types between phylogenetically distant species has been observed even across continents. We also observed a vast diversity of species of *Streptomyces* with the detected gene for the phytotoxin thaxtomin, the main determinant of common scab disease. Those strains were previously unknown to be phytopathogenic or even reported as plant beneficial. Finally, we developed and successfully tested an approach for selecting biocontrol actinobacteria based on the ability to inhibit the respective phytopathogen and grow rapidly *in vitro*. We demonstrated that the successful biocontrol strain possesses multiple genes for the production of siderophores and antibiotics and significantly modified soil bacterial community *in situ* towards the plant-beneficial state.

Abstrakt

Actinobacteriota představují jeden z nejvýznamnějších bakteriálních kmenů v rostlinném a půdním prostředí. Actinobacteriota jsou známá svou schopností produkovat sekundární metabolity, a proto mohou různými způsoby ovlivňovat jiné bakterie a rostliny. Táto práce byla zaměřena na posouzení souvislostí mezi produkcí sekundárních metabolitů a interakcemi aktinobakterií.

V první části jsme hodnotili, jak aktinobakterie z kyselé půdy ovlivňují kultivovatelné půdní bakterie. Získali jsme sbírku kmenů aktinobakterií z kyselé půdy, ve které dominuje dříve nekultivovatelná skupina. Izolovali jsme zástupce této skupiny a popsali jsme novou čeleď *Treboniaceae*, ale také několik dalších potenciálně nových druhů známých rodů. Analýza genomu a metabolomu nového druhu *Trebonia kvetii, sp.nov. gen.nov*., prokázala jeho schopnost produkovat neobvyklé a potenciálně nové bioaktivní metabolity. Navrhli jsme pokus, ve kterém byl vždy jeden aktinobakteriální kmen pěstován společně s celým půdním bakteriálním společenstvem tak, aby byla umožněna volná výměna metabolitů. Pro tuto interakci byly vybrány tři kmeny pocházející z různých linií. Každý kmen významně a specificky ovlivňoval kultivovatelné bakterie a také produkci metabolitů, z nichž byla také část indukovana společnou kultivací.

Ve druhé části práce jsme zkoumali fytopatogenní a biokontrolní aktinobakterie ovlivňující obecnou strupovitost bramboru. Ostrovy patogenity (PAI, Pathogenicity Islands) nesoucí virulentní geny, které určují schopnost způsobit strupovitost, byly studovány ve vztahu ke geografickému a fylogenetickému původu fytopatogenních kmenů. Oba faktory ovlivnily typ PAI. V některých případech byl pozorován horizontální přenos genů podobných typů PAI mezi fylogeneticky vzdálenými druhy i mezi kontinenty. Pozorovali jsme také obrovskou diverzitu druhů *Streptomyces* s detekovaným genem pro fytotoxin thaxtomin, který představuje hlavní determinantu strupovitosti. Dříve nebylo známo, že by tyto kmeny byly fytopatogenní, byly dokonce uváděny jako prospěšné pro rostliny. Nakonec jsme vyvinuli a úspěšně otestovali přístup pro selekci biokontrolních aktinobakterií na základě rychlého růstu *in vitro* a schopnosti inhibovat příslušný fytopatogenní kmen. Prokázali jsme, že úspěšný biokontrolní kmen má více genů pro produkci sideroforů a antibiotik, a významně modifikuje půdní bakteriální společenstvo *in situ* směrem ke stavu prospěšnému rostlině.

List of Abbreviations

PCR – polymerase chain reaction

DNA – deoxyribonucleic acid

RNA – ribonucleic acid

rRNA – ribosomal RNA

CDS – coding DNA sequence

OTU – operational taxonomic unit

SA – *Streptacidiphilus*

ST – *Streptomyces*

TR – *Trebonia*

CC – co-culture

PC – pure culture

SC – soil control

CS – common scab

PAI – Pathogenicity Island

Introduction

Actinobacteriota is one of the major phyla in many natural environments, including soil and rhizosphere (Barka et al., 2016). They are mostly known as producers of numerous bioactive secondary metabolites thus are expected to play an important role in interactions in soil and plant environments (Berdy, 2005). Indeed, many different types of interactions are performed by actinobacteria including symbiosis and pathogenesis to humans, animals and plants. The great phylogenetic variability of the phylum also represents a basis for rare actinobacteria from unusual environments. Consequently, the thesis aimed at *assessing relationships between secondary metabolites and interactions of actinobacteria in plant and soil environments*

The thesis is divided into two conceptual parts:

Part 1. *The role of rare actinobacteria in bacterial interactions in acid soil dominated by rare actinobacterial taxon.*

Actinobacteria from taxonomic lineages distinct from the genus *Streptomyces* are believed to be a promising source of novel metabolites, which is especially important due to the spread of antibiotic resistance diminishing the effect of commonly used antibiotics (Frieri et al., 2017). Unusual environments may not only become a source of novel bacteria, but a specific local gene pool shared horizontally may add to the diversity of secondary metabolites produced even by known species (Polz et al., 2013). That led to a search for unexplored sites in the Czech Republic, where actinobacteria were studied by both molecular and cultivation techniques. At a site near Opatovicky fishpond near Trebon, 16S rRNA gene libraries of actinobacteria were dominated by previously uncultured taxonomic group putatively belonging to a new suborder within the class Actinobacteria and named Trebon Clade (TC) (Kopecky et al., 2011). Eventually, TC was found dominating at several other acidic soil sites in the Czech Republic.

Actinobacteria belonging to TC represent a deep branching rare lineage and we hypothesized that these bacteria are specialized for acidic environments and possibly affect the local bacterial communities by unknown secondary metabolites. Therefore, the first task was to isolate and characterize a representative strain of the TC and possibly also other rare actinobacteria from acidic forest soil. The second task was to design and

conduct a co-cultivation experiment testing the effect of selected actinobacterial strains on the total soil bacterial community and metabolite pool.

Part 2. *Characterization of phytopathogenic and biocontrol actinobacteria from the tuberosphere of healthy and diseased potatoes coming from different geographical regions.*

Only a few actinobacteria were described to be phytopathogenic, one of which belonging to *Streptomyces* causes the common scab of potatoes (Barka et al., 2016). The main determinant for their pathogenicity is a secondary metabolite, phytotoxic dipeptide thaxtomin, with biosynthetic genes located on the Pathogenicity Island (PAI) together with other optional virulence genes (Li et al., 2019). As all of the known disease-causing *Streptomyces* species also have non-pathogenic members (Healy&Lambert, 1991; Dees et al., 2013), we hypothesized that the PAI type is not correlated to taxonomy, but to the site of origin of the pathogen, as the confirmation of horizontal gene transfer (HGT).

The first task was to isolate actinobacteria from the tuberosphere of potatoes coming from different geographical locations, while the second task was to analyze pathogenicity genes and by comparing their sequences in a phylogenetic tree to evaluate the hypothesis about locally specific variants and hosts. Finally, actinobacterial strains effectively suppressing phytopathogenic streptomycetes were also tested by *in vitro* and pot assay experiment.

Literature review

Microbial interactions – ecological view

In natural environments, organisms live within an ecological community, where populations of different species interact directly and indirectly to form the basis for many ecosystem processes (Lang&Benbow, 2013). Microbial interactions can be described based on partners involved as *microbe-microbe*, *plant-microbe*, *animal-microbe,* and so on. Moreover, ecological interactions can be defined as either *intra-specific* (within one species) or *inter-specific* (between different species), *facultative* or *obligatory*. Based on positive (+), negative (-), or neutral (0) outcomes for each interaction partner, all ecological interactions can be classified into 6 types (Table 1) (Faust&Raes, 2012). These basic types also encompass basic types of "symbiosis", when organisms live in a close spatial association, - parasitism, mutualism and commensalism (Leung&Pouling, 2008).

Table 1. Ecological types of pairwise interactions

Interactions Type	***Species A***	***Species B***
Mutualism/Cooperation	+	+
Commensalism	+	0
Neutralism	0	0
Amensalism	-	0
Parasitism/Predation	-	+
Competition	-	-

positive (+), negative (-) or neutral (0) outcomes of interaction

In *mutualism* and *cooperation* both partners benefit from the interaction. For example, syntrophy is a mutualistically beneficial cross-feeding where one species feeds on the metabolic by-products of another species, which otherwise inhibit the producer (Morris et al., 2013). In *commensal* interactions one partner benefits from another, which remains unaffected. For instance, propionic acid bacteria use lactic acid produced by lactic acid bacteria (Tshikantwa et al., 2018). Oppositely, in *amensalism,* an unaffected organism negatively impacts another organism. An example of *amensalism* is the inhibition of microorganisms by metabolites of non-affected bacteria, such as alcohols or

acids. *Predation* in the microbial world is usually associated with protozoa grazing on bacteria (Hilbi et al., 2007) or *Bdellovibrio* and like organisms (BALOs) preying on Gram-negative bacteria (Mookherje, Jurkevitch, 2021). Examples of *parasitism* include multiple bacterial and fungal phytopathogens in the plant-microbe system, such as phytopathogenic *Streptomyces scabies* causing common scab disease of potato (Loria et al., 2006).

Competition occurs directly through *interference competition* or indirectly through *resource exploitation* (Holomuzki et al., 2010). An example of interference competition is the production of antibiotics against competitors (Cornforth and Foster, 2013). Exploitation competition suggests competition for limited substrate, nitrogen, and iron (Lockwood, 1992). For example, the production of siderophores with a high affinity for iron by *Pseudomonas* is an example of exploitation competition that can be used for the biocontrol of fungal phytopathogens (Sass et al., 2019).

Apart from the described basic ecological interactions, there are particular cases of within-species interactions, where one interacting individual benefits and the other loses fitness. For example, intra-species predation, which occurs between higher organisms, is called *cannibalism*, while in the world of bacteria we may encounter the terms *altruism* and *cheating.* An example of *altruism* is the autolysis of mycelium of filamentous actinobacteria under adverse conditions to give nutrients to other cells for the formation of sporogenous aerial mycelium (Barka et al., 2016). *Cheating* occurs when part of the population cells loses the ability to produce but uses energetically-costly „common“ goods, such as siderophores or antibiotics (Mitri&Foster, 2013).

In the real environment, the questions about what type of interactions would dominate and how different species co-exist in the various situations are yet to be solved. According to the Inclusive fitness theory, cooperation should prevail within a single cell genotype, and competition - between cells of different genotypes provided limited resources (Gardner&Foster, 2008). This is valid for biofilms, where segregated clusters of clone-mates exhibit cooperative traits, while spatially mixed different species have antagonistic behavior (Nadell et al., 2016). The dominance of competitive interactions was also demonstrated between different species of cultivable aquatic bacteria where the productivity of pairwise bacterial mixtures was lower in most cases than the predictive sum of productivities of monocultures (Foster&Bell, 2012).

Yet, several studies indicated the importance and abundance of positive interactions in nature. For example, data based on metabolic models only, without

consideration of secondary metabolites, motility, and predation, predicted that bacteria typically form close cooperative loops resulting in indirect benefit to all species involved (Freilich et al., 2011). Cordero et al., 2012 showed that non-clonal genetically-different ecological populations of *Vibrio* also act cooperatively, where few genotypes produce broad-range antibiotics against other populations while all other genotypes within the population are resistant.

The dominance of neutral interactions was revealed in a pairwise study of actinobacteria on other microbes (Yan et al., 2021), while in a study specifically on *Streptomyces* strains, the pairwise comparisons revealed more inhibitory interactions (45%) than cooperative (19%) (Vetsigan et al., 2011). In addition, most interactions were reciprocal, especially if strains originated from the same soil grain (Vetsigan et al., 2011). That would suggest that closely related strains within the same genus would be more competitive than within actinobacteria in general, which would have more neutral interactions. That was also observed in another study, in which the inhibitory outcomes were predicted by a close phylogenetic distance of interacting partners, especially regarding overlapping BGCs. Further to that, around one-third of tested streptomycetes induced antibiotic production in the presence of another species in co-culture (Westhof et al. (2020).

The uniqueness and specificity of metabolic response were demonstrated for every given pairwise interaction between actinomycetes which gives space for the induction of cryptic biosynthetic gene clusters (BGCs). Notably, not only antibiotics but also siderophores and a plethora of unknown compounds were induced by the presence of a competitor (Traxler et al. (2013). Therefore, these findings demonstrated the weakness of using a pairwise approach for assessing interactions in complex natural communities and the importance of specific metabolome for higher-order interactions. Moreover, it was modeled that in order to be stable, firstly, a bacterial community should have a strong antibiotic production with intermediate levels of antibiotic degradation. Secondly, the greater number of antibiotics the higher number of species that can coexist in the community (Kelsic et al., 2015). Therefore, in a complex microbial community, we can expect specific metabolic interactions and dynamic oscillation between production-degradation of inhibitory compounds, where higher diversity of inhibitory compounds supports higher microbial diversity.

Quorum Sensing

Another commonly described type of interactions, firstly described as intra-specific cell-to-cell communication is represented by Quorum Sensing. QS is the mechanism, by which bacteria secrete, sense, and respond to small diffusible signal molecules in a density-dependent manner to control many important processes, such as virulence, biofilm formation, production of antibiotics, etc. (Geethanjali et al., 2018). Bacteria secrete so-called auto-inducer molecules (AI), which bind to a specific receptor protein and activate target genes after reaching a special threshold concentration during the growth. Firstly, QS was discovered in *Vibrio fischeri*, a bacterial symbiont of a squid, which triggered bioluminescence as anti-predator protection only at a high cell density (Nealson et al., 1970). Later, QS was discovered to influence different processes in many bacteria. It is believed that the QS phenomena are widespread among bacteria, although not systematically studied (Brotherton et al., 2018; Polkade et al., 2016).

More examples of QS regulation include (Zhao..., 2016; Polkade et al., 2016):

- Production of secondary metabolites including antibiotics and siderophores (Polkade et al., 2016)
- Synthesis of different virulence factors (Castillo-Juárez et al., 2015)
- Conjugative plasmid transfer (Fuqua and Winans, 1994)
- Biofilm formation (Rahbari et al., 2021)
- Exopolysaccharide biosynthesis (Beck von Bodman and Farrand, 1995)
- Bioluminescence (Nealson et al., 1970)
- Swarming motility (Eberl et al., 1996)
- Genetic competence (Ibanez de Aldecoa et al., 2017)
- Sporulation (Magnuson et al., 1994)
- Antibiotic resistance by the production of efflux pumps (Zhao et el., 2020)

These triggered processes would most probably succeed only at high cell densities. For example, if the synthesis of virulence factors or antibiotics started before pathogenic bacteria reached high cell density, host defense systems would sense the presence and eliminate intruders. Interestingly, genes for QS systems receptors are often, although not always, encountered adjacent to BGCs they regulate, like BGCs for antibiotic production (Brotherton et al., 2018).

Generally, two models of QS communication systems differing between Gram-positive and Gram-negative bacteria were distinguished (Romero et al., 2011; Polkade et al., 2016). In Gram-negative bacteria, QS signals, commonly represented by N-acyl homoserine lactones(AHL) (also called AI-1 (Gospodarek et al.,2009)) and synthesized by AHL synthases (*luxI*), at high threshold concentrations bind to the transcriptional regulator (*luxR*), which alters expression of target genes. Thus, AHL-based systems are one-component systems, where the sensor protein bears a regulatory function. In many Gram-positive bacteria, the QS signals are often small post-translationally modified peptides also called Auto Inducing Peptides (AIP) secreted through an ABC exporter system and sensed by a two-component signal-transduction system (Romero et al., 2011).

However, this binary division of QS systems is far from complete and is based on only first findings, like the *luxI/R* AHL-based system in *V. fischeri*. For example, the peptides signaling system was also reported to be encoded in genomes of many Gram-negative bacteria (Michiels et al., 2001). *V. harveyi* and *V. cholerae* utilize circuits of QS systems consisting of 3 and 2, respectively, different QS inter-dependent systems with two-component signal transduction regulators (Ng&Bassler, 2009). Moreover, both species reacting to furanosyl borate diester (autoinducer 2 or AI-2), which was proposed to serve as a universal signal as homologs of *LuxS* for the synthesis of AI-2 have been found in over 30 Gram-positive and Gram-negative bacteria (Tarighi & Taheri, 2011). Another example is *Pseudomonas aeruginosa* that has 4 interconnected QS systems with 2 AHLs, 1 unique Pseudomonas quinolone signal (PQS), and 1 unique so-called integrated QS signal (IQS, chemically 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde) (Lee&Zhang, 2015). The chemical diversity of some QS signals described earlier and further is presented in Figure 1.

Phytopathogenic *Ralstonia solanacearum* (Proteobacteria) also uses unique fatty acid like molecules R-methyl 3-hydroxymyristate (3-OH MAME) and Methyl 3-hydroxypalmitate (3-OH PAME) as QS signals that regulates the production of virulence factors and sensed by a two-component sensor system (Kai et al., 2015). Gram-Positive phylum Actinobacteria utilize γ-butyrolactone (GBL) systems (Polkade et al., 2016). Interestingly, GBLs are structurally similar to AHLs typical for Gram-negative bacteria, also sensed by one-component system and diffuse freely via membranes, while peptides do not (Michiels et al., 2001; Polkade et al., 2016).

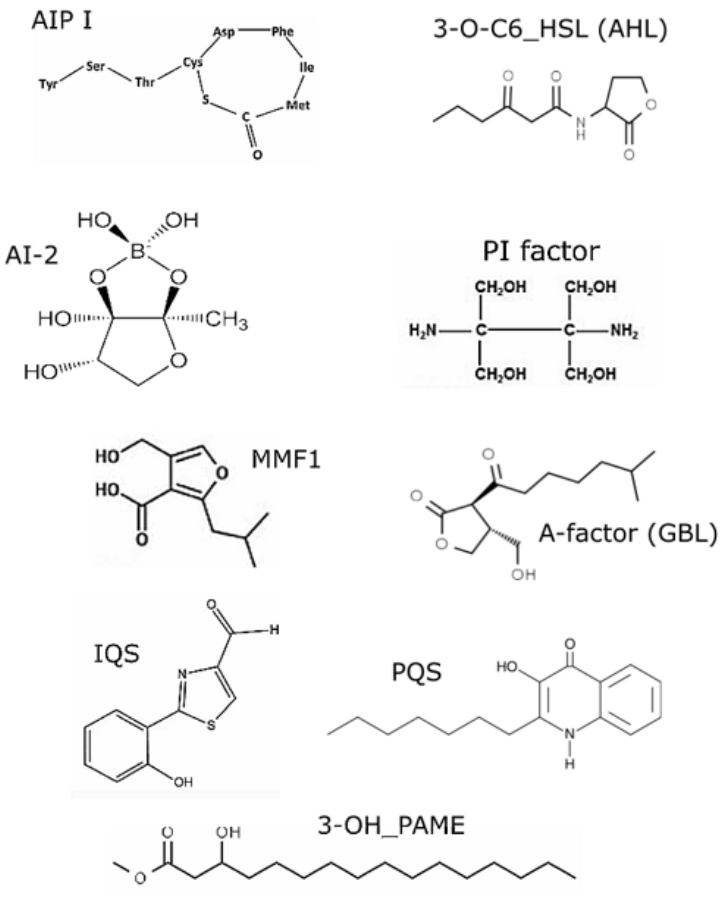


Figure 1. Chemical diversity of some known QS signals

The first described and the most known QS GBL is an autoregulatory factor (A-factor) of *Streptomyces griseu*s controlling the production of streptomycin and sporulation (Takano, 2006). In phytopathogenic *S. scabiei* GBL controls virulence and pathogenicity (Lin et al., 2018), similarly to AHL-based QS systems in many Gram-negative bacteria. Again, even within the actinobacterial genus *Streptomyces,* there is no common schema for QS systems. Some streptomycetes produce several different GBLs with different functions and even non-GBL QS signals, while others only a single GBL signal (Polkade et al., 2016). For instance, a unique non-lactone QS signal called PI Factor (2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol) regulates pimaricin biosynthesis in *S. natalensis* (Recio et al., 2004). QS in *S. coelicolor* is based not only on GBLs but also utilizes methylenomycin furans (MMFs) (Willey and Gaskell, 2011).

Although the QS signal system was first described as a cell-to-cell intra-species communication mechanism, there is more evidence that QS signals and receptors can serve for inter-species and even inter-kingdom signaling. For example, AI-2 synthesized by *luxS* is proposed to serve as a universal signal because it is synthesized by both Gram-

positive and Gram-negative bacteria (Zhao et al., 2016). *P. aeruginosa* does not synthesize AI-2, yet eavesdrops on AI-2 signal produced by nonpathogenic microflora in CF sputum and modulates its own gene expression (Duan et al., 2003). The QS signal *cis*-2-dodecenoic acid from the genus *Burkholderia serves not only* for regulation of its own virulence but also triggers morphological changes in the fungus *Candida albicans (Deng et al., 2010). The AHL from Sinorhizobium meliloti increases nodule numbers in plant Medicago truncatula (Veliz-Vallejos et al., 2014).*

Finally, Eukaryotes were reported to produce cyclic dipeptides (CDPs) mimicking *AHLs, thereby* affecting QS-regulated behavior in symbiotic prokaryotes (Zhao et al., 2016). Phytopathogenic streptomycetes also produce unique CDPs that act on plants as phytotoxins, inducing programmed cell death and tissue necrosis (Li et al., 2019). Some CDPs, like cyclo-phenyl-proline, were reported to act like auxin for plants (Ortiz-Castro et al., 2011), but at the same time as inter-species QS signal between *Vibrio* species inducing the expression of pathogenicity genes (Park et al. 2006) and as QS inhibitors (QSI) mimicking AHL (Holden et al., 2002). Therefore, CDPs may have multiple roles, all of which somehow are related to performing signaling functions at different levels including high inter-kingdom interactions.

*I*nter-species communication can occur via receptors for signals from other species without an appropriate synthase. For instance, *P. aeruginosa* has two different full (synthase and receptor) AHL-based systems (*lasI/R* with 3OC12-HSL signal, *RhlI/R* with C4-HSL signal) and so-called orphan or a solo *luxR*-type receptor (called *QscR*), which can react to multiple different AHLs and induce virulence (Chungani&Greenberg, 2014). *In silico* analysis of actinobacterial genomes were detected 991 protein sequences from 53 species that contained at least one *LuxR* domain (Santos et al.,2012), thereby possibly reacting to AHLs of Gram-negative bacteria. Interestingly, some actinobacterial species had a single sequence of *LuxR* homolog (e.g., *Mycobacterium leprae*), while others had over 50 (e.g., *Streptomyces* sp.). Again, an ecological function to sense other bacteria may be suggested, as streptomycetes are typical inhabitants of soil, heterogeneous in time and space environment, where multiple interference and exploitative competitors are present. Even QS signal receptor SCB1 for intrinsic GBL of S. *coelicolor* is able not only to sense its own GBL product, but also C6-HSL produced by *E.coli* and other Gram-negative bacteria (Yang et al., 2009).

Finally, some organisms are able to disrupt QS of competing species in the process termed Quorum Quenching (QQ) (Zhao et al., 2016). Firstly, QS signal can be destroyed

or modified to become inactive by enzymes, like acylases and lactonases (Polkade et al., 2016). For example, lactonase from *Agrobacterium tumifaciens* is able to inactivate lactone-based signals (such as AHLs and GBLs), and was demonstrated to eliminate pathogenicity of *S. scabiei* on potato by disrupting the synthesis of phytotoxin thaxtomin (Lin et al., 2018). *Variovarax paradoxus* synthesizes acylase to linearize AHL lactone rings to use for carbon and nitrogen source (Leadbetter&Greenberg, 2000). Interestingly, some bacteria with AHL degradation activity like *Bacillus* and *Ralstonia* have non-AHL QS signal systems, while others can degrade own autoinducers (Waters&Bassler, 2005).

Secondly, QS receptor can be blocked by another molecule mimicking QS signal, so-called QS inhibitors (QSIs). Thus, red algae *Delisea pulchra* produces brominated furanones to prevent colonization by bacteria by interfering with the AHL-based QS system (Hentzer et al., 2002). The cyanobacterium *Leptolyngbya crossbyana* produces hydroxy-GBL derivatives honaucins, which inhibits QS and subsequently bioluminiscence in *V. fischeri* (Choi et al., 2012). Cinnamic acid and linear dipeptides from marine streptomycetes attenuate QS resulting in inhibition of virulence of *P. aeruginosa* (Naik et al., 2013). Another streptomycete, *S. parvulus*, uses antibiotic actinomycin and possibly cyclodipeptide cyclic (4-OH-Pro-Phe) as QSIs against QS-controlled production of antibiotic violacein by *Chromobacterium violaceum* and pigment prodigiosin of *Serratia proteamaculans* without the growth inhibition of these bacteria (Miao et al., 2017).

Many other different secondary metabolites produced by bacteria and plants have been as well reported as QSIs, e.g. lumichrome, brominated alkaloids, kojic and tumanoic acids, manoalide (Zhao et al., 2016), cyclic dipeptides, cyclodepsipeptides, phenylethyl-butyramides, fatty acids and phenol derivatives (Zhao et al., 2019). It is estimated that 5-15% of cultivable rhizospheric bacteria possess lactonases (Polkade et al., 2016). A study of cultivable marine bacteria showed that more isolates had QQ activity with deeper depth (37% at 90m, while 47% at 2000m), where there is less organic carbon, more competition and AHLs may be used as a carbon source in oligotrophic environments (Muras et.al., 2018).

Overall, many actinobacterial genera (*Arthrobacter*, *Microbacterium*, *Micromonospora*, *Mycobacterium*, *Nocardioides, Rhodococcus* and *Streptomyces*) were reported to utilize QQ activity (Devaraj et al., 2017; Polkade et al., 2016). Crude extracts of 30% (8 out of 30) of actinobacterial isolates showed anti-QS and subsequent antibiofilm activity without any antibacterial activity (Mulya&Watyrangi, 2021).

Multiple examples of anti-biofilm activity of crude extracts from streptomycetes is reviewed in Azman et al., 2019. Thus, Actinobacteria may be a rich source of QQ activity, although not studied systematically regarding this aspect. Furthermore, unknown bioactivity and ecological functions may be expected from known compounds, e.g. signaling role of compounds firstly described as antibiotics. This is especially important for medicine and agriculture as preventing biofilm formation is a promising strategy for fighting multi-drug resistant pathogens (Frieri et al., 2017) and for suppressing bacterial plant diseases (Qi et al., 2022).

Actinobacteria as main producers of secondary metabolites

The phylum Actinobacteria belongs to the largest bacterial phyla found in many natural terrestrial and aquatic environments. Many Actinobacteria are Gram-positive with high G+C DNA content (Barka et al., 2016), which means more than 70% like in *Streptomyces* and *Frankia,* but there are also Actinobacteria with as low G+C DNA content as 51% in some corynebacteria(Ventura et al., 2007). The phylum *Actinobacteria* includes six classes: Acidimicrobiia , Actinomycetia, Coriobacteriia, Nitriliruptoria, Rubrobacteria and Thermoleophilia. The largest class Actinomycetia, formerly class Actinobacteria (Nouioui et al., 2018), according to the latest updates comprises 34 orders with 60 families, while the other five classes contain only 20 families (Salam et al., 2020; Table 2). The most commonly described actinobacterial genus is *Streptomyces* and many other so-called „rare actinobacteria“ are characterized by complex morphological differentiation and mycelial lifestyle (Goodfellow et al., 2012).

Apart from taxonomical and physiological diversity, Actinobacteria demonstrate a great variety of lifestyles and include human pathogens (e.g. *Mycobacterium spp., Nocardia spp., Corynebacterium spp., Tropheryma spp., Propionibacterium spp.*), free-living soil inhabitants (e.g. *Streptomyces spp.*), nitrogen-fixing plant symbionts (*Frankia spp.*) (Ventura et al., 2007), phytopathogens (e.g. *Streptomyces scabies,* etc.) (Loria et al., 2006) and obligatory symbionts of insects (Barka et al., 2016).

Table 2. Current taxonomical outline of the phylum Actinobacteria based on Salam et al., 2020

Class	***Order***	***Family***
Acidmicrobiia	*Acidimicrobiales*	*Acidimicrobiaceae*
	Iamiales	*Iamiaceae*
		Ilumatobacteraceae
Actinomycetia	*Actinomycetales*	*Actinomycetaceae*
		Arcanobacteriaceae
	Acidothermales	*Acidothermaceae*
	Actinocatenisporales	*Actinocatenisporaceae*
	Antricoccales	*Antricoccaceae*
	Aquipuribacterales	*Aquipuribacteraceae*
	Beutenbergiales	*Beutenbergiaceae*
	Bifidobacteriales	*Bifidobacteriaceae*
	Bogoriellales	*Bogoriellaceae*
	Brevibacteriales	*Brevibacteriaceae*
	Catenulisporales	*Catenulisporaceae*
		Actinospicaceae
	Cellulomonadales	*Cellulomonadaceae*
		Actinotaleaceae
		Jonesiaceae
		Oerskoviaceae
		Promicromonosporaceae
	Corynebacteriales	*Corynebacteriaceae*
		Dietziaceae
		Gordoniaceae
		Hoyosellaceae
		Lawsonellaceae
		Mycobacteriaceae
		Nocardiaceae
		Segniliparaceae
		Tomitellaceae
		Tsukamurellaceae
	Cryptosporangiales	*Cryptosporangiaceae*
	Demequinales	*Demequinaceae*
	Dermabacterales	*Dermabacteraceae*
	Dermatophilales	*Dermatophilaceae*
		Arsenicicoccaceae
		Dermacoccaceae
		Intrasporangiaceae
		Kytococcaceae
		Ornithinimicrobiaceae
	Frankiales	*Frankiaceae*
	Geodermatophilales	*Geodermatophilaceae*

	Glycomycetales	*Glycomycetaceae*
	Jatrophihabitantales	*Jatrophihabitantaceae*
	Jiangellales	*Jiangellaceae*
	Kineosporiales	*Kineosporiaceae*
	Microbacteriales	*Microbacteriaceae* *Tropherymataceae*
	Micrococcales	*Micrococcaceae*
	Micromonosporales	*Micromonosporaceae*
	Motilibacterales	*Motilibacteraceae*
	Nakamurellales	*Nakamurellaceae*
	Phytomonosporales	*Phytomonosporaceae*
	Propionibacteriales	*Propionibacteriaceae* *Actinopolymorphaceae* *Kribbellaceae* *Nocardioidaceae*
	Pseudonocardiales	*Pseudonocardiaceae*
	Ruaniales	*Ruaniaceae*
	Sporichthyales	*Sporichthyaceae*
	Streptomycineae	*Streptomycetaceae* *Allostreptomycetaceae*
	Streptosporangiales	*Streptosporangiaceae* *Nocardiopsaceae* *Thermomonosporaceae*
Coriobacteriia	*Coriobacteriales*	*Coriobacteriaceae* *Atopobiaceae*
	Eggerthellales	*Eggerthellaceae*
Nitriliruptoria	*Nitriliruptorales*	*Nitriliruptoraceae*
	Egibacterales	*Egibacteraceae*
	Egicoccales	*Egicoccaceae*
	Euzebyales	*Euzebyaceae*
Rubrobacteria	*Rubrobacterales*	*Rubrobacteraceae*
Thermoleophilia	*Thermoleophilales*	*Thermoleophilaceae*
	Gaiellales	*Gaiellaceae*
	Solirubrobacterales	*Solirubrobacteraceae* *Baekduiaceae* *Conexibacteraceae* *Parviterribacteraceae* *Patulibacteraceae*

Actinobacteria are the main decomposers of complex polymers in nature along with fungi as they have multiple extracellular enzymes such as chitinases, laccases, cellulases etc. (Buresova et al., 2019). Moreover, by chitin-degradation ability they can participate in antogonistic interactions with fungi as fungal cell wall contains chitin,

therefore acting as biocontrol agents against phytopathogenic fungi (Gomes et al., 2001). Many actinobacteria are generalists capable of using multiple nutrient sources, which enables them to adapt to diverse environments and growth conditions. As generalists they usually have complex morphology and large genomes, making them the most important producers of natural products (Bergeijk et al., 2020). Many actinobacteria with complex morphology, such as *Nocardia, Actinoplanes, Micromonospora, Streptovericillium, Streptomyces*, and *Saccharopolyspora*, have linear chromosomes (Redenbach et al., 2000). Strain-specific genes and BGCs for secondary metabolism incorporated via HGT are localized in unstable end regions of linear actinobacterial chromosomes or on mobile genomic islands relatively distant from the origin of replication in circular chromosomes, although they can migrate towards the chromosomal core region under the selective pressure (Cornell et al., 2018).

Above complex mycelial lifestyle, linear large genome organization and utilization of recalcitrant organic matter, Actinobacteria are mostly famous for their enormous capacity to produce various bioactive secondary metabolites important in medicine and agriculture. Apart from siderophores, volatiles and pigments, they produce a vast spectrum of antimicrobials, anticancer and antiviral compounds, immunosuppressants, anthelmintics, and herbicides (Bergeijk et al., 2020). Of more than 22 000 described microbial bioactive compounds, 38% (>8 000) comes from fungi, 45% (10 000) from actinobacteria, and 17% (around 4000) from other unicellular bacteria. Among actinobacteria, 75% of known bioactive SM are produced by *Streptomyces*, while 2500 bioactive compounds are produced by 50 rare actinobacterial genera (Berdy, 2005; Figure 2).

Two thirds of clinically used antibiotics are derived from actinobacteria (Barka et al.,2016). Furthermore, actinobacteria produce all major chemical classes of antibiotics: macrolides, aminoglycosides, polyenes, anthracyclines, polyethers, tetraciclines, cloramphenicol, nucleosides, beta-lactams, peptides, polyketides, macrolytic lactones (Anandan et al., 2016). First 7 classes are produced exclusively by actinobacteria, almost all glycopeptides – only by rare actinobacteria. In addition, rare bacteria produce the most complicated and versatile compounds (Berdy, 2005). Many actinobacterial genera have a high number of BGCs for SMs, a vast majority of which are not shared with other strains (Doroghazi&Metcalf, 2013). For this reason, rare and uncultivable actinobacteria, especially from unusual environments with extreme physical characteristics like pH, temperature, and pressure, are considered a promising source for novel secondary

metabolites (Berdy, 2005; Lam, 2006; Lewis, 2010). For instance, rare marine actinobacteria have high numbers of unique BGCs for SMs in their genomes (Schorn et al., 2016), and up to 75% of species-specific genes, especially for SMs, may be located in genomic islands (Penn et al., 2009). Therefore, the niche-specific gene pool shared by HGT can be especially enriched with novel SMs in such environments. As new antibiotics are in a special demand to counteract the constant spreading of resistant pathogens (Berdy, 2005; Frieri et al., 2017; Qi et al., 2022), isolation of uncultivable actinobacteria from unusual conditions and analysis of their genomic and physiological characteristics have not only theoretical but also great practical value.

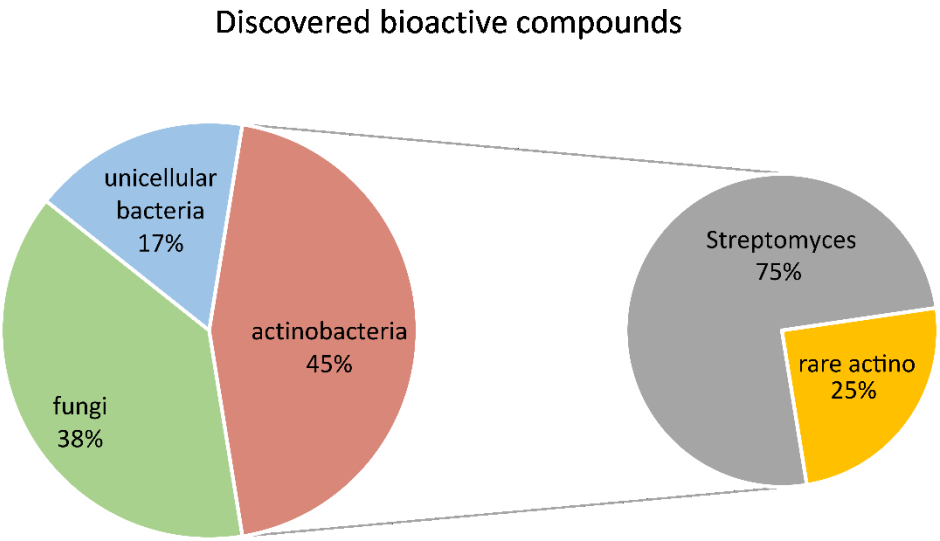


Figure 2. Distribution of bioactive metabolites based on data from Berdy, 2005

Trebon Clade, a novel actinobacterial lineage dominating in acidic environments

A novel actinobacterial taxon, preliminary defined as a new order, named Trebon Clade (TC) was described as dominating (85-96% of actinobacteria in the 16S rRNA clone library) in waterlogged acidic soil near the town Trebon in the Czech Republic (Kopecky et al., 2011). In other studies (Sagova-Mareckova et al., 2011) on the bacterial community of acidic soils in other locations also in the Czech Republic, pyrosequencing of eDNA sample from the humic horizon revealed a 10.6% proportion of actinobacteria with a 34% share of TC within it, while eRNA analysis indicated 22.2% share of actinobacteria with 62% of TC members. Furthermore, several sequences of the TC were previously found at different environmental sites characterized also by acidic conditions: pine forest soils (Sagova-Mareckova et al., 2011; Hui et al., 2012; our unpublished data),

acidic mine lake sediments (Lu et al., 2010), wetlands and paddy fields (Liu et al., 2014) and extremely acidic rivers (Garcia-Moyano et al., 2007).

Four related strains were previously isolated and classified as belonging to a new putative family 'Ellin5129 group' within the class Actinomycetia without detailed characterization (Joseph et al., 2003). One isolate of the TC was suggested to belong to a new order or family within the order *Streptosporangiales* (Sait et al., 2002), which at present contains three families, namely *Nocardiopsaceae* , *Streptosporangiaceae,* and *Thermomonosporaceae* (Goodfellow et al., 2015). Both studies were devoted to the cultivation of widespread and previously uncultured soil bacteria and used oligotrophic media with recalcitrant organic sources, long incubation time, and lower pH. Another member of the TC was isolated from acidic lignite mine lake sediment and described as *Thermomonosporaceae*-related without further description (Lu et al., 2010). Kopecky et al. (2011) proposed the TC placement at the level of a putative new order within the class Actinomycetia, sharing a common ancestral node with the order *Streptosporangiales* (Figure 3).

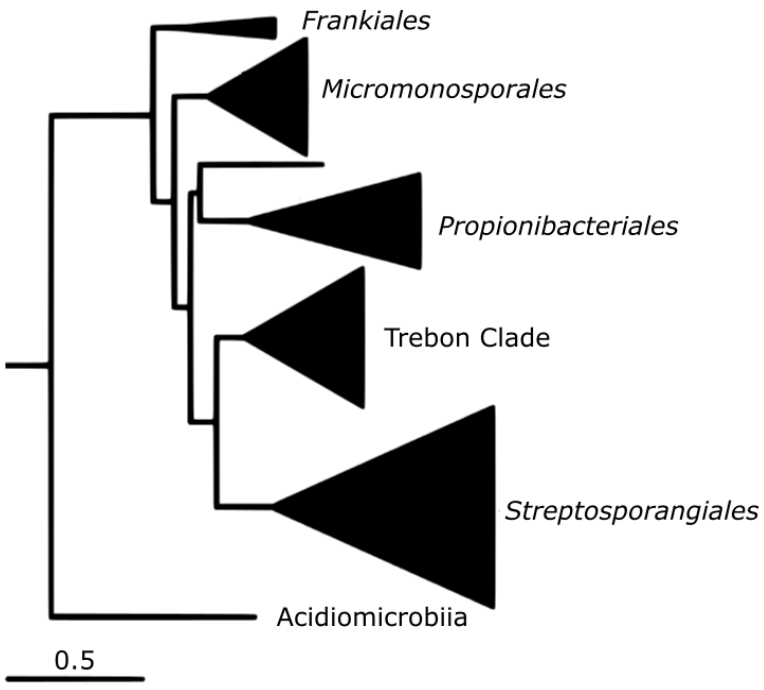


Figure 3. Phylogenetic position of Trebon Clade (85 clones) and neighboring actinobacteria (289 type strains) based on 16S rRNA sequences (adapted from Kopecky et al., 2011).

As the TC is phylogenetically distant from the known actinobacterial taxa and adapted to life in acidic and extremely acidic environments, its potential as a producer of novel secondary metabolites is enormous. As several not-characterized isolates were obtained in previous studies, it is possible to isolate the members of the TC group from environments, where TC dominates for detailed genetic and metabolic characterization. Moreover, being dominant or one of the dominant groups in the actinobacterial community in acidic soils, this clade probably plays an important role in soil-forming processes and interactions with co-inhabiting microorganisms via its specific pool of metabolites and enzymatic activities.

Actinobacterial interactions in plant environment

As producers of various bioactive compounds, especially antibiotics, actinobacteria are found in close association with eukaryotic hosts, especially plants and insects. The most famous example of actinobacterial-insect interactions is cultivating *Pseudonocardia* or *Streptomyces* by fungus-growing leaf-cutter ants to protect their fungal gardens against another fungus *Escovopsis* by the production of antibiotics targeting only that fungal pathogen (Kalthenpoth, 2009). A similar association is described between *Streptomyces* and fungus-growing bark-beetle (Scott et al., 2008). Beewolf wasps cultivate *Streptomyces* for the protection of larval cocoons against pathogens also by specific antibiotic production (Kroiss et al., 2010). These examples support an inhibitory role of antibiotics at sufficient for natural environment concentrations.

Most of the actinobacterial-plant interactions are beneficial, although there is a small number of actinobacterial phytopathogens important for agriculture. For the ability to aggressively colonize roots, solubilize phosphate and produce antimicrobials, plant hormones, siderophores, degradative chitinolytic and other enzymes, and QQ molecules, actinobacteria are considered to be PGP bacteria and effective biological control agents, especially of fungal phytopathogens (Bouizgarne&Aouamar, 2014; Barka et al., 2016).

There are multiple examples of root-colonizing *Streptomyces* and endophytic rare actinobacteria that produce antimicrobial and especially antifungal compounds *in situ* (Behie et al., 2017). Many inoculated actinobacteria were demonstrated to be effective plant protectors against fungal and bacterial pathogens (Hiltunen et al., 2017; Behie et al., 2017; Ling et al., 2020). Another plant-protection mechanism provided by root-colonizing streptomycetes is promoting nodulation by nitrogen-fixing plant symbiont

Rhizobium spp. (Tokala et al., 2002) and the ability to fix nitrogen by many non-symbiotic actinobacteria, including *Arthrobacter sp*., *Agromyces sp*., *Corynebacterium autitrophicum*, *Mycobacterium*, *Micromonospora sp*., *Propionibacteria,* and *Streptomyces sp.* (Sellstedt&Richau, 2013). Nitrogen-fixing symbioses between non-leguminous plants and the actinobacterial genus *Frankia* is another important example of beneficial plant-actinobacterial relationships (Sellstedt&Richau, 2013; Barka et al., 2016).

The importance of microbial interactions for plant health is especially seen when comparing disease suppressive and conductive soils against root infections by soil-borne pathogens (Exposito et al., 2017). Fungal and bacterial disease-suppressive soils, in some cases, have a higher abundance of several keystone taxa, including rare actinobacteria (Siegel-Hertz et al., 2018) and streptomycetes (Rosenzweig et al., 2012; Zheng et al., 2021). For instance, proteobacterial *Pseudomonas* and actinobacterial *Streptomyces* and *Gaiella* negatively correlate with phytopathogenic bacterium *Ralstonia solanacearum* and increase the complexity of bacterial networks associated with the resilient microbiome of healthy plants against bacterial wilt (Zheng et al., 2021). Higher bacterial diversity with complex bacterial networks with Actinobacteria as dominant keystone taxa were also observed for soil after manure application (Wang et al., 2022), which is an effective method to suppress many bacterial and fungal plant diseases (Larkin, 2013). As greater numbers of antibiotics *in situ* may result in a higher bacterial diversity (Kelsic et al., 2015), highly abundant or introduced actinobacteria may not only reduce phytopathogens directly by SMs and degradative enzymes production, but also by guiding microbial community toward a more diverse healthy state of the soil.

Phytopathogenic actinobacteria are not so common as saprophytic and beneficial for plants and limited to several non-spore forming genera *Clavibacter*, *Leifsonia*, *Rathayibacter*, *Curtobacterium*, *Rhodococcus*, *Arthrobacter*, and spore-forming *Streptomyces* (Bouizgarne&Aouamar, 2014). However, in particular areas, they represent major pathogens of certain crops affecting the quality and yield (Barka et al., 2016). Plant pathogenic *Streptomyces* and *Rhodococcus* have very wide host ranges, while *Clavibacter, Rathayibacter,* and *Leifsonia*, formerly belonging to the same genus *Clavibacter*, are host-specific (Hogenhout, Loria, 2008; Li et al., 2018). Different host-specific subspecies of *Clavibacter michiganensis* cause tomato canker, potato ring rot, wilt and blight in maize, leaf spots in wheat, stunting in alfalfa, while *L.xyli* causes ratoon stunting in sugarcane (Barka et al., 2016). A nematode-vectored *Rathayibacter toxicus*

causes a gumming disease of grasses and the production of a highly potent toxin fatal to livestock (Agarkova et al., 2007). *Curtobacterium flaccumfaciens* is the causal agent of bacterial wilt of beans (Guimaraes et al., 2001). Phytopathogenic *Rhodococcus fascians* causes leafy galls and plant deformations in over 100 genera of plants, including herbaceous and woody plants (Savory et al., 2017). Of over 500 species of *Streptomyces*, only 10 species are believed to be truly phytopathogenic: 9 species (*S. scabiei*, *S. acidiscabies*, *S. europaeiscabiei*, *S. luridiscabiei*, *S. niveiscabiei*, *S. puniciscabiei*, *S. reticuliscabiei*, *S. stelliscabiei*, *S. turgidiscabies*) causing common scab (CS) of potato and *S. ipomoeae* causing soft rot of sweet potatoes (Labeda, 2011). CS also affects other root crops such as radish, turnip, carrot and beet (Bouizgarne&Aouamar, 2014). The main virulence determinant is dipeptide phytotoxin thaxtomin A produced by CS-causing streptomycetes and thaxtomin C by *S. ipomoeae* (Huguet-Tapia et al., 2016). In studies of Bouchek-Mechiche et al., 2006, *S. reticuliscabiei,* genetically closest to *S. turgidiscabies*, was reported to lack thaxtomin A production and cause superficial netted scab of potato and severe necrosis of fibrous roots leading to significant yield losses. Other phytotoxins like FD-891 and borrelidin were reported to be associated with non-CS phytopathogenic streptomycetes (Bignell et al., 2014). There are also reports on other phytopathogenic species of streptomycetes plugging xylem vessels in maples (*S.parvus*, *S.sparsogenes*, *Streptomyces sp.*) and degrading lignocellulose in Douglas firs (*S.flavovirens*) (Barka et al., 2016).

There is strong evidence for horizontal gene transfer (HGT) involved in pathogenicity. To name a few examples, many genera usually have non-pathogenic i.e. saprophytic, plant-growth-promoting, and endophytic members, in some cases differing from phytopathogenic members only on the strain level (Barka et al., 2016; Savory et al., 2017; Nordstedt et al., 2021). Plasmid-mediated transfer of virulence factors was demonstrated for *Rhodococcus* (Savory et al., 2017), and some but not all strains of *Clavibacter michiganensis* subsp. *michiganensis* (Thapa et al., 2017). In addition, the large mobile pathogenicity island (PAI) bearing multiple genes for virulence in *Lefsonia* resulted from the integration of the conjugative plasmid into the chromosome (Monteiro-Vitorello et al., 2004). Moreover, phytopathogenic *Leifsonia* shares higher percent homology with virulence factors of phytopathogenic *Clavibacter* than with non-pathogenic *Leifsonia* members (Nordstedt et al., 2021). Genes for the main pathogenicity determinant phytotoxin thaxtomin are also located within large mobile PAI in scab-causing streptomycetes and in the region flanked by multiple putative transposase genes

and insertion sequences (IS) in sweet rot-causing *S. ipomoeae* (Guan et al., 2012). Furthermore, the TR2 subregion from PAI of some scab-causing *Streptomyces* also contains a conjugative element, which is capable of self-mobilization and transferring of the whole PAI to a non-pathogenic streptomycete (Zhang&Loria, 2017).

Curiously in particular cases, phytopathogens can share some factors with PGPR bacteria, which affect plants and may play a dual role. For example, phytopathogenic *Rhodococcus* has three of the *fas* genes, responsible for plant growth-promoting cytokinins (Savory et al., 2017) and biosynthesizes auxin indole-3-acetic acid, commonly produced by both phytopathogens and PGPR bacteria (Vandeputte et al., 2005). Moreover, the transfer of such dual PGPR genes from PGPR bacteria to phytopathogens can switch them to virulence factors, e.g. adhesion factor from PGPR *Pseudomonas* transferred to *Erwinia carotovora* increased natural virulence of this phytopathogen (Stritzler et al., 2018).

Scab-causing streptomycetes

Common scab (CS) is a worldwide economically important disease of potato (Li et al., 2019) and the most frequent bacterial disease in imported seed potatoes in Europe (Flores-Gonzalez et al., 2008). The symptoms are dark superficial, raised, or deep-pitted lesions on the tuber surface (Figure 4).

Figure 4. Potato tuber with common scab symptoms

CS pathogens have a wide host and tissue range and can infect turnip, raddish, beet and also monocot and dicot plants, resulting in root and shoot stunting and tissue necrosis (Loria et al., 2006).

The main determinants of CS-associated pathogenicity are phytotoxins thaxtomins, exclusively produced by phytopathogenic streptomycetes (Huguet-Tapia et al., 2016). Thaxtomins are cyclic dipeptides (CDPs) (2,5-diketopiperazines) formed from the condensation of 4-nitrotryptophan and phenylalanine. Thaxtomin A and B were first thaxtomins discovered in *S.scabies* broth in 1989, while thaxtomin C is produced by *S. ipomoeae* (King&Calhoun, 2009). Thaxtomin D is an intermediate product before thaxtomin A (Li et al., 2019). There are 11 thaxtomins known to differ only in the presence or absence of N-methyl and hydroxyl groups (King&Calhoun, 2009; some are presented in Figure 5). Yet, thaxtomin A is believed to be the major product produced by the CS-causing phytopathogens (Li et al., 2019).

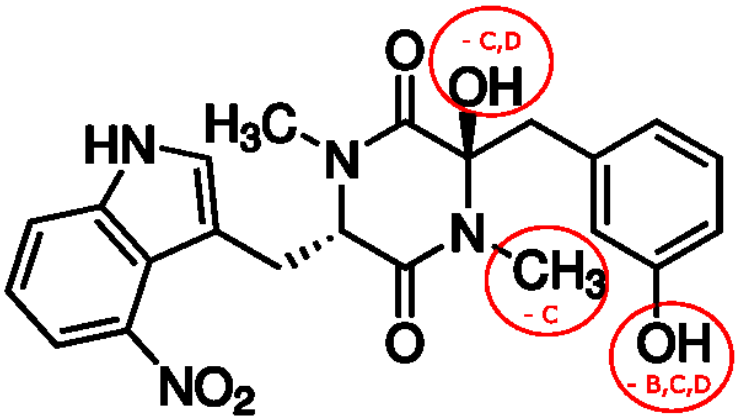


Figure 5. Structure of the major thaxtomin A. In red circles - radicals lacking in thaxtomins B,C,D

Other phytotoxins may act synergetically with thaxtomin such as polyketide macrolides concamycins, coronafacic acid-like metabolites (Bignell et al., 2014) and lipopeptidic rotihibins (Planckaert et al., 2021). Apart from phytoxic activity, concamycins were firstly described for the antiviral (Prchalova et al., 2014) and antifungal activities and were isolated from the mycelium of *S.diastatochromogenes* (Kinashi et al., 1984). Moreover, multiple siderophores, antimicrobials, and other SMs may assist the successful infection and pathogenicity. For instance, the genome of *S. scabiei* 87-22 encodes 46 BGCs for SMs with multiple polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), hybrid PKS-NRPSs, terpenes, ribosomally

synthetized and post-translationally modified peptides (RiPPs), siderophores, a betalactone and butyrolactone, melanin and ectoin (Deflandre et al., 2022). Many of these SMs may act antagonistically, e.g. many RiPPs are bacteriocins inhibiting related strains (Heilbronner et al., 2021), many products of PKS and NRPS are antibiotics (Walsh, 2004), and siderophores may mediate competition for iron in limiting conditions (Shiessl et al., 2017). Furthermore, the addition of cello-oligosaccharides induces the production not only of thaxtomin but also desferrioxamine, scabichelin and turgichelin siderophores, bottromycin and concanamycin antimicrobials and osmoprotector ectoin (Deflandre et al., 2022).

Biosynthesis of thaxtomin is reviewed in several papers (King&Calhoun, 2009; Li et al., 2019; Loria et al., 2008). Shortly, BGC of thaxtomin biosynthesis consists of 6 genes *txtABCDEH* and a regulator *txtR* gene (Fig.5). First, TxtD nitric oxide synthase (NOS) produces nitric oxide which is used by *Txt*E cytochrome P450 monooxygenase to produce nitrotryptophan. Then, two NRPSs, *Txt*A and *Txt*B, utilize nitrotryptophan and phenylalanine to produce CDP thaxtomin D, which is methylated by catalytic domains found in *Txt*A and *Txt*B and hydrolyzed by the cytochrome P450 monooxygenase *Txt*C to produce thaxtomin A. *S. ipomoeae, which produces thaxtomin C,* lacks *txtC* gene in BGC and appropriate hydroxy-radical in chemical structure (Figure 5). *txt*H encodes a protein important for the proper function of NRPS enzymes (Li et al., 2019).

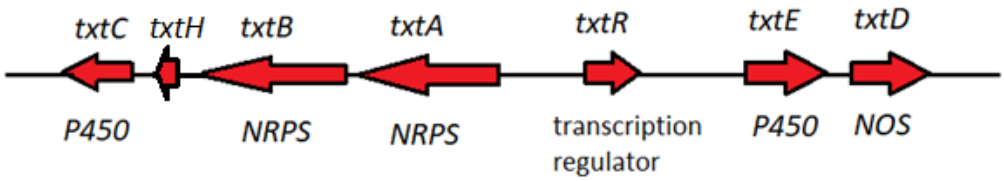


Figure 6. Schematic organization of the biosynthetic gene cluster for the thaxtomin production: *P450* – monooxygenases, *NOS* – nitric oxide synthase, *NRPS* – non-ribosomal peptide synthases

*Txt*R is a transcriptional regulator of thaxtomin production (Joshi et al., 2007), which is controlled at multiple levels by multiple regulators in this organism. Firstly, thaxtomin production is induced by cello-oligosaccharides, cellobiose and cellotriose (Loria et al., 2008; Deflandre et al., 2022). Cellobiose serves as a ligand of the *Txt*R regulator itself (Joshi et al., 2007). Moreover, both *txtR* and *txtB* genes expression is

controlled by the CebR repressor, which reduces its DNA-binding activity after binding cellobiose and cellotriose ligands. Interestingly, CebR is also a known repressor of cellulose and cello-oligosaccharide utilization in nonpathogenic streptomycetes (Francis et al., 2015). In addition, cello-oligosaccharides are transported to the cell by the ABC transport system, which has a higher affinity to cellotriose, a more effective inducer of thaxtomin production than cellobiose. Cellotriose, but not cellobiose can serve as a signal of actively growing plant tissues favorable asinfection sites, while other cello-oligosaccharides may come from dead plant material (Johnson et al., 2007).

Secondly, TxtR is regulated by several global regulators of the *bld* family (namely, *bld*A, *bld*C, *bld*D, *bld*G, and *bld*H), controlling morphological changes and secondary metabolism in streptomycetes (Barka et al., 2016; Bignell et al., 2014). The *bld*A gene encodes the only leucyl-tRNA required for the translation of a rare codon TTA found in *txt*R gene. Other *bld* family genes also affect *txt*R expression, some of which are *bld*A-dependent transcription regulators. For example, *S. scabies bld*H (also called *adpA*) gene also contains a rare TTA codon. Furthermore, there is a feedback loop between BldH and BldA regulators (Bignell et al., 2014). Interestingly, transcriptor regulator BldH is under the control of the GBL-based QS system (Horinouchi, 2002). Therefore, a complex network of different regulators governs thaxtomins production.

The BGCs for the thaxtomin production and other virulence factors are situated on a large mobile pathogenicity island (PAI), which can be horizontally transferred and integrated by site-specific recombination at short palindromic sites (Huguet-Tapia et al., 2014). There are two separate regions on PAI flanked by short palindromic attachment sites, toxigenic region (TR) with thaxtomin BGC (*txt* genes) and conserved 105 kb long colonization region (CR) with other virulence genes, such as *nec1* and *tomA* (Li et al., 2019) (Figure 6). The 666-bp *nec1* gene has low GC content (52%) in comparison with streptomycete genomic DNA (72%), therefore possibly transferred laterally from another organism. *Nec*1 gene coding for necrotizing secreted protein is saponinase homolog of fungal phytopathogens, which necrotizes and colonizes plants. Although saponins are plant glycosides, protecting plants against fungi by acting on eukaryotic membrane sterols, saponin hydrolysis may result in interference with plant defense resistance. Likewise, *tom*A encodes tomatinase acting on tomato saponin alpha-tomatine, and the degradation product suppresses the induced defense response in plants (Loria et al., 2006).

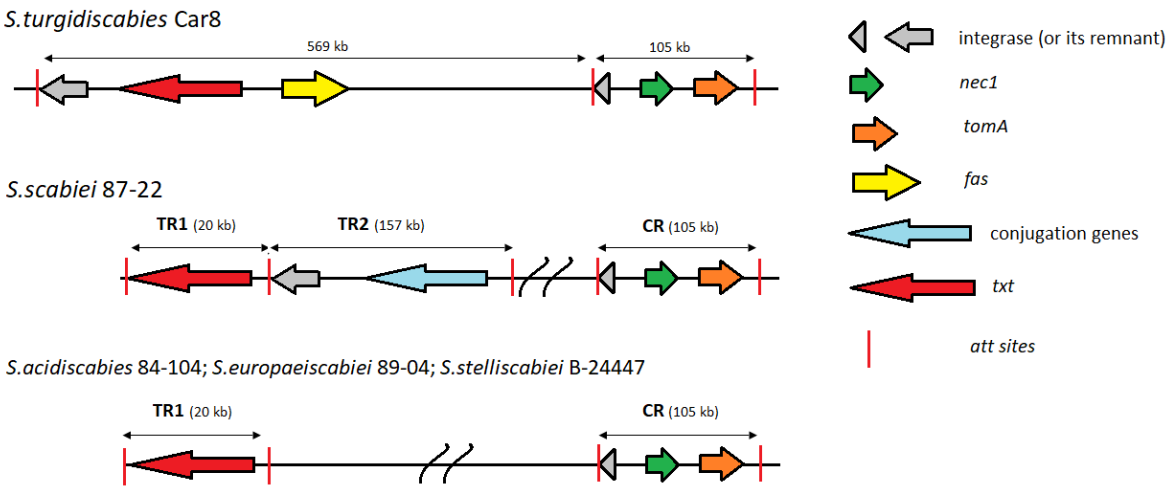


Figure 7. Organization of the pathogenicity islands (PAIs) of some scab-causing streptomycetes. Regions are flanked by the palindromic attachments sites (*att*). TR - toxigenic region; CR - colonization region. Only some virulence and mobility genes are shown.

TR of *S. scabiei* 87-22 PAI contains two regions, conserved 20 kb TR1 with *txt* genes and 157 kb TR2 with integrative and conjugative elements (Zhang et al., 2016) (Figure 6). While TR1 confers pathogenicity, TR2 is required for mobilization to non-pathogenic recipients. Interestingly, the frequency of HGT is increased in the presence of non-pathogenic streptomycete or antibiotic mitomycin (Chapleua et al., 2016). *S. acidiscabies* 84-104, *S. europaeiscabeiei* 89-04, 96-14, NCPPB4086, and *S. stelliscabiei* NRRL B-24447 lack TR2 (Zhang et al., 2016). TR of *S. turgidiscabies* Car8 additionally contains genes for integrase and fasciation (*fas*) operon encoding cytokinins genes homologous (67-84% aa similarity) to those in the linear plasmid of *Rhodococcus fascians* causing leafy gall on different plants(Kers et al., 2005; Loria et al., 2006; Zhang et al., 2016; Li et al., 2019). Hence, a general rule is that all scab-causing streptomycetes have conserved TR1 and CR, with *S. turgidiscabies* having different TR but with conserved 20kb bp long *txt* BGC (Li et al., 2019).

Yet, these schematic structures of PAI are not absolute and there are examples of *S. scabiei* lacking TR2 (Chapleua et al., 2016) or *S. turgidiscabies* having multiple copies of *tomA* cluster (Huguet-Tapia et al., 2016). Even in the same species of scab-causing *S. turgidiscabies* isolated from potatoes originated in Finland, the wide genetic variability of PAIs types regarding *nec*1, *tom*A, *fas* genes were observed (Aittamaa et al., 2010). Among all scab-causing streptomycete species, there were isolates missing some non-essential virulence genes. For instance, only 87% out of 1471 isolates had full CR with

nec1 and *tomA* genes (Wanner, 2009). A study of scab-causing streptomycetes from Norway showed that the *nec1*−*/tom*A+ PAI genotype predominated in *S. turgidiscabies* (63% of isolates), whereas *nec1*−*/tomA*− and *nec1*+*/tomA*+ (41%) were detected most frequently in the *S. europaeiscabiei* isolates (Dees et al., 2013). Dees et al. (2013) found no pattern in the geographical distribution of scab-causing species, which could be found together even in the same lesion. Yet, the specific PAI type and dominant species were typical for one field location, neighboring a field dominated by another PAI type or species, thereby having patchy geographical distribution, in another study (Wanner, 2009). Furthermore, some *S. turgidiscabies* strains causing netted scab of potato lacked genes for thaxtomin production utilizing instead another toxin, fridamycin E (Natsume et al., 2018). Therefore, these data support conclusions made for *S. turgidiscabies* that PAI consists of a mosaic of regions that may undergo independent evolution and can be transferred independently (Aittamaa et al., 2010).

Although many independent virulence factors (e.g. *nec1*, *tom*A, *fas* operon) other than *txt* are not essential for the pathogenicity, they may be present on PAI in different combinations and may contribute to virulence phenotype and infection process differently. Once transferred, these genes may be further diversified. Therefore, it is important to describe scab-causing actinobacteria from different geographical locations not only taxonomically, but with the respect to the PAI type, and probably the exact sequence differences in individual virulence genes.

Part I. The role of rare actinobacteria in bacterial interactions in acid soil dominated by rare actinobacterial taxon

Hypothesis: Actinobacteria from rare lineage dominating acidic soil affect the local bacterial community by specific metabolite pool

Goal: To assess the changes in the metabolite pool and cultivable bacterial community composition driven by interactions with three selected acidophilic actinobacteria strains

Tasks:

1. To isolate representative(s) of the Trebon Clade (**Publication II**) and other rare actinobacteria from acid soil
2. To choose 3 strains for a co-cultivation experiment and to establish a setup, which allows the assessment of interactions between the original soil bacterial community and the selected strains while allowing an exchange of diffusible metabolites
3. To assess changes in the cultivable part of the bacterial community in response to the presence of the interacting strain by *16S rRNA* gene amplicon sequencing
4. To assess changes in the diffusible metabolite pool and its inhibitory activities in response to the interaction of the three selected actinobacteria strains and soil bacterial community by LC/MS-MS of medium extracts

Materials and Methods

Isolation of acidophilic actinobacteria

Upper Ah and lower Go horizon of waterlogged acidic soil located in the south of the Czech Republic near the town of Trebon (48° 58′ 27.2″N 14° 46′ 11.5″ E) were used for bacterial isolation using a standard dilution plating on VL55 medium (Sait et al., 2002), humic acid-vitamin agar medium (HV) (Hayakawa&Nonomura, 1987), R2A medium (Reasoner&Geldreich, 1985) and the mineral agar Gauze 1 (G) (Gauze et al., 1984) supplemented with cycloheximide (300 mg/L) against fungi and incubated at 25°C up to 48 days. Both soil horizons were previously characterized by the dominance of the TC clones in actinobacterial clonal library both in winter and summer seasons (Kopecky et al., 2011). Soil DNA from both freshly-collected horizons was extracted by a modified phenol-chloroform extraction method (Sagova-Mareckova et al., 2008) to assess the total soil microbial community by sequencing of 16S rRNA amplicons (V4 region) before plating. For details about bioinformatics pipeline refer to the section 1.2.1. The diversity, richness, rarefaction curve was assessed using *R* package *vegan 2.5.7.* (Oksanen et al., 2013).

Cultivation conditions have been chosen based on the data of the only isolated strain of TC, derived during the study of previously uncultured bacteria isolated from pasture soil using VL55 medium with xylan (Sait et al., 2002), long incubation time (up to three months) at 25°C (Joseph et al., 2003). HV medium is used for the selective isolation of rare soil actinobacteria, including *Streptosporangium* and *Thermomonospora* belonging to the order *Streptosporangiales* (Hayakawa&Nonomura, 1987). R2A medium (Reasoner&Geldreich, 1985) was reported to be effective for isolating Actinobacteria (Liu et al., 2019). Mineral Gauze 1 is used for the selective isolation of *Streptomyces* (Gauze et al., 1984).

Further description of the site may be found in Kopecky et al., 2011 and our published paper (Rapoport et al., 2020).

Isolated strains were purified and cultured routinely at 28 °C in R2A medium (Reasoner&Geldreich, 1985) with pH adjusted to 5.5 (further referred to as R2A55). The potassium hydroxide test was performed for Gram differentiation (Suslow et al., 1982). Only Gram-positive isolates have been selected for further analysis (DNA extraction, amplification and sequencing of 16S rRNA region for taxonomy placement).

DNA extraction, 16S rRNA phylogeny, strains selection

Bacterial DNA was extracted using DNeasy PowerLyzer Microbial Kit (Qiagen) from liquid cultures according to the protocol. 16S rRNA gene was amplified with primers 16Seu27f and pH' (Cermak et al., 2008) and sequenced by Sanger method (Macrogen). The sequences were trimmed based on the default quality scores by Pregap4 of the Staden software package (http://staden.sourceforge.net/) with manual curation. Initial classification to the genus level was performed by the naïve Bayesian classifier provided by the Ribosomal Database Project (RDP) (Cole et al., 2009). The genera assigned with less than 97% confidence, were manually assigned based on the phylogenetic tree inferred from 16S rRNA sequences with all type strains of actinobacteria downloaded from the SILVA rRNA database project (Quast et al., 2013). This maximum-likelihood phylogenetic tree was built in FastTree 2.1.11 under the generalized time-reversible and discrete gamma models (Price et al., 2009) based on the alignment performed by accurate aligner SINA 1.2.11 according to the global SILVA alignment for rRNA genes (Pruesse et al., 2012). The phylograms were finalized using Figtree v.1.4.2 (http://tree.bio.ed.ac.uk/) and *R* package *ggtree 2.4.1*. (Yu, 2020).

DNA extraction, genome analysis and physiological characterization of TC isolate (15TR583T)

The isolate 15TR583T was cultured at 28 °C in liquid R2A55 medium for 2 weeks. The high quality genomic DNA of strain 15TR583T was extracted by phenol-chloroform protocol (Hopwood at al., 1985), modified by the addition of achromopeptidase (250 U ml−1) to the lysis buffer. DNA was sequenced by both short-read Illumina MiSeq and long-read Oxford Nanopore technologies and assembled de-novo by hybrid-assembly pipeline in SPAdes v3.11.0 (Bankevich et al., 2012). Annotation with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 4.7 was performed automatically during submission to the GenBank. The biosynthetic gene clusters (BGCs) for secondary metabolite production were identified with antiSMASH 5.1.2 (Blin et al., 2021). Genome-based phylogenetic trees were constructed in web-servers the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff& Göker, 2019) and KBase (Arkin et al., 2018). Additionally, reads were submitted to the comprehensive genome analysis service at PATRIC (the Pathosystems Resource Integration Center) web-server (Wattam et al., 2017). Annotation and subsequent subsystem analysis of

proteins performing specific biological processes was performed in RAST (Rapid Annotation using Subsytems Technology) web-server (Overbeek et al., 2014). Standard chemotaxonomic and physiological tests included pH, temperature, salt tolerance description, utilization of carbohydrate and nitrogen souces, enzymatic biotyping, cell wall and fatty acids characterization (Rapoport et al., 2020). Detailed description of chemotaxonomic methods can be found in Rapoport et al., 2020 (Publication II) (https://doi.org/10.1099/ijsem.0.004388).

Interactions: Experimental design

Strains isolated from the lower G0 horizon, *Streptomyces sp*. 15TR67b (ST), *Streptacidiphilus sp.* 15TR831 (SA), *Trebonia kvetii* 15TR583T (TR), were selected for the interaction experiment. SA and TR had separated phylogenetic positions from the known type strains, higher abundances within cultivable (SA) and uncultivable Actinobacteriota (TR), and both were isolated on VL55 medium after more than 1 month of incubation. ST was chosen as model typical streptomycete, isolated after 1 week of incubation on the typical medium for the selective isolation of streptomycetes (Gauze 1) and clustering in phylogenetic tree (16S rRNA) with known species *S.niveus.* ST also demonstrated antibacterial and antifungal activities on the plate during isolation.

Bacterial interactions were studied in an experiment where the three strains were co-cultivated with the total soil bacterial community on the same plate with a free exchange of soluble metabolites (Fig. 1-0).

Each 90-mm Petri dish was filled with VL55 medium supplied with cycloheximide (300 mg/ml) (Sait et al., 2002) and divided into three zones along the diameter i.e. two 40 mm-wide zones for inoculation and a central 10 mm-wide inter-zone with no growth for collection of metabolites. Petri dishes were inoculated with a pure culture of one of the strains (pure culture, PC), the strain on one side and soil suspension on the other side (co-culture, CC), or with just soil suspension on both sides of the Petri dish (soil control, SC).

The inoculation procedure started with the inoculation of a pure culture zone with one of the three strains. After 24 hours of incubation at 25 °C, the soil suspension zone was inoculated with 100 µl of soil suspension of freshly collected lower mineral horizon G0 of acidic waterlogged soil (pH 3.1-3.5) diluted to 103. The dilution was selected to receive individual separated bacteria colonies (around 100 colonies per zone). Plates were

sealed and incubated at 25 °C for 14, 21, and 28 days. Relatively long incubation time and lower temperature were chosen to allow slowly-growing bacteria to develop colonies.

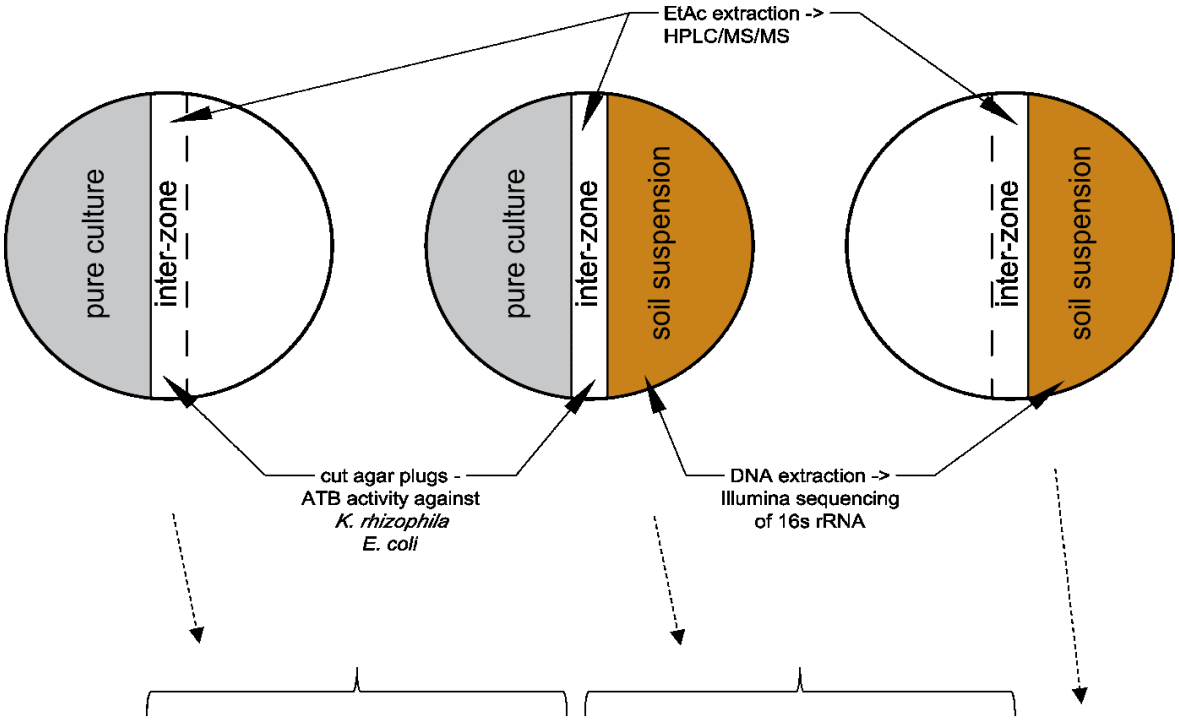


Plate Type		pure culture (PC)			co-culture (CC)			soil control (SC)
Strain		*Trebonia*	*Streptomyces*	*Streptacidiphilus*	*Trebonia*	*Streptomyces*	*Streptacidiphilus*	
Days	14	14TR	14ST	14SA	i14TR	i14ST	i14SA	14sc
	21	21TR	21ST	21SA	i21TR	i21ST	i21SA	21sc
	28	28TR	28ST	28SA	i28TR	i28ST	i28SA	28sc

Figure 1-0. Experimental design. Interactions of actinobacterial strains with cultivable soil bacteria on Petri dishes. Table presents abbreviations used for each treatment (n=6)

For each treatment (each time point, for each strain and for each plate type) (Fig.1-0), 6 biological replicates were collected for analysis. The soil suspension zone of agar plates was used for DNA extraction to assess how the individual strains influenced the structure and diversity of the cultivable bacterial community, thereby comparing co-culture (CC) and the soil control (SC) plates. DNA was also extracted from the soil sample used for initial inoculation in triplicate (soil sample, SS). Ethyl Acetate extracts of the inter-zones were used to assess the diffusible metabolites by LC-MS/MS to compare the metabolite production between the individual strains (PC), co-culture (CC) and the soil control (SC). Finally, agar plugs from the inter-zones were used in inhibition tests (see below) to assess the inhibitory activity of the diffusible metabolites.

DNA extraction and sequence processing of cultivable and total soil bacterial community

Soil DNA was extracted in 3 replicates by a modified phenol-chloroform extraction method (Sagova-Mareckova et al., 2008) to assess the total soil microbial community before plating.

DNA of cultivable soil community was extracted from the dried agar with grown bacterial colonies. At each time point, soil suspension zones of each agar plate were aseptically cut, transferred to a sterile Petri dish, dried at 65C for 24 h, and stored at -70C before DNA extraction. Dried agar was homogenized to powder with a bead-beater with 3 x 2.8mm metal beads, 3450 rpm, 3 min (MiniBeadBeater-16/607EUR, BioSpec Products, USA). Total DNA from the agar powder was extracted by the modified phenol-chloroform extraction method (Sagova-Mareckova et al., 2008) and evaluated on a 1% agarose electrophoresis and NanoPhotometer (IMPLEN 2207, Germany). DNA extraction from the dried homogenized agar has been chosen for giving a robust high yield with constant DNA fragment length after a previous comparison with extraction from raw agar, not-homogenized dried agar, and scraping the agar surface with an inoculation loop.

Bacterial 16S rRNA V4 region was amplified using universal primers with overhang adapters (CS1-515F 5'-ACACTGACGACATGGTTCTACAGTGCCAGCMGCCGCGGTAA and CS2-806R 5'-TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT) (Kozich et al., 2013). Construction of amplicon libraries and sequencing was performed on the MiSeq sequencer (Illumina, San Diego, USA) at the DNA Services Facility, Research Resources Center, University of Illinois (Chicago, USA). The resulting paired sequence reads were processed with the Mothur v. 1.39.5 software (Schloss et al., 2009) according to the MiSeq standard operation procedure (Kozich et al., 2013) with OTUs clustering (97%) by USEARCH v10.0.240 (Edgar, 2013). Reference alignment from the Silva database (Quast et al., 2013) was used for sequence alignment, the non-redundant subset of Silva Small Subunit rRNA Database, release 132 (Yilmaz et al., 2014) adapted for use in Mothur (https://mothur.org/w/images/3/32/Silva.nr_v132.tgz) was used as the reference database for the taxonomical assignment. Sequences of plastids, mitochondria, and those not classified in the domain Bacteria were discarded. Finally, the abundance table was normalized to the same (minimum) total reads per sample, to correct for varying

sample size. The resulted normalized OTU table was further used for statistical analysis and data visualization.

LC-MS/MS of soluble low-molecular-weight metabolites

Inter-zones were cut to small pieces by a sterilized scalpel, placed into 50ml-vials with 25 ml of ethyl acetate (EtAc), mixed, and left overnight at room temperature for extraction. The supernatant with soluble organic compounds was placed in a -20ºC freezer for subsequent analysis. Before LC-MS/MS measurement, supernatants were concentrated by evaporation and dissolved in 50% methanol to a concentration of 2 mg/ml. LC-MS/MS analyses were performed on the 6546 LC/Q-TOF system (Agilent, USA). One µL of the sample was loaded onto the Acquity UPLC CSH C18 LC column (50 mm × 2.1 mm I.D., particle size 1.7 µm, Waters) kept at 40 °C and eluted with a two-component mobile phase, A and B, consisting of 0.1% formic acid and acetonitrile, respectively. The analyses were performed under a linear gradient program (min/%B) 0/5, 1.5/5, 15/70, 18/100 followed by a 1-min column clean-up (100% B) and 2-min equilibration (5% B), at the flow rate of 0.4 ml min-1. The mass spectrometer settings were as follows. The capillary voltage at the AJS ESI source, 3500 V; nozzle voltage, 200 V; gas temperature, 250 °C; drying gas flow rate, 8 L min-1; nebulizer, 35 psi; sheath gas temperature, 400 °C; sheath gas flow, 12 L min-1; fragmentor, 140 V; skimmer, 65 V; The column effluent was monitored in the Auto MS/MS ESI positive ion mode in the m/z range from 50 to 1700. The maximal number of precursors for MS/MS experiments per cycle was 3; MS spectra and MS/MS spectra were collected at 5 and 6 Hz, respectively. The cycle time was 0.8 s. The collision energies for the collision-induced dissociation (CID) fragmentation were dependent on the ion m/z according to the following equation: E = (4*m/z)/(100+6) eV. EtAc extracts from the inter-zone of non-inoculated VL55 agar plates served as a control.

Resulted output data in vendor format were converted into an open-format (.mzML) using ProteoWizard v.3.0 (Kessner et al., 2008) and analyzed in the Global Natural Product Social Molecular Networking (GNPS) server (Wang et al., 2016) with classical molecular network tool with the default settings for initial spectra identification against GNPS Public Spectral Libraries. The resulted network was inspected with Cytoscape v.3.8.0 (Shannon et al., 2003), node table was exported for further processing in R (R Core Team, 2021). GNPS Network Annotation Propagation (NAP) tool (Silva et al., 2018) was used for in silico predictions of molecular structures through propagation

of structural annotations based on spectral similarity. The resulted molecular network was visualized with Cytoscape v.3.8.0 (Shannon et al., 2003) with ChemViz2 plug-in to draw molecular structures. Drawn structures were selected in the following priority: Reference Match (with MZerror<5 ppm) > top Fusion scoring match > top Consensus match > top MetFrag match. The node table was parsed in R to add the number of plates interacting with each strain, where a particular node was detected. The adjusted node table was used to draw pie charts of shares on each node in Cytoscape. Several subnetworks were selected for the illustration so that a minimum number of plates interacting with individual strain was 5. For the selected subnetworks chosen, the SMILEs were searched against ChemSpider (http://www.chemspider.com/ Pense&Williams, 2010) to derive IUPAC name and related information. If a top match was not in accordance with our samples, second best match was chose, and the reason is described in the results section.

For quantification and further processing with Feature-Based Molecular Networking (FBMN) tool in GNPS, spectra were processed in MZMine v.2.53 (Pluskal et al., 2010) according to the manual for FBMN (https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking-with-mzmine2/). Three compounds (cycloheximide, novobiocin, OH-C8-HSL), among compounds identified with classical molecular network tool, were selected as references for parameters adjustment for peak detection in MZMine. Antifungal cycloheximide was present in all samples, as it was added to the agar medium initially. Antibiotic novobiocin was present only in samples with the producing strain. Quorum-sensing (QS) molecule OH-C8-HSL was present in some samples. Peak widths, mzerror, and noise level were assessed based on the extracted ion chromatograms (XICs) of the reference compounds visualized using xcms v.3.13.8 (Smith et al., 2006), R package for metabolomics. Total ion current (TIC) was used to assess the overall quality of extraction and retention time (RT) tolerance for the peak alignment. MS peaks were detected with the noise threshold 10000 (MS1) and 500 (MS2). Initial chromatograms were built with m/z tolerance 0.05 and 20 ppm. Chromatograms were deconvoluted using Wavelets algorithm (S/N threshold 6, peak duration 0.05-2 min, RT wavelet ranges 0.02-0.5, Coefficient/Area threshold 10), aligned (RT tolerance 0.5 min, mz tolerance 0.005 m/z or 5 ppm), and gap-filled. The resulted feature quantification table and MS/MS spectral summary were analyzed by the FBMN tool in GNPS for features identification. Library hits were filtered by MZerror < 5 ppm and identified spectra were manually inspected by Mirror Match tool in GNPS server. Annotated quantification table from FBMN was imported into R

v.4.0.2. All features detected in agar control samples (plates with VL55 medium only) were deleted from all other samples. The resulted quantification table was subjected to statistical analysis and visualization in R and Mothur environments.

Inhibition tests

Antagonistic activities of soluble metabolites from all plates were tested using the agar diffusion test against type strains of G+ bacterium Kocuria rhizophila (CCM 552 =ATCC 9341) and G- E. coli (CCM 3988 =ATCC 10536). Cylindrical agar blocks were cut out from the inter-zone using a sterile plastic pipette tip (7mm in diameter). The blocks were placed on Petri dishes inoculated with test strain overnight cultures diluted to standard OD. The cultures were cultivated at the appropriate conditions (B1 medium: beef extract - 10 g, peptone – 10 g, NaCl – 5g, agar - 20 g; 37°C). The inhibition zone was measured after 18 hours of incubation.

Statistical analysis and data visualization

Statistical analyses and visualization were performed with R software using built-in functions and external libraries: ggplot2, ggpubr, rstatix, vegan, rcompanion, ggtree, ape, igraph, dplyr, xcms, phyloseq, vegan. Final figures were finalized in Inkscape (Inkscape Project. (2020). Inkscape. Retrieved from https://inkscape.org). For univariate variables, the normality of distributions was checked using Shapiro-Wilk Normality Test. As individual univariate response variables violated the normality test, non-parametric tests were used: Scheirer-Ray-Hare test (2-way non-parametric ANOVA analog), Kruskal-Wallis test (one-way ANOVA analog), and Dunn's test with Benjamini-Hochberg p-value adjustments for pairwise post hoc comparisons among treatments.

For assessing microbial and compounds diversity, dissimilarity distances were calculated based on the Bray-Curtis dissimilarity index (Bray&Curtis , 1957) from the OTU table and LC-MS/MS feature quantification table and subjected to Nonmetric multidimensional scaling (NMDS) in Mothur with subsequent visualization in R using ggplot2 (Wickham, 2016). The effect of Strains and Days of incubation were further analyzed by permutational multivariate analysis of variance (PERMANOVA) using R package vegan 2.5.7 (Oksanen et al., 2020) based on Bray-Curtis distance and 10000 permutations. The assumption of PERMANOVA were checked by the analysis of multivariate homogeneity (PERMDISP) (Anderson, 2006) to test if groups differed in their dispersion. Metastats (White et al., 2009) analyses were used to detect differentially

represented OTUs and LC-MS/MS features between soil control and interacting plates in Mothur with subsequent visualization in R using ggplot2.

For detecting and visualizing differentially abundant taxa detected on plates with strains and on soil control plates, LefSe analysis (Segata et al., 2011) was performed on Huttenhower lab Galaxy server (https://huttenhower.sph.harvard.edu/galaxy/) with the default settings (threshold on the logarithmic LDA score for discriminative features was set to 2, alpha values for the factorial Kruskal-Wallis test among classes and for the pairwise Wilcoxon test between subclasses was set to .05). Pairwise (soil control vs interacting plate) LefSe results were transformed to presence/absence table using splist2presabs() function from fuzzySim 3.0 R package (Barbosa, 2015) for further visualization in Excel.

The proportion of cultivable OTUs was calculated as the number of OTUs detected in current sample divided by all OTUs detected in soil.

UpSet plots of common and unique LC-MS/MS features were visualized using UpsetR 1.4.0 (Larsson, 2020) in R.

Mantel test for the similarity between Bray-Curtis distance matrices calculated from OTU table and Feature quantification table was performed by R package ape 5.4.1 (Paradis et al., 2004) using 10 000 permutations. Heatmap of known compounds was produced using pheatmap 1.0.12 (Kolde, 2019).). Library hits for the same compounds were merged after inspection for visualization purposes (e.g.nodes detected as Novobiocin_40eV CCMSLIB00005723234 and Novobiocin_50eV CCMSLIB00005723235 was named as Novobiocin). Compounds detected in each sample and reported as contamination from plastic were deleted from the heatmap.

Rank-based Spearman correlation between OTU abundances and LC-MS/MS feature quantities were calculated using R package Hmisc 4.4.2 (Harrell, 2020). Strong correlations (>0.6) were visualized and the assortativity coefficient was calculated using igraph 1.2.6 (Csárdi, Nepusz, 2006).

Graphical outputs were finalized in Inkscape 0.92 (2020) if needed.

Results

Acidophilic actinobacteria from gleysol

The number of 16S rRNA gene sequences were 19 201 in organic horizon Ah and 37 395 in mineral horizon G0. Observed genera-based richness were 553 and 476 genera in Ah and G0 respectively, while rarefied richness were 553 and 410 genera respectively (Fig.1-1). Shannon diversity index was 4.93 for Ah horizon and 4.28 for G0 horizon. Pielou's evenness was 0.79 for Ah and 0.69 for G0 horizons.

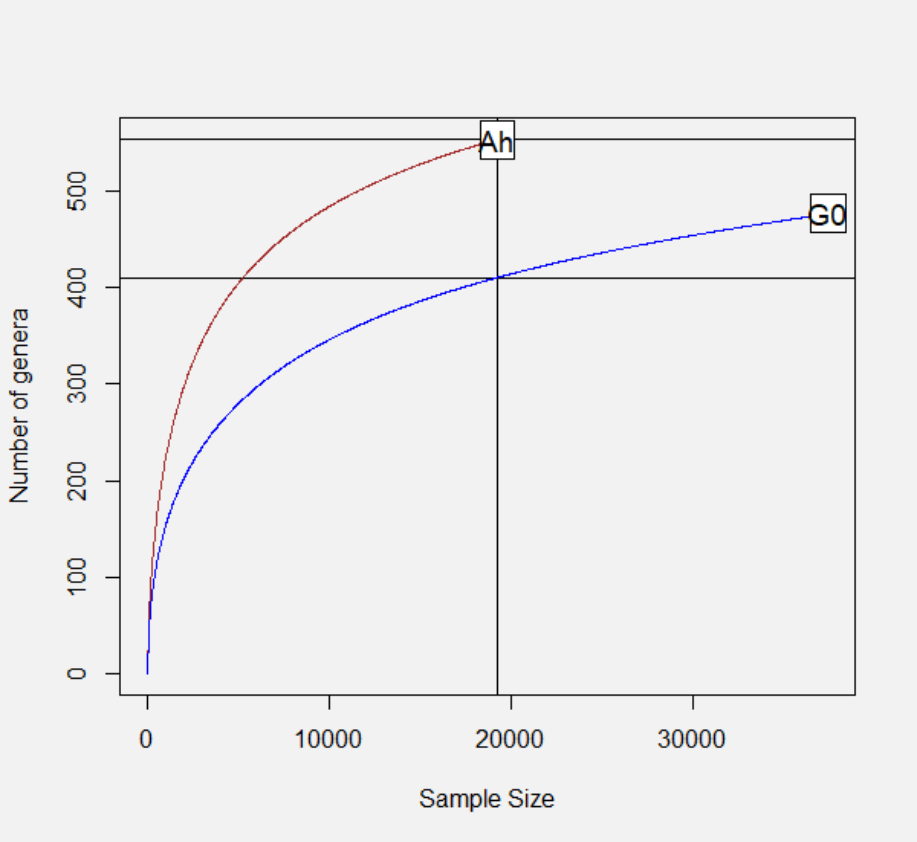


Figure 1-1.Rarefaction curve of genera detected in 16S rRNA amplicon library in upper Ah and lower G0 horizons of acid waterlogged soil.

The most abundant soil phyla were Proteobacteria (36% and 31% of relative abundance of bacteria in Ah and G0 horizons respectively). Phylum Actinobacteria was the second most abundant in organic horizon Ah (21% of relative abundance bacterial 16S rRNA library) and the third in mineral horizon G0 (15%). Phylum Acidobacteria was the third most abundant in Ah (12%) and the second in G0 (16%) (Fig.1-2).

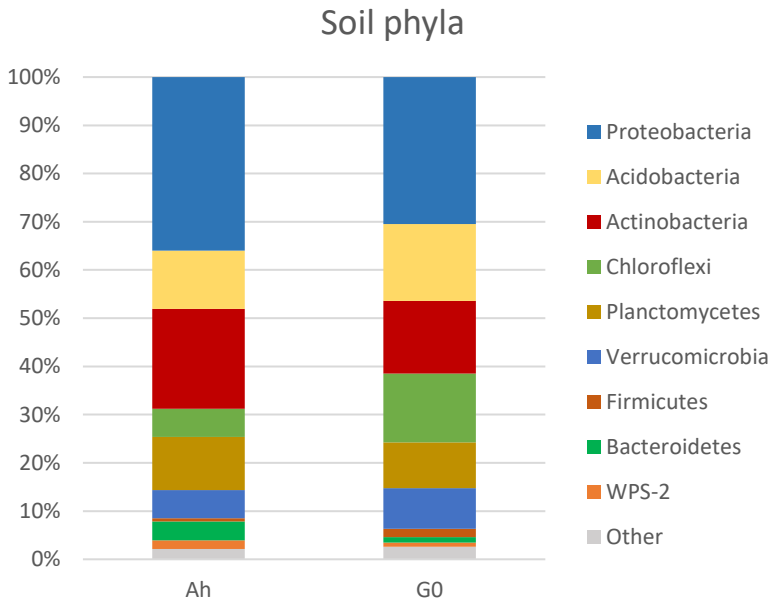


Figure 1-2. Relative abundance of bacterial phyla based on Illumina MiSeq 16S rRNA genes in upper Ah and lower G0 horizons of acid waterlogged soil sample

Trebonia and *uncultured Gaiellales* were the most abundant genera of the phylum Actinobacteria in both Ah (5,39% and 2,95% respectively) and G0 (8.03% and 1,69%) horizons. Genus *Mycobacterium* was the top third most abundant actinobacterium in Ah (1.87%), but not in G0 (0.44%). Other top actinobacterial genera included representatives of the class Thermoleophilia (*Conexibacter*, *Solirubrobacter*, unclassified *Solirubrobacteraceae, 67-14*), Rubrobacteria (*Gaiella*, *Gaillales unclassified*), Acidimicrobiia (uncultured), and Actinomycetia (*Streptacidiphilus, Crossiella, Allostreptomyces, Nocardioides, unclassified Micrococcaceae* and *Micromonosporaceae*) (Fig.1-3).

Genus *Trebonia* was the most dominant among all genera of all phyla in both horizons (5,39% and 8,03% of relative abundance in Ah and G0 respectively). However, next top most abundant genera in Ah horizon were *Nitrobacter* of the phylum Proteobacteria (3,95%), *Subgroup_6* of Acidobacteria (3,56%), *Conexibacter* of Actinobacteria (2,95%) and uncultured genus of Acidobacteria (2,25%), while in G0 horizon uncultured genus of Acidobacteria (6,26%), *AD3* of Chloroflexi (5,54%), *Subgroup_2* of Acidobacteria (4,52%), unclassified genus of *Xantobacteriaceae* of Proteobacteria (4,51%), *Candidatus Udaeobacter* of Verrucomicrobia (4,38%) and *HSB_OF53-F07* of Chloroflexi (4,27%). (Fig.1-4)

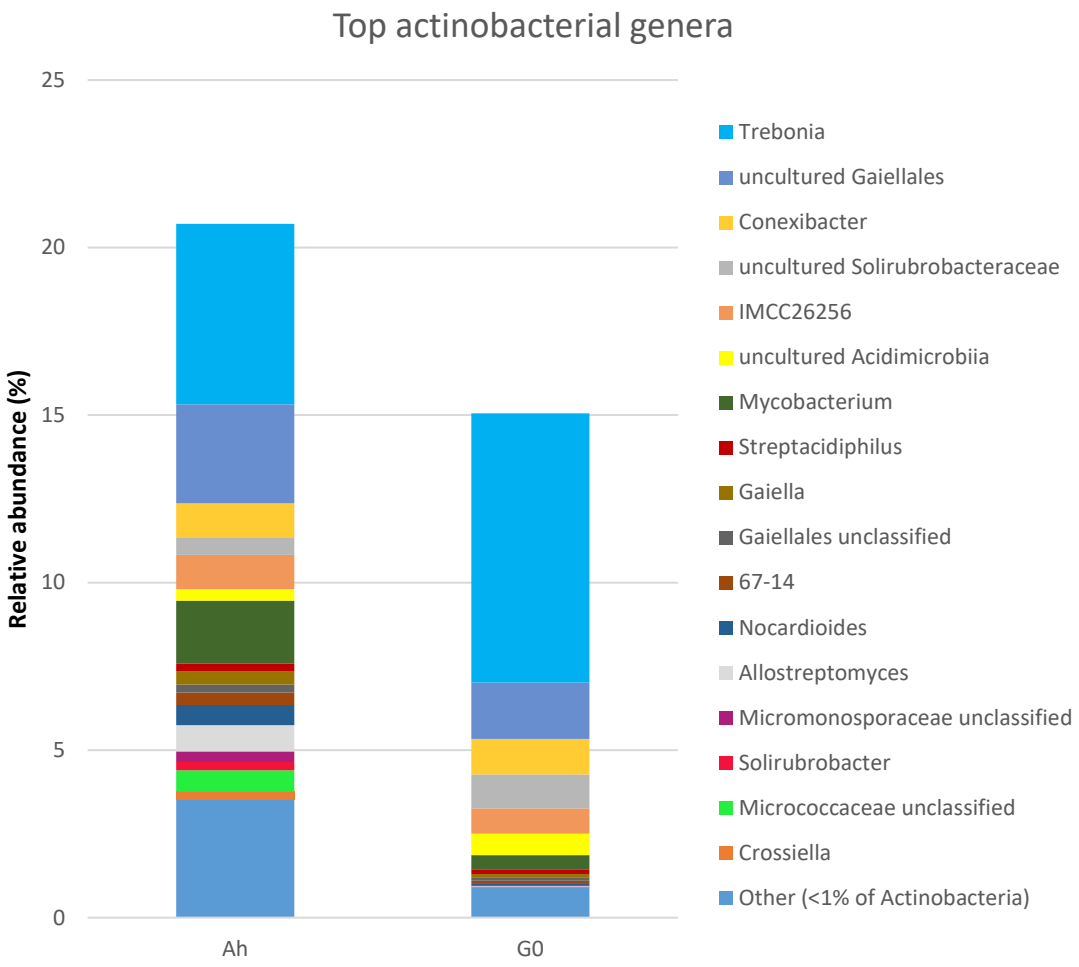


Figure 1-3. Relative abundance of the most abundant genera within the phylum Actinobacteria based on Illumina MiSeq 16S rRNA genes in upper Ah and lower G0 horizons of acid waterlogged soil sample

In upper Ah horizon 26 genera make up 50% and 161 genera 90% of the total bacterial community, while in G0 horizon 13 genera make up 50% and 83 genera 90% of the relative abundance of bacteria (Fig.1-5).

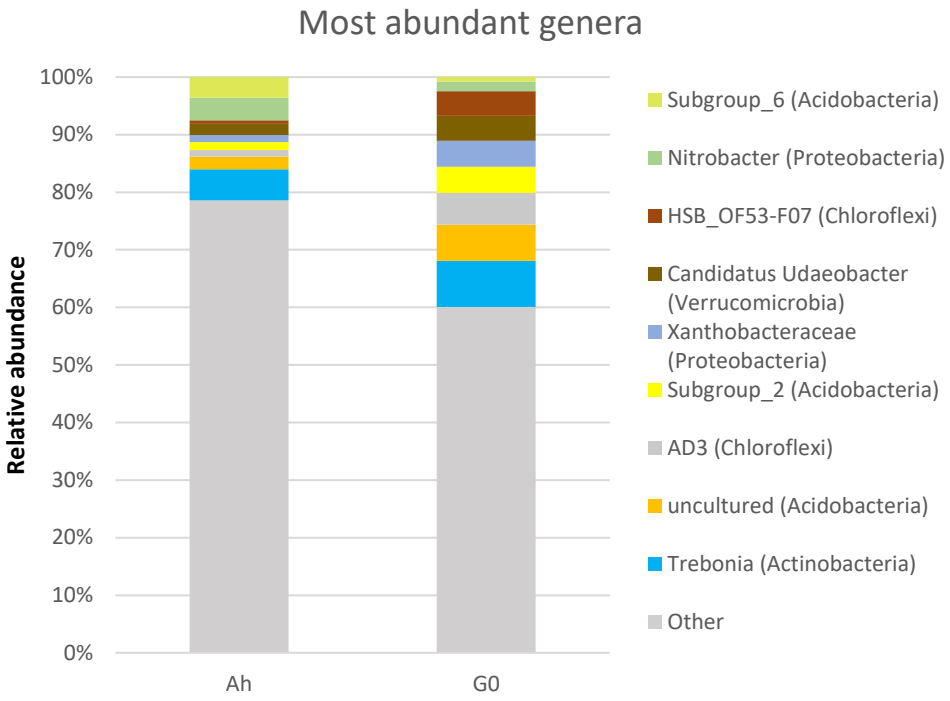


Figure 1-4. The top 10 most abundant genera in upper Ah and lower G0 horizons of soil based on the relative abundance of 16S rRNA amplicon sequences

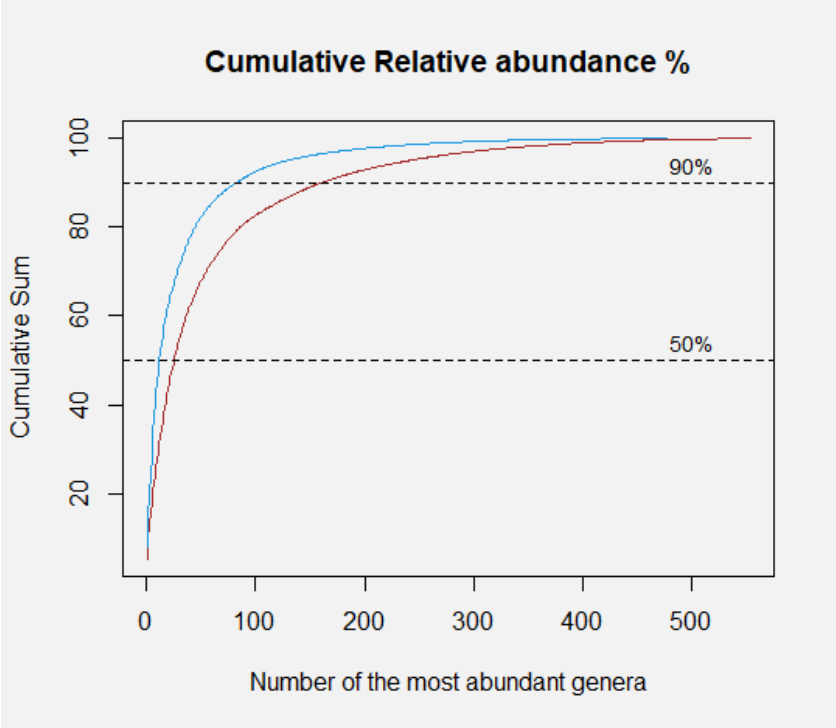


Figure 1-5. Cumulative curve of the relative abundance of bacterial genera in upper Ah (brown) and lower G0 (blue) horizons of acid waterlogged soil based on 16S rRNA amplicon sequencing

Totally, 1476 bacterial strains were isolated, with 759 being Gram-positive based on KOH test, from which 341 pure cultures of actinobacterial strains were recovered. The highest number of actinobacteria was isolated on VL medium from Ah (133 isolates) and G0 (109 isolates) horizons (Fig.1-6).

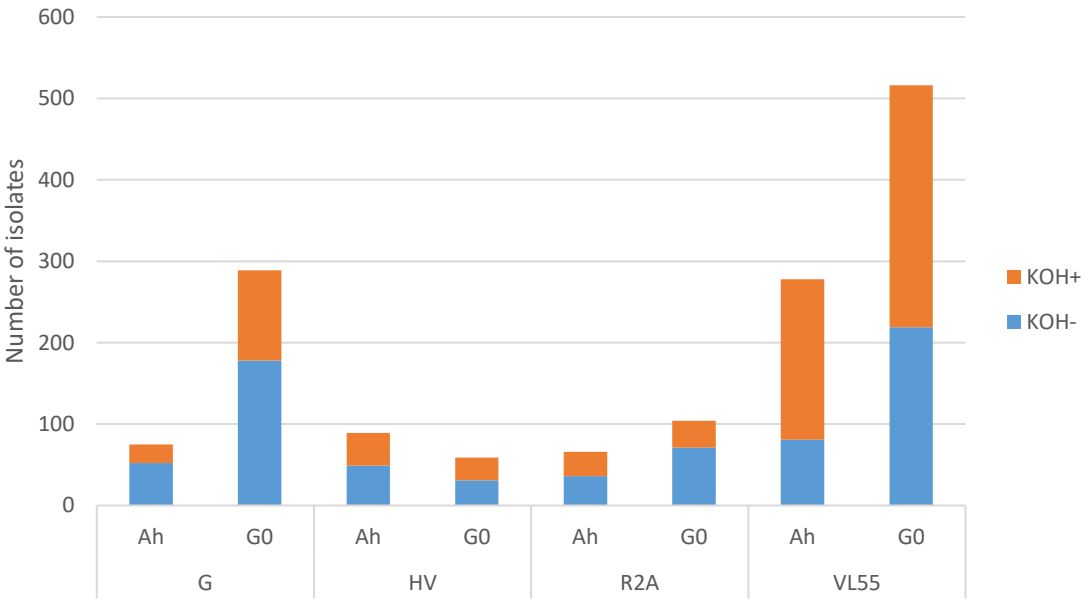


Figure 1-6. Number of Gram-positive (red) and Gram-negative (blue) isolates defined by KOH-test isolated from upper (Ah) and lower (G0) horizons on different selective media (G, HV, R2A, VL)

Proportion of isolated genera varied based on the horizon and media (Fig.). Isolated actinobacteria belonged to the 16 genera of the class Actinomycetia: *Actinomadura, Actinospica, Allostreptomyces, Catenulispora, Kitasatospora, Kribbella, Micrococcus, Micromonospora, Nocardia, Plantactinospora, Pseudonocardia, Rhodococcus, Streptacidiphilus, Streptomyces, Streptosporangium, Trebonia.*

The most numerous isolates from VL55 medium from both horizons belonged to *Catenulispora* and *Streptacidiphilus,* while isolates belonging to *Streptomyces* were isolated mainly from Ah horizon and *Actinospica* from G0 horizon. *Streptosporangium* was isolated only on HV medium (Fig.1-7).

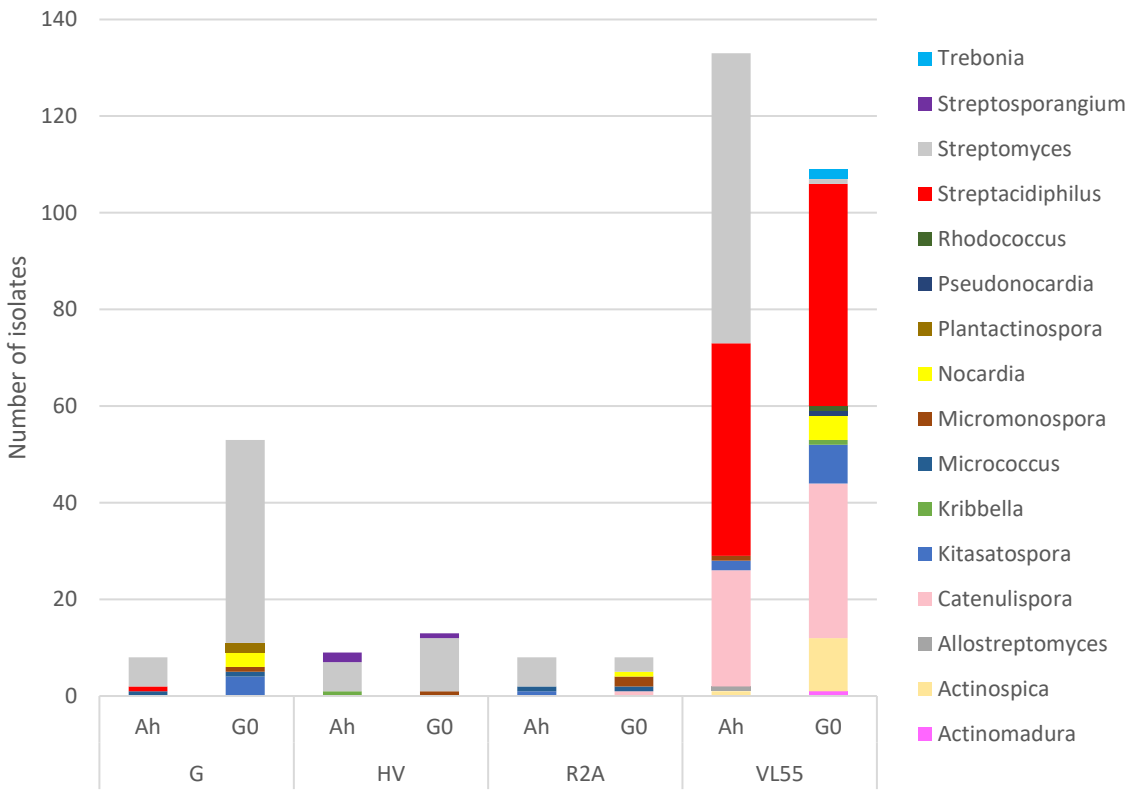


Figure 1-7. Genera of isolated Actinobacteria recovered from upper (Ah) and lower (G0) horizons of acid forest soil on different media (G, HV, R2A, VL)

Genera of isolated Actinobacteria in soil 16S rRNA amplicon library comprised only 7,1% of the total bacterial relative abundance (one third of all soil actinobacteria) in Ah and 8,5% (half of all soil actinobacteria) in G0 horizons. *Actinospica* had 0,04% and 0,12%, *Allostreptomyces* had 0,79% and 0, 02%, *Streptacidiphilus* had 0,23% and 0, 13%, and *Streptomyces* had 0,17% and 0,053% of the total relative abundance in Ah and G0 respectively. (Fig.1-8).

Strains isolated from the same horizon on the same media after similar incubation time tended to form phylogenetically close clusters. Within the order *Catenulisporales*, genus *Actinospica* had two clusters of isolates with smaller (8-16 days) and longer (26-48 days) incubation time, while genus *Catenulispora* formed two larger clusters of strains coming mostly whether from G0 or Ah horizons. Within the order *Streptomycetales*, genus *Kitasatospora* formed one homogenous cluster of strains isolated from G0 horizon after 48 days of incubation on VL55 and another heterogeneous cluster of strains isolated from different horizons and after different time of incubation. Within the genus Streptomyces there were also two clusters – one heterogeneous of strains isolated mostly

on media other than VL55 and one homogeneous cluster of strains all isolated from Ah horizon on VL55 after small incubation time. (Fig.1-9)

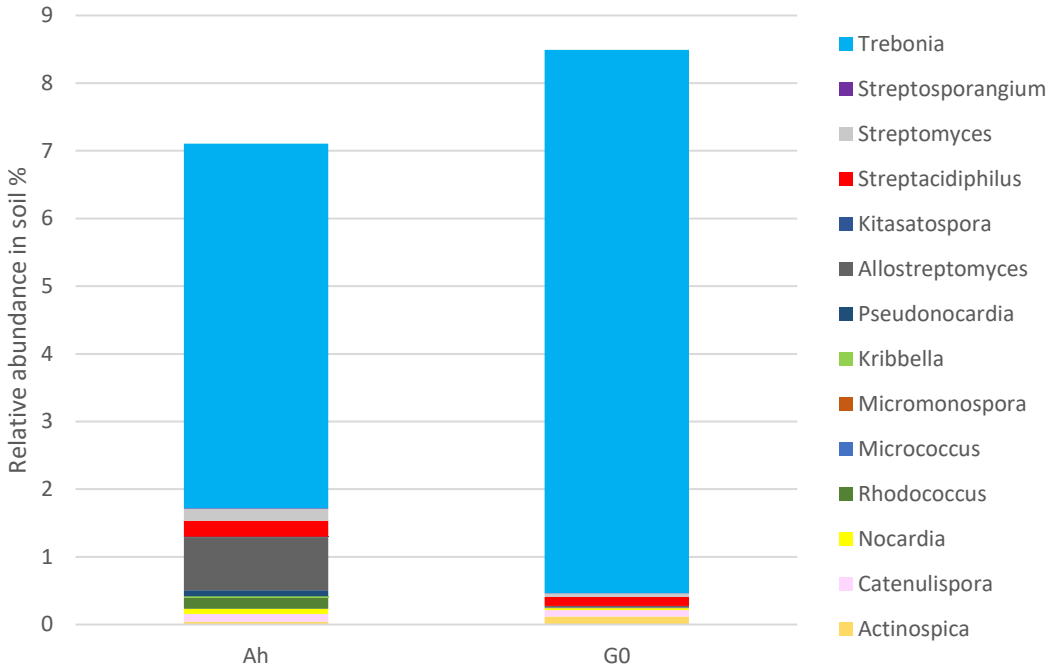


Figure 1-8. Relative abundance of the cultured actinobacterial genera (of isolated actinobacteria) detected in 16S rRNA amplicon libraries from upper Ah and lower G0 horizons of soil sample

Within the genus *Actinospica* all isolates formed separate cluster distinct from the only 3 previousely described type species *A.acidiphila, A.durhamensis,* and *A.robiniae* (Fig.1-10). Strain TR7_C11 from the cluster had 97,37% of the 16S rRNA (1031 nt) sequence similarity with the closest match *A. acidiphila* (NR_042363.1) based on ncbi blast search.

Majority of the isolates belonging to the *Catenulispora* (44 out of 57) formed a cluster distinct from the type species of *Catenulispora*. (Fig.1-10). Strain TR5_G10 from the cluster had 98,1% of the 16S rRNA (1051 nt) sequence similarity with the closest match *C. acidiphila* DSM 44928 (NR_074457.1) based on ncbi blast search.

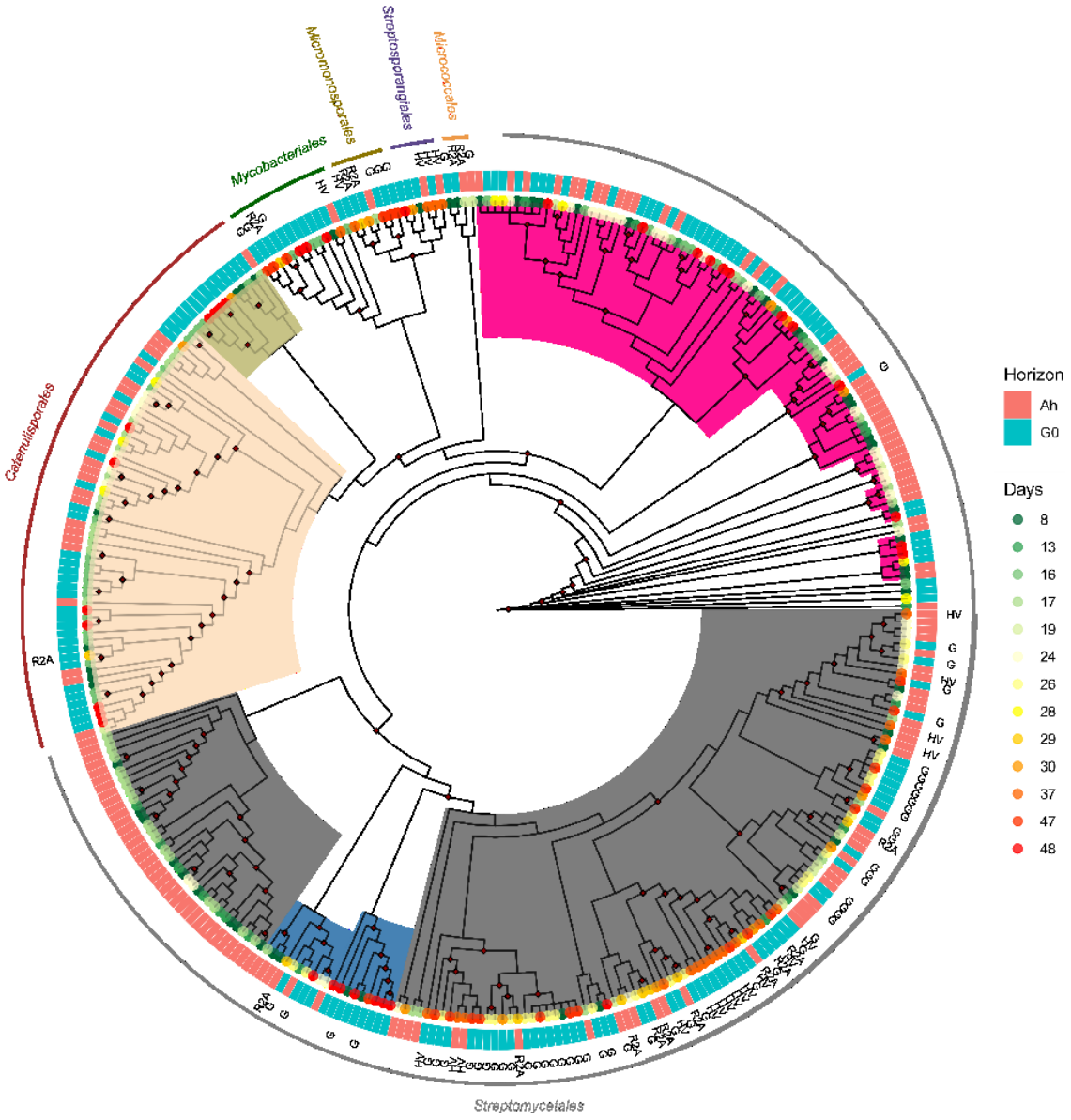


Figure 1-9. Phylogenetic tree of 341 actinobacterial strains isolated from acid soil on VL55 (not labeled) or on HV, G, R2A (labeled at outer circle). This cladogram was inferred using the maximum-likelihood method (FastTree) under GTR+GAMMA model based on partial (1162-1175 bp) 16SrRNA gene alignment. Tips colored based on Days of incubation. Outer circle is colored based on soil Horizon used for isolation. Red dots on inner nodes points out low local support values (<80%) of branching based on 1000 resampled alignments. Clades of the most numerous genera are highlighted: *Streptomyces* (grey), *Kitasatospora* (blue), *Streptacidiphilus* (pink), *Actinospica* (green), *Catenulispora* (sand).

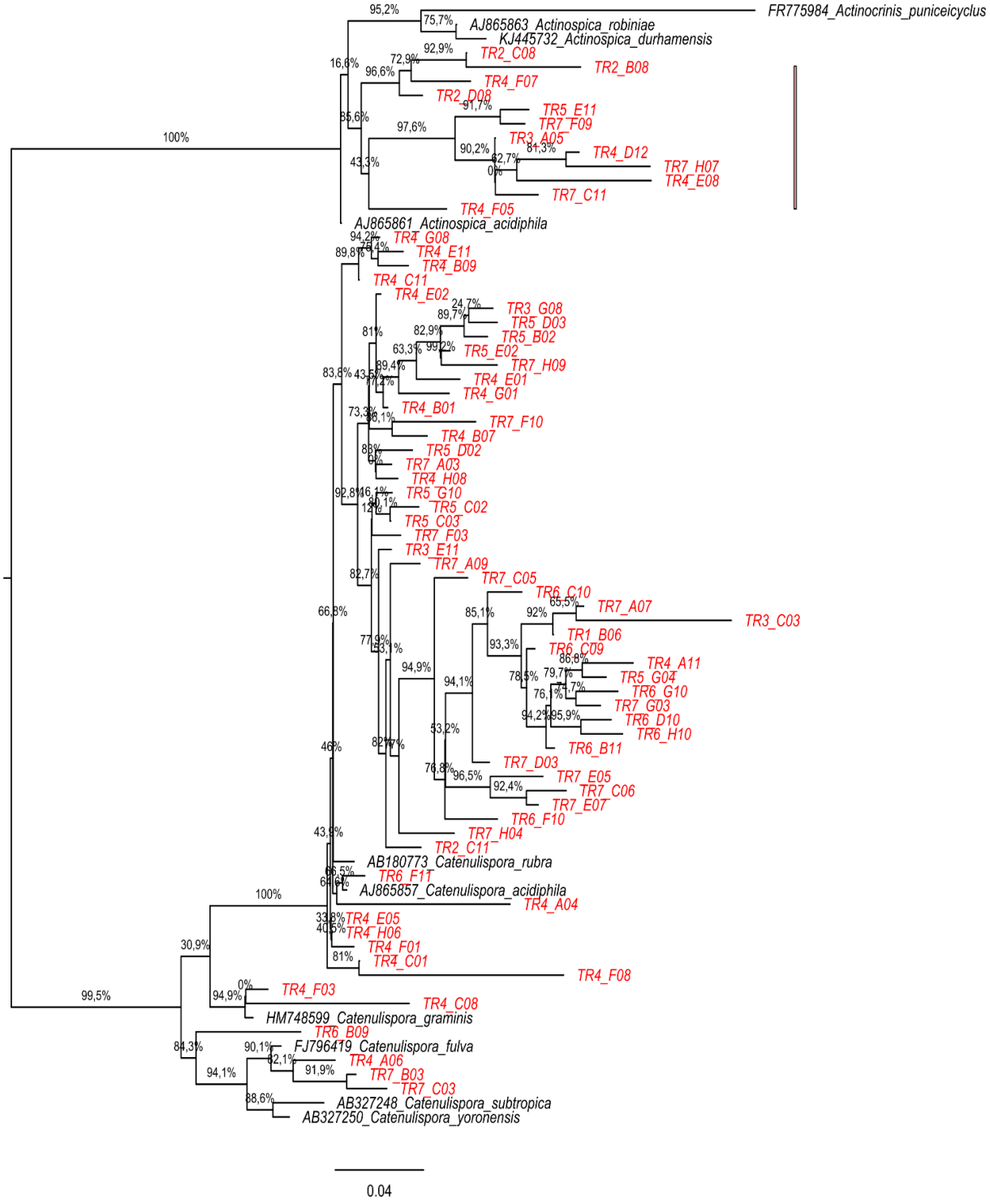


Figure 1-10. Subtree of isolates (in red) belonging to the order *Catenulisporales* from the phylogenetic tree based on the partial 16S rRNA gene sequences with all described type species within Actinobacteria. Phylogeny was inferred using the maximum-likelihood method (FastTree) under GTR+GAMMA model based on partial (1162-1175 bp) 16SrRNA gene alignment.

The majority of strains within the genus *Streptomyces* (95 out of 135) clustered with described type species, while 40 isolates clustered into 4 clusters of possibly novel species isolated exclusively on VL55 medium (Fig.1-11). Overall, 40 out of 61 streptomycetes isolated on VL55 belonged to possible novel species, including 35 isolates forming distinct clade and all isolated at early incubation time from Ah horizon (Fig.1-11, Fig.1-12). Based on the ncbi blast results, strain TR3_B10 from the upper cluster had 98,7% of the partial (921 nt) 16S rRNA sequence similarity with the closest type species *S. cocklensis* NR_108501.1, while strain TR2_F10 from the lower cluster had 98.22% sequence similarity of the partial (951 nt) 16S rRNA with the closest type species *S. bryophytorum.* Both strains had 97.94% of pairwise sequence similarity based on ncbi BLAST results.

All except one strain within *Kitasatospora* clustered with type species. Strain TR3_B04, isolated on VL55 from G0 horizon, had distinct position on 16S phylogenetic tree and had 96.95% sequence similarity of the partial (1016 nt) 16S rRNA with the closest match *K. viridis* (NR_043023.1) of ncbi BLAST search.

All except one strain within *Streptacidiphilus* formed 3 separate clusters distinct from the described type species (Fig.1-11, Fig.1-13). Strain 15TR831, isolated from G0 horizon on VL55, from the most numerous cluster of strains has been selected for the interaction experiment (Fig.1-13). Strain 15TR831 had 98.15% of 16S rRNA (1051 nt) sequence similarity with the closest ncbi BLAST match *Streptacidiphilus melanogenes* (NR_044033.1).

Strain 15TR67, isolated from the G0 horizon on G medium and clustering together with the described type species *S.niveus* (Fig.1-14), has been selected for the interaction experiment. Strain 15TR67 had 99.5% of 16S rRNA (830 nt) sequence similarity with the closest ncbi BLAST match S. niveus (NR_115784.1).

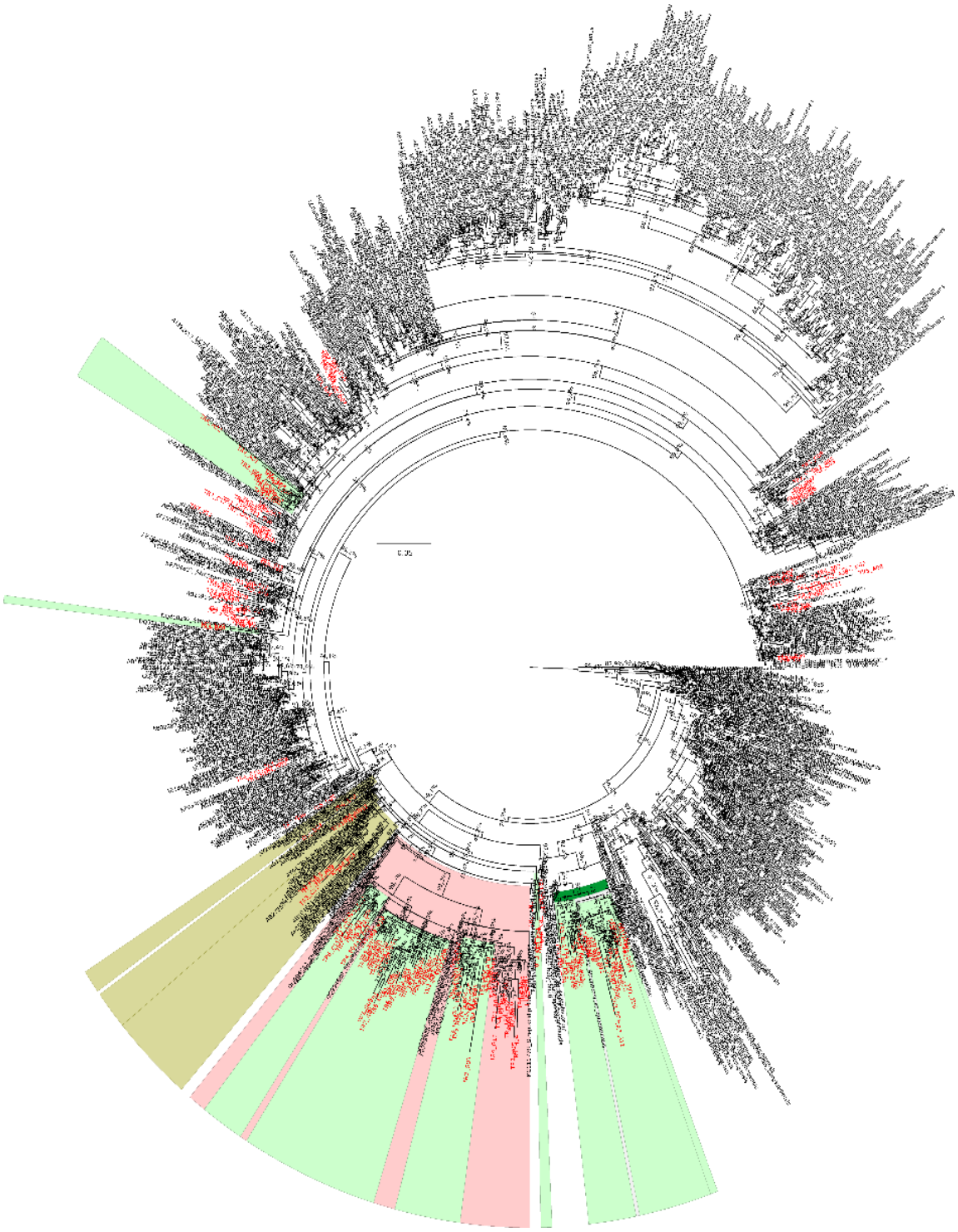


Figure 1-11. Maximum-Likelihood (Fasttree) phylogenetic tree based on 16S rRNA partial gene alignment of all type Streptomycetaceae family: genus *Streptomyces* (not highlighted), *Kitasatospora* (highlighted in olive), *Streptacidiphilus* (highlighted in pink). Strains isolated in our study have red labels, while described type species have black labels. Clusters of possible new species are highlighted in light green. Cluster of Streptomyces isolated at early incubation time from Ah horizon is highlighted in dark green.

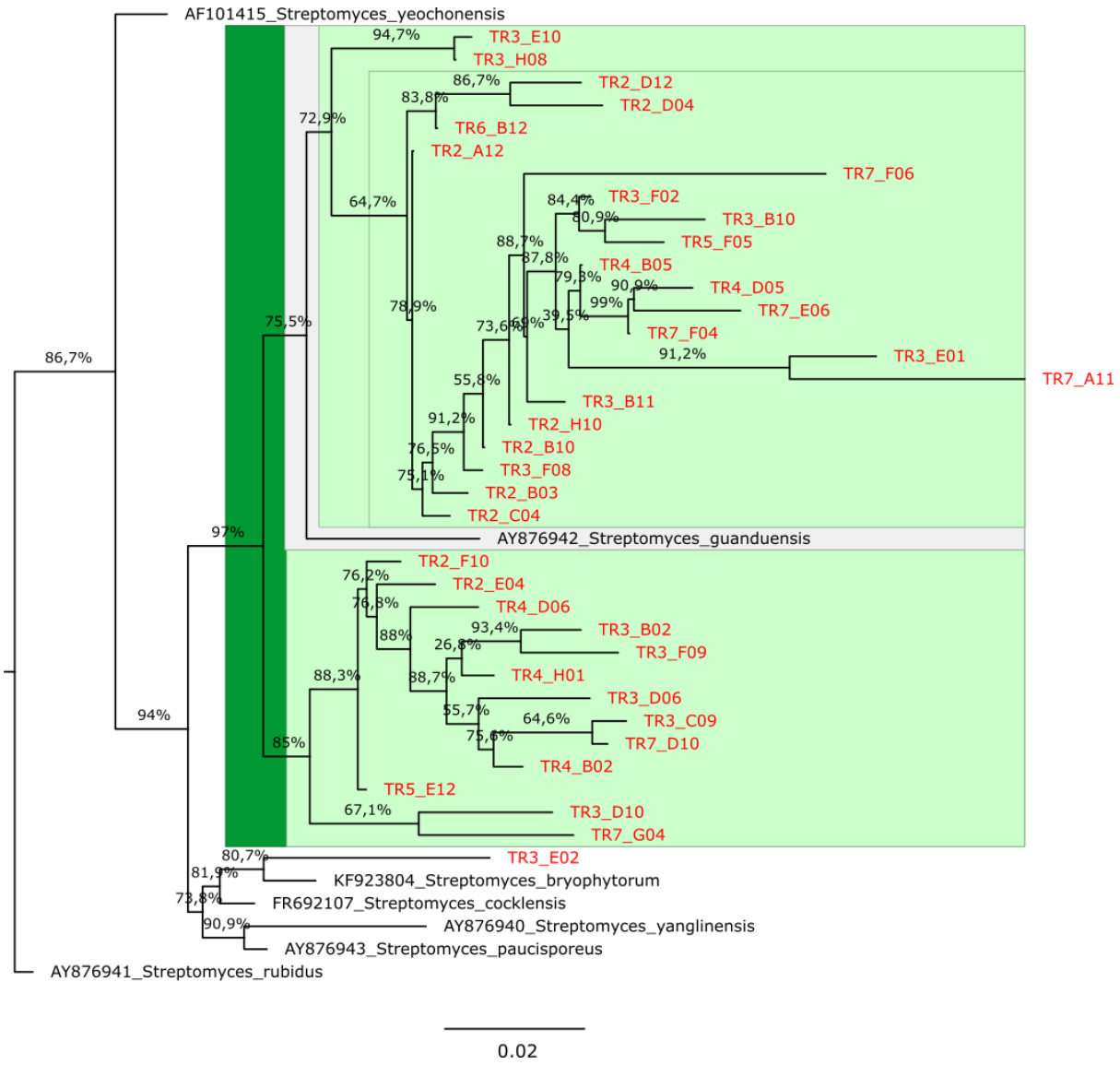


Figure 1-12. Subtree of strains (in red) within *Streptomyces* isolated from Ah horizon on VL55 after short incubation time based on the partial 16S rRNA gene sequences with all described type species within Actinobacteria. Subtree is selected from the phylogenetic tree in Fig.1-11

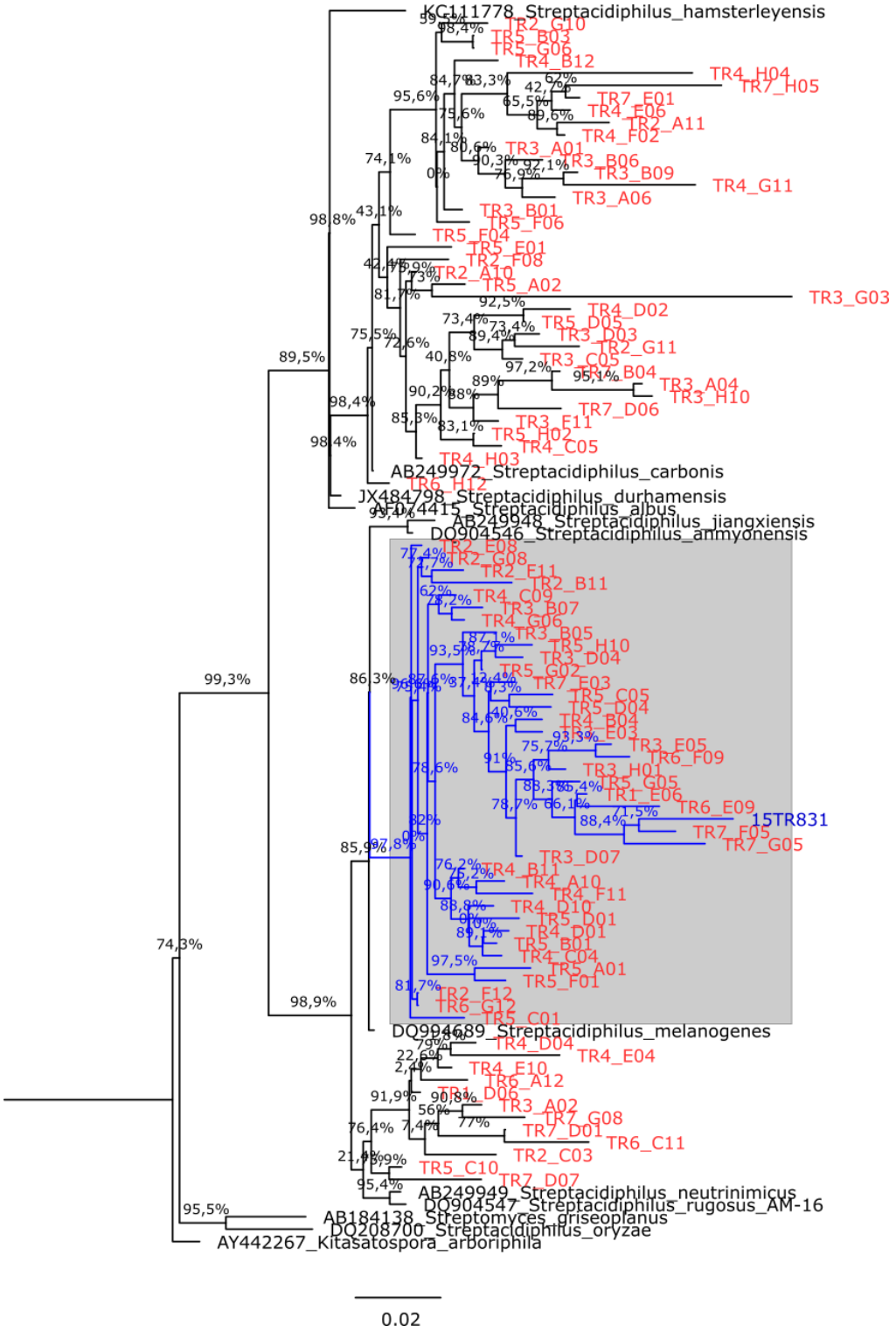


Figure 1-13. Subtree of isolates (in red) belonging to *Streptacidiphilus* from the maximum-likelihood (FastTree) phylogenetic tree based on the partial 16S rRNA gene sequences with all described type species within Actinobacteria . Cluster of the strain 15TR831 chosen for the interaction experiment (see section 2.2) is colored in blue.

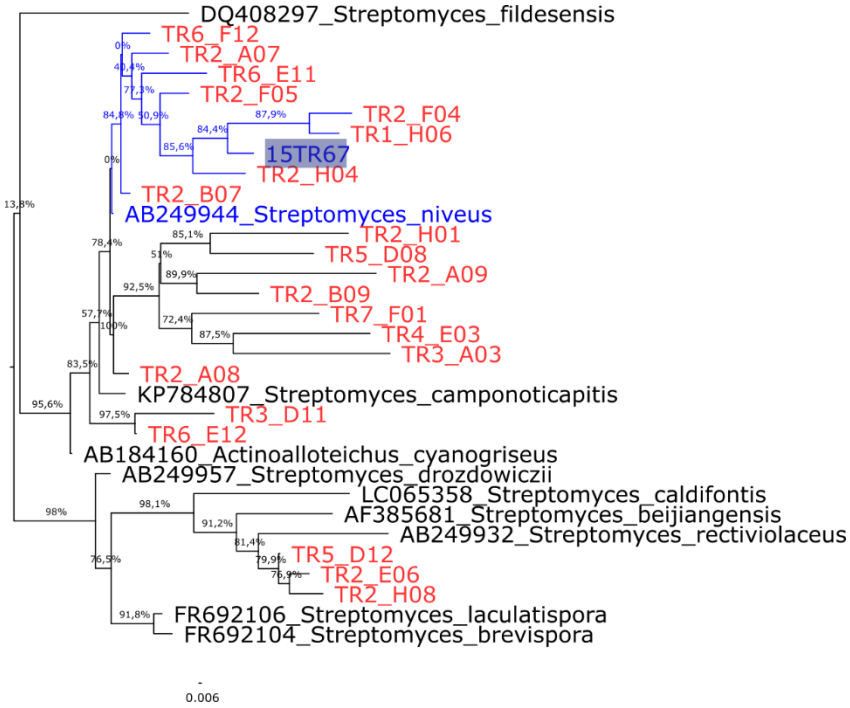


Figure 1-14. Subtree of isolates belonging to *Streptomyces* from the phylogenetic tree based on the partial 16S rRNA gene sequences with all described type species within Actinobacteria. Cluster of the strain 15TR67 chosen for the interaction experiment (see section 2.2) is colored in blue.

Strain 15TR583 from TC clade, Trebonia kvetii gen.nov., sp.nov.

Phylogenetic analysis based on 16S rRNA gene of the strain 15TR583, isolated from the G0 horizon on VL55, indicated its phylogenetic position within Trebon Clade (TC) actinobacteria (Fig. 1-14). The closest 16S rRNA (1510 nt) gene match was *Actinocorallia longicatena* IMSNU (NR_042033.1) with 92.36% similarity (ncbi BLAST). The strain 15TR583 has been selected for the genome sequencing and detailed polyphasic taxonomic characterization.

A novel species *Trebonia kvetii*, genus *Trebonia* and family *Treboniceae* within the suborder *Streptosporangiales* were established and described in details in **Publication II** (Rapoport et al., 2020). Briefly, the strain 15TR583T has morphological (areal and substrate mycelium type, spores), chemotaxonomical features (peptidoglycan type, major menaquinones, fatty acid pattern, phospholipid type and whole-organism sugar type) and phylogenetic position (based on 16S rRNA and whole genome sequences) different from other members of the suborder *Streptosporangiales* (families *Nocardiaceae*, *Streptosporangiaceae* and *Nocardiopsaceae*).

The hybrid assembly consisted of 93 contigs with a total of 9234338 bp, G+C content of 69.66%, the largest contig of 1889528 bp, N50 value of 1103890 bp and L50 of 4. GenBank assembly accession GCA_007827045.1 (BioProject PRJNA497400). The closest type strain genome determined by TYGS (type strain genome) server was *Thermomonospora echinospora* DSM43163 with 10.5 - 16.6% of digital DNA-DNA hybridization (dDDH) values. The strain 15TR583 had a separate position on a genome-based phylogenetic tree with the 20 closest type strain genomes (Fig.1-15).

Annotation with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) detected a total of 8619 genes including: 8145 protein coding sequences, 59 tRNAs, 4 5S, 16S and 23S rRNAs, 3 non-coding RNAs, and 400 pseudogenes. Annotation with RAST (Rapid Annotations using Subsystems Technology) server detected 9011 features with 16 genes as possibly missing. Only 16% of features were classified into subsystems. An overview of subsystem feature counts is presented in Table 1-1. No gram-postive nor gram-negative cell wall components were detected. 104 features were detected in the subsystem Metabolism of Aromatic Compounds, including genes for salicylate ester degradation (1 feature), quinate degradation (1), biphenyl degradation (14), p-hydroxybenzoate degradation (3), catechol branch of beta-ketoadipate pathway (8), salicylate and gentisate catabolism (20), homogentisate pathway of aromatic compound degradation (18), central meta-cleavage pathway of aromatic compound degradation (12), aromatic amin catabolism (10) and gentisate degradation (17). 100 features were detected in the subsystem Fermentation with genes for butanol biosynthesis (53 features), acetolactate synthase subunits (2), fermentations to lactate (3), and acetyl-CoA fermentation to butyrate (42). Among features responsible for stress response, genes for uptake of selenate and selenite (3 features) and bacterial hemoglobins (5) were detected. Among genes conferring resistance to antibiotics and toxic compounds were genes for copper homeostasis (5 features), mercuric reductase (3), resistance to chromium compounds (2) and tetracycline (4) and fluoroquinolones (2) resistance and beta-lactamase (3). Features in the category Invasion and intracellular resistance were limited to mycobacterium virulence operon involved in protein synthesis (SSU(4 feautures) and LSU(3) ribosomal proteins), in DNA transcription (2), and in quinolinate biosynthesis (3). Several genes for polysaccharide degradation were detected: two cellulases, one exocellobiohydrolase, eight xylanases, one chitinase and other genes for chitin utilization, and genes for lignin degradation.

Figure 1-14. Maximum -likelihood phylogenetic tree of 16S rRNA gene sequences of the type-strains of type species in the order *Streptosporangiales*, the strain Trebonia kvetii 15TR583 , related isolates (in bold), and environmental clones available from the GenBank database. Branch labels indicate supporting bootstrap value(%) and the scale bar phylogenetic distance.

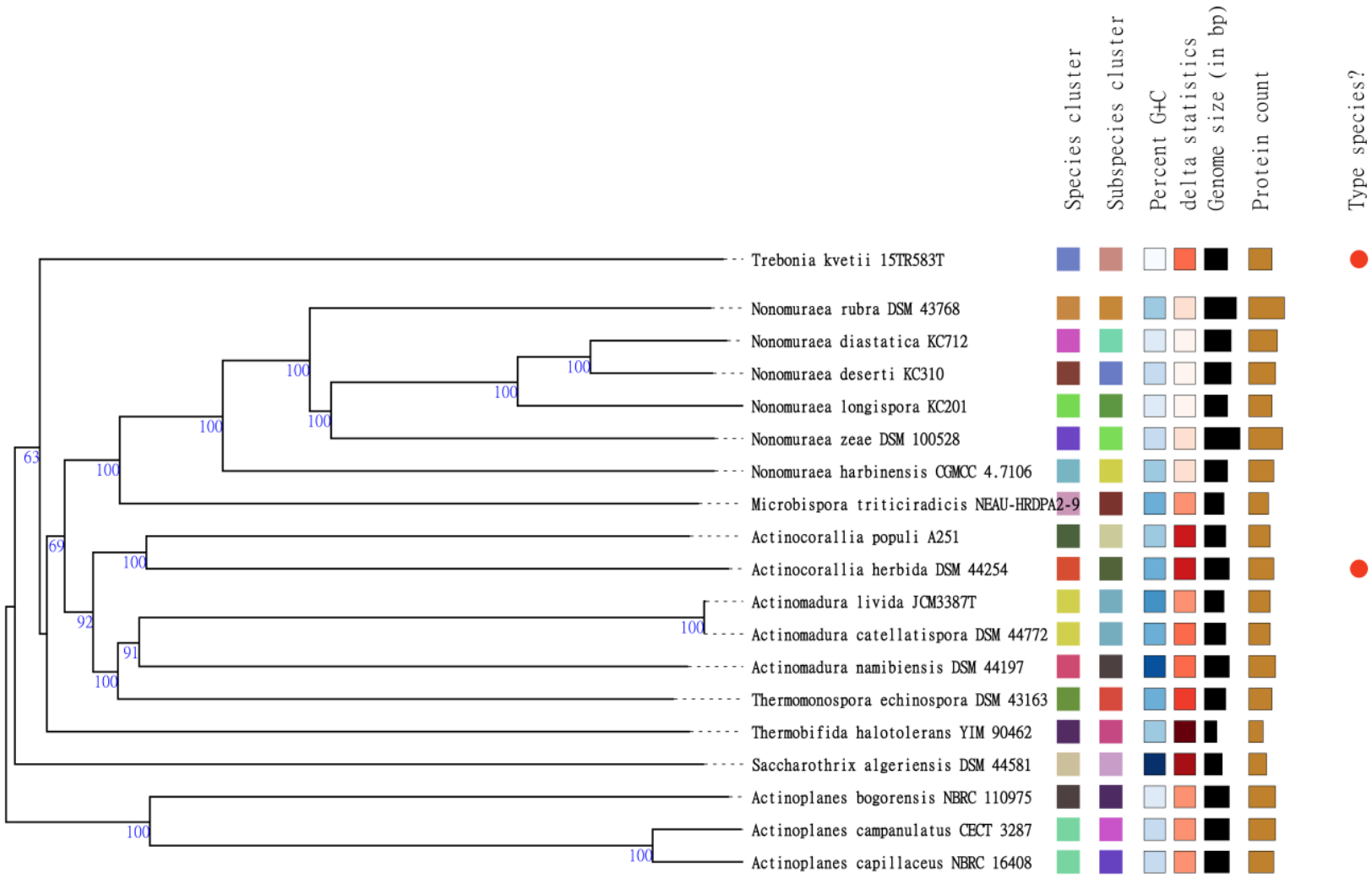


Figure 1-15. GBDP (Genome Blast Distance Phylogeny) based on genome data of the 20 closest type strain genomes from TYGS server. Branch support was inferred from 100 pseudo-bootstrap replicates each.

Table 1-1 Subsystem Feature Counts detected by RAST annotation server in the genome of *Trebonia kvetii* 15TR583

Subsystem Category		Subcategory	
Cofactors, Vitamins, Prosthetic Groups, Pigments	248	Biotin	45
		Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	15
		Quinone cofactors	12
		Tetrapyrroles	7
		Riboflavin, FMN, FAD	25
		Pyridoxine	13
		NAD and NADP	16
		Folate and pterines	95
		Coenzyme F420	4
		Coenzyme A	16
Cell Wall and Capsule	38	Capsular and extracellular polysacchrides	28
		Cell Wall and Capsule - no subcategory	10
Virulence, Disease and Defense	31	Resistance to antibiotics and toxic compounds	19
		Invasion and intracellular resistance	12
Potassium metabolism	10	Potassium metabolism - no subcategory	10
Photosynthesis	0		
Miscellaneous	55	Plant-Prokaryote DOE project	14
		Miscellaneous - no subcategory	41
Phages, Prophages, Transposable elements, Plasmids	0		
Membrane Transport	29	ABC transporters	14
		Protein translocation across cytoplasmic membrane	3
		Cation transporters	10
		Uni- Sym- and Antiporters	2
Iron acquisition and metabolism	2	Iron acquisition and metabolism - no subcategory	2
RNA Metabolism	76	RNA processing and modification	19
		Transcription	37
		RNA Metabolism - no subcategory	20
Nucleosides and Nucleotides	84	Pyrimidines	19
		Purines	54
		Nucleosides and Nucleotides - no subcategory	8
		Detoxification	3
Protein Metabolism	221	Protein folding	9
		Selenoproteins	3
		Protein biosynthesis	152
		Protein processing and modification	23
		Protein degradation	34
Cell Division and Cell Cycle	0		
Motility and Chemotaxis	0		
Regulation and Cell signaling	21	Regulation and Cell signaling - no subcategory	16

		Programmed Cell Death and Toxin-antitoxin Systems	5
Secondary Metabolism	11	Bacterial cytostatics, differentiation factors and antibiotics	1
		Plant Hormones	10
DNA Metabolism	100	DNA repair	80
		DNA Metabolism - no subcategory	13
		DNA replication	5
		DNA uptake, competence	2
Fatty Acids, Lipids, and Isoprenoids	201	Phospholipids	49
		Fatty acids	88
		Fatty Acids, Lipids, and Isoprenoids - no subcategory	53
		Isoprenoids	11
Nitrogen Metabolism	11	Nitrogen Metabolism - no subcategory	11
Dormancy and Sporulation	1	Dormancy and Sporulation - no subcategory	1
Respiration	127	ATP synthases	7
		Electron accepting reactions	32
		Electron donating reactions	57
		Respiration - no subcategory	31
Stress Response	41	Osmotic stress	6
		Oxidative stress	14
		Detoxification	6
		Stress Response - no subcategory	18
Metabolism of Aromatic Compounds	104	Peripheral pathways for catabolism of aromatic compounds	19
		Metabolism of central aromatic intermediates	58
		Metabolism of Aromatic Compounds - no subcategory	27
Amino Acids and Derivatives	361	Glutamine, glutamate, aspartate, asparagine; ammonia assimilation	26
		Histidine Metabolism	6
		Arginine; urea cycle, polyamines	58
		Lysine, threonine, methionine, and cysteine	41
		Amino Acids and Derivatives - no subcategory	2
		Branched-chain amino acids	102
		Aromatic amino acids and derivatives	56
		Proline and 4-hydroxyproline	11
		Alanine, serine, and glycine	59
Sulfur Metabolism	23	Sulfur Metabolism - no subcategory	17
		Organic sulfur assimilation	6
Phosphorus Metabolism	34	Phosphorus Metabolism - no subcategory	34
Carbohydrates	479	Central carbohydrate metabolism	192
		Aminosugars	10
		Di- and oligosaccharides	40

One-carbon Metabolism	68
Organic acids	5
Fermentation	100
Sugar alcohols	10
Polysaccharides	6
Monosaccharides	48

The Genome Annotation in the Pathosystems Resource Integration Center (PATRIC) server resulted in 8,815 CDS including 4,904 hypothetical proteins and 3,911 proteins with functional assignments. Genes annotated into subsystems of proteins that together implement a specific biological process or structural complex is presented in Fig.1-16. 35 genes for antimicrobial resistence (AMR) were detected based on PATRIC's curated collection of representative AMR gene sequence variants (Wattam et al., 2017). A circular graphical display of the distribution of the genome annotations with detected AMR is presented in Fig.1-17 .

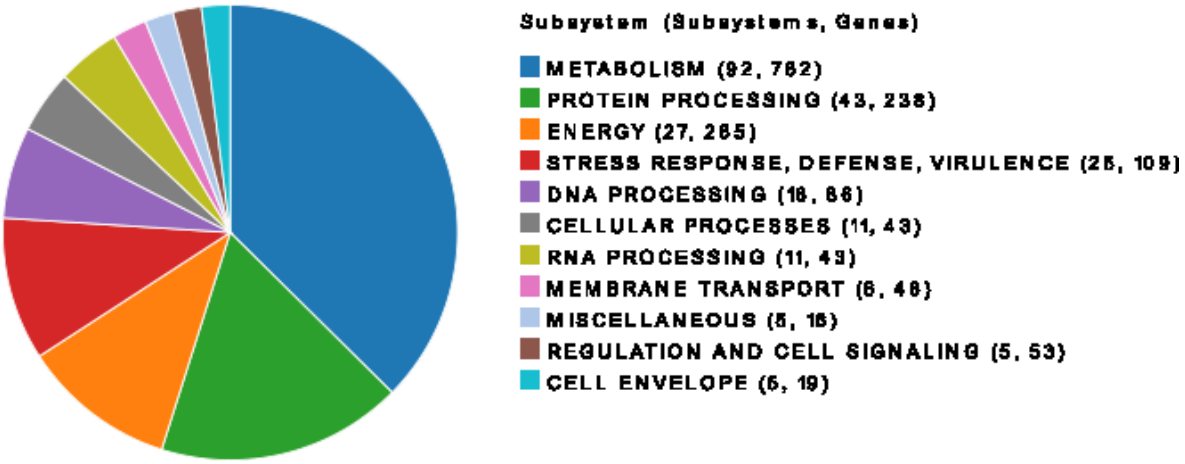


Figure 1-16. Subsystem analysis based on the genome annotation of *Trebonia kvetii* 15TR583

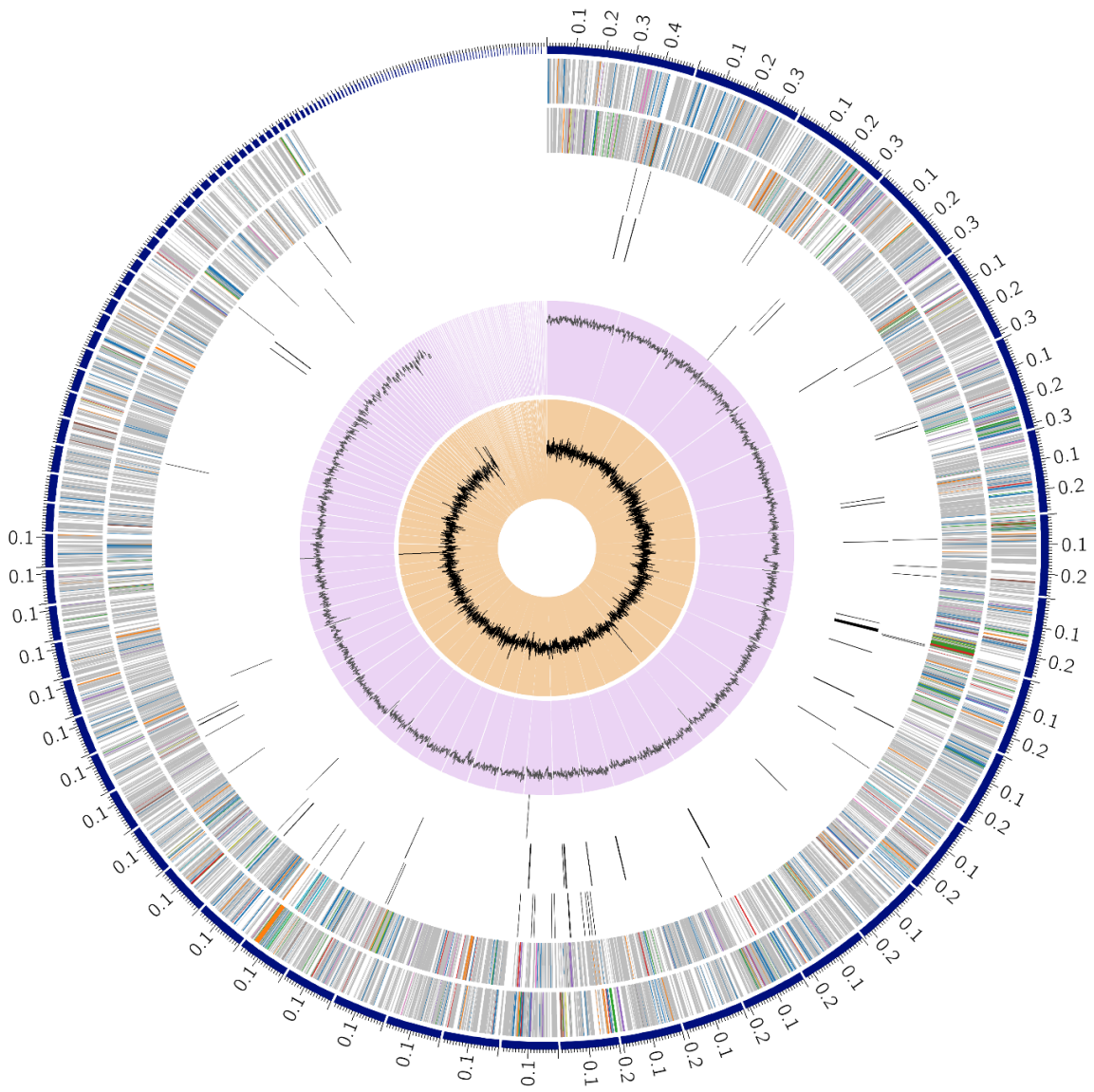


Figure 1-17. A circular graphical display of the distribution of the genome annotations of *Trebonia kvetii* 15TR583. This includes, from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to know virulence factors, GC content and GC skew. The colors of the CDS on the forward and reverse strand indicate the subsystem that these genes belong to (pie chart in Fig.1-16).

11 biosynthetic gene clusters (BGCs) for secondary metabolite production were identified with antiSMASH 6.1.1: two clusters for ribosomally synthesized and post-translationally modified peptides (RiPPs), two betalactones, indole, NRPS-like,

resorcinol, terpene, ranthipeptide, type I and type III polyketides. Only one cluster had 100% similarity match with the known cluster for thesynthesis of alkylresorcinol from *Streptomyces griseus* NBRC13350, one cluster had 38% similarity with BGC for hopene synthesis from *Streptomyces coelicolor A3(2)*, while 5 clusters did not have matches and 4 clusters had 4-12% of similarities with known BGCs (Table 1-2).

Interactions of actinobacteria with cultivable bacteria from acid gleysol

The number of 16S rRNA gene sequences ranged between 52,501 and 185,717, with a median of 64,548 sequences per agar plate, and 48,467±1086 sequences for soil sample (SS).

The most abundant phyla in soil were different from those of plates (Fig.1-18).

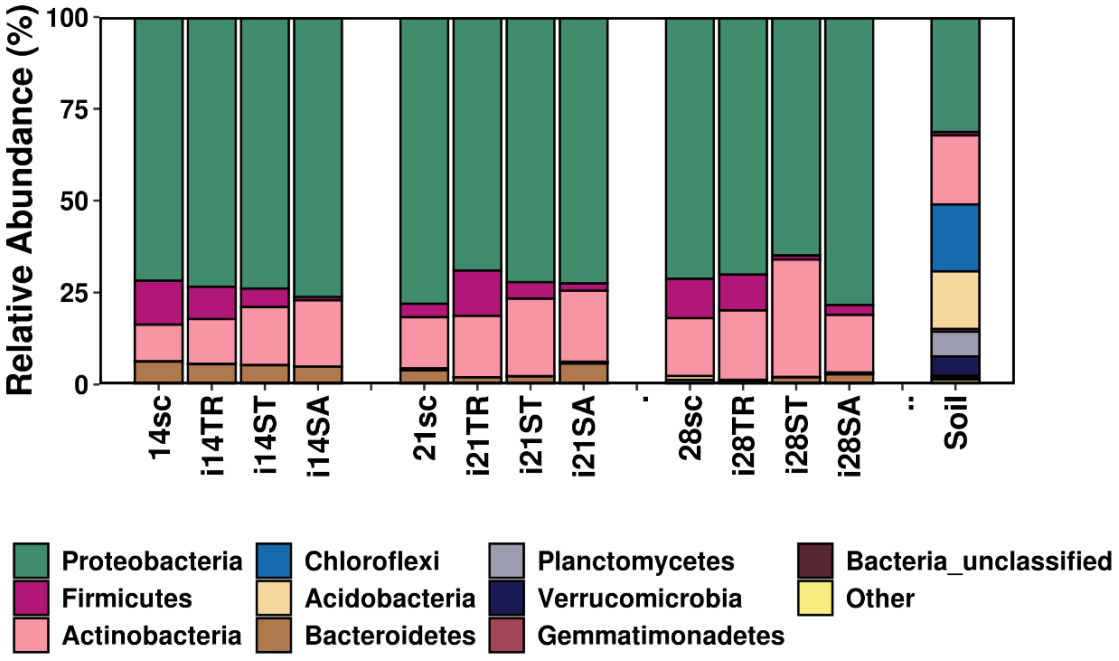


Figure 1-18. Relative abundance of phyla detected on plates after 14, 21, 28 days of incubation with different strains (SA, ST, TR strains) or without interacting strains (sc – soil control), and in Soil sample used for inoculation.

Table 1-2. Summary of *antiSMASH* 6.1.1 analysis of the genome of *Trebonia kvetii* 15TR583T

Source	Cluster Type	Location	Most similar known cluster	Biosynthetic class	Similarity	MIBiG accession
NZ_RPFW01000001	T1PKS	7,218 - 55,206				
NZ_RPFW01000001	terpene	431,654 - 458,342	hopene from *Streptomyces coelicolor A3(2)*	Terpene	38%	BGC000066
NZ_RPFW01000002	T3PKS	75,725 - 116,813	alkylresorcinol from *Streptomyces griseus subsp. griseus NBRC 13350*	Polyketide	100%	BGC0000282
NZ_RPFW01000001	resorcinol	280,893 - 322,722	nocathiacin biosynthetic gene cluster from *Nocardia sp. ATCC 202099*	RiPP:Thiopeptide	4%	BGC0000609
NZ_RPFW01000002	indole	325,945 - 349,106				
NZ_RPFW01000001	betalactone	606,345 - 635,092	ashimides biosynthetic gene cluster from *Streptomyces sp.*	NRP	8%	BGC0001961
NZ_RPFW01000002	ranthipeptide	885,655 - 907,23				
NZ_RPFW01000003	NRPS-like	75,991 - 119,395	kistamicin A biosynthetic gene cluster from *Nonomuraea sp. ATCC 55076*	NRP + Polyketide	12%	BGC0001635
NZ_RPFW01000004	RiPP-like	660,539 - 671,375				
NZ_RPFW01000005	betalactone	443,372 - 475,833				
NZ_RPFW01000009	RiPP-like	51,101 - 61,898	aurantimycin A biosynthetic gene cluster from *Streptomyces aurantiacus JA 4570*	NRP + Polyketide	6%	BGC0001519

While in soil communities top phyla with the relative abundance above 1% were represented by Proteobacteria (31% of relative abundance), Actinobacteria (19%), Chloroflexi (18%), Acidobacteria (16%), Planctomycetes (7%), Verrucomicrobia (5%), in plate communities averaged across all conditions top phyla (>1%) were represented by Proteobacteria (73%), Actinobacteria (17%), Firmicutes (6%) and Bacteroidetes (3%). The full list of phyla with average relative abundancies can be found in Table 1-3.

The length of incubation affected significantly the relative abundance of Bacteroidetes, Acidobacteria and Actinobacteria, while the presence of interacting strain significantly changed the relative abundance of only Acidobacteria (Table 1-4). In particular, the relative abundance of Acidobacteria was significantly lower in plates interacting with ST than with SA or soil control (SC) plates (post-hoc Dunn's test, p p=.041).

The average proportion of cultivable OTUs varied between 9.42 and 12.5% among all plates (Fig.1-19). The proportion of cultivable OTUs was affected significantly only by incubation time for plates interacting with SA (KW, $H(2)=19.5$, $p < .001$), but not by co-cultivation with the strains. Only plates co-cultivated with SA had significantly higher cultivable percentage of OTUs after 28 days than after 14 days of incubation (12.8±0.8% vs 10.1±0.6% resp.) (post-hoc Dunn's test, p=.006).

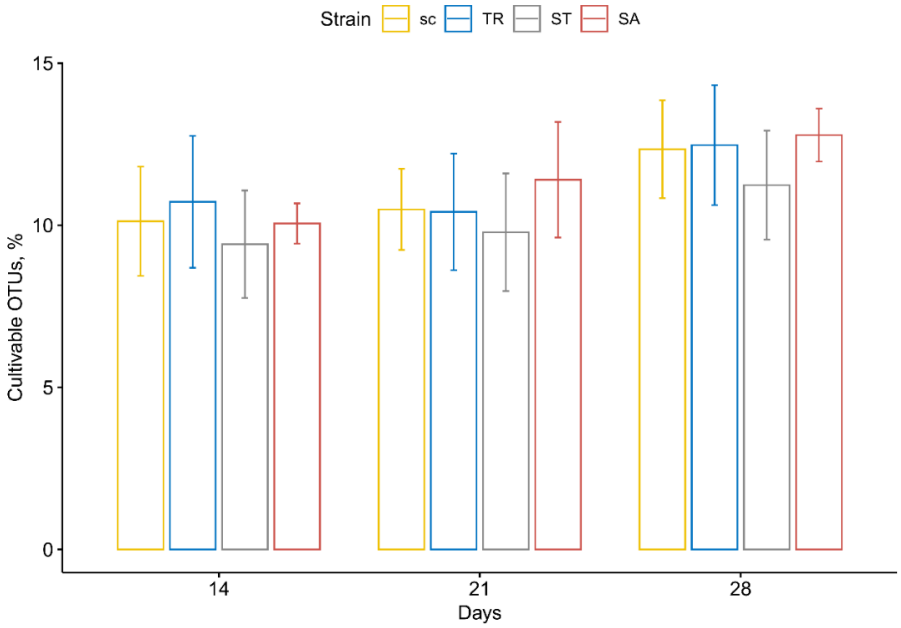


Figure 1-19. The average proportion of cultivable OTUs detected on Petri dishes with different co-cultivated strains (TR, SA, ST) and in soil control (SC) plates. Error bars represent SD (n=6).

Table 1-3. The relative abundance of phyla (%) averaged across soil samples (n=3) and across plates (n=72)

	Soil	Plates
Proteobacteria	31,322	73,087
Actinobacteria	18,78	17,976
Chloroflexi	18,237	0,002
Acidobacteria	15,638	0,646
Planctomycetes	6,701	0,002
Firmicutes	0,926	5,037
Verrucomicrobia	5,23	0,003
Bacteroidetes	0,778	3,239
Gemmatimonadetes	0,589	0,001
Bacteria_unclassified	0,514	0,005
Chlamydiae	0,274	<0,001
WPS-2	0,248	<0,001
Dependentiae	0,183	0
Patescibacteria	0,162	<0,001
Armatimonadetes	0,152	<0,001
Cyanobacteria	0,101	0
Elusimicrobia	0,089	0
Nitrospirae	0,036	<0,001
FCPU426	0,017	0
GAL15	0,012	0
Fibrobacteres	0,004	0
FBP	0,003	0
Omnitrophicaeota	0,002	0
Latescibacteria	0,001	<0,001
Spirochaetes	0,001	0
WS4	0,001	0
Rokubacteria	0	<0,001
Fusobacteria	0	<0,001
Deinococcus-Thermus	0	<0,001
Entotheonellaeota	0	<0,001

Table 1-4. Summary of the results of the two-factor non-parametric Scheirer-Ray-Hare test (H). The test was performed to detect the effect of incubation time (Days) and the presence of interacting strain (Strains) on the relative abundance of the main detected phyla.

Variable	Factor	df	H	P value	Significance
Firmicutes	Strain	3	5.0201	0.17033	
	Days	2	2.2044	0.33214	
	Strain:Days	6	2.8041	0.833	
Bacteroidetes	Strain	3	4.677	0.19703	
	Days	2	21.4135	0.00002	***
	Strain:Days	6	4.3932	0.62363	
Acidobacteria	Strain	3	8.4163	0.03815	*
	Days	2	28.2441	0	***
	Strain:Days	6	2.4402	0.8751	
Proteobacteria	Strain	3	2.21	0.52999	
	Days	2	1.0945	0.57853	
	Strain:Days	6	4.3944	0.62346	
Actinobacteria	Strain	3	6.6366	0.08443	
	Days	2	6.595	0.03697	*
	Strain:Days	6	4.6719	0.58652	

The presence of SA inhibited higher number of OTUs over time simultaneously propagating even higher numbers of OTUs over incubation time. On the contrary, ST inhibited a higher number of OTUs than stimulated over the incubation time. At each time point, TR influenced the lowest number of OTUs than SA and ST (Fig. 1-20. Metastats, $p<.05$). Comparison between all CC and SC plates by LEfSe (Linear discriminant analysis Effect Size) method revealed that the highest number of taxa (20) of different hierarchical levels were statistically enriched in plates interacting with SA, while only 2 genera were enriched in plates interacting with ST. Soil control (SC) plates were enriched in comparison with all co-culture plates (CC) in Acidobacteria. No taxa were enriched in plates co-incubated with TR in comparison with the other strains regardless the incubation time (Fig.1-21).

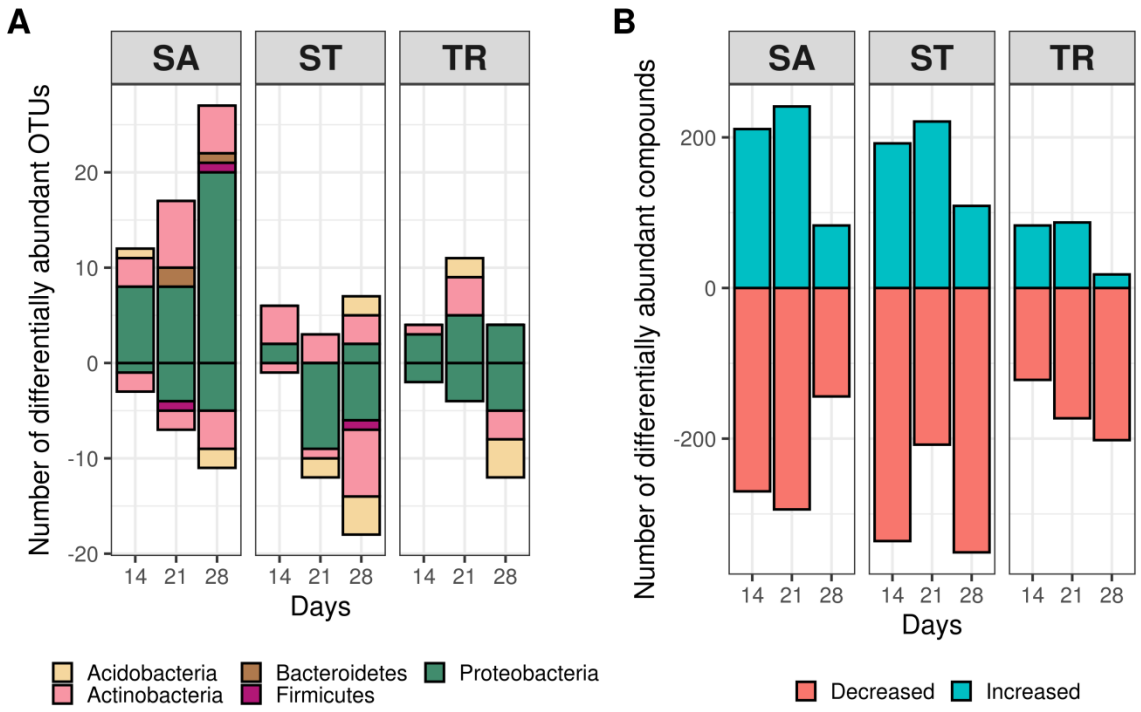


Figure 1-20. Number of OTUs (A) and LC/MS-MS features (B) detected by metastats analysis ($p<.05$) enriched (above 0) and inhibited (below 0) by the co-cultivation with SA, ST, TR strains

However, pairwise comparisons of the relative abundance of taxa at each time point between plates interacting with each strain and SC revealed different patterns of enriched and inhibited taxa depending on the co-cultured strain and incubation time. Higher number of taxa (64) were enriched than inhibited (34) by co-cultured strains. Regardless the incubation time, SA showed stimulating effect for 58 taxa and inhibited only 9 taxa, ST stimulated 10 taxa and inhibited 17 taxa, TR stimulated 7 taxa and inhibited 11 taxa. SA had stimulating effects on actinobacterial orders *Catenulisporales*, *Propionibacteriales* and *Streptomycetales*, on *Alphaproteobacteria* including orders *Azospirales*, *Dongiales*, *Elsterales*, *Sphingomonadales* and several genera of different families of *Rhizobiales*, on gammaproteobacterial orders *Salinisphaerales* and *Xanthomonadales*, family *Rhodocyclaceae* and several genera of *Burkholderiaceae* family. ST stimulated Actinobacteria, mainly *Catenulisporales*, while inhibiting Acidobacteria and alphaproteobacteria, including several genera of *Xanthobacteriaceae* family. TR stimulated *Catenulisporales* and proteobacterial families *Pseudomonadaceae* and *URHD0088* inhibiting *Rhodospirillales* and *Rhizobiales* (Table 1-5).

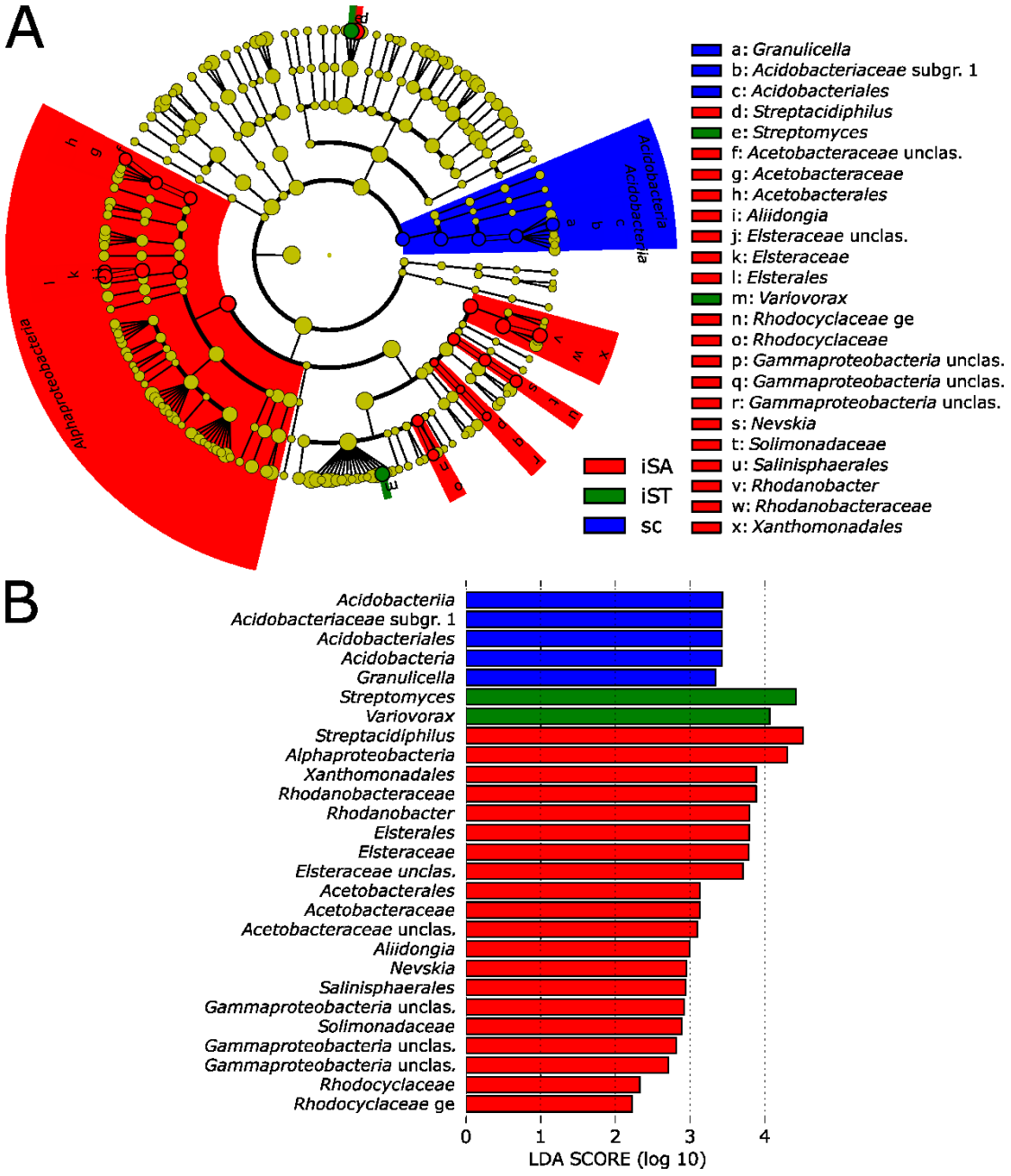
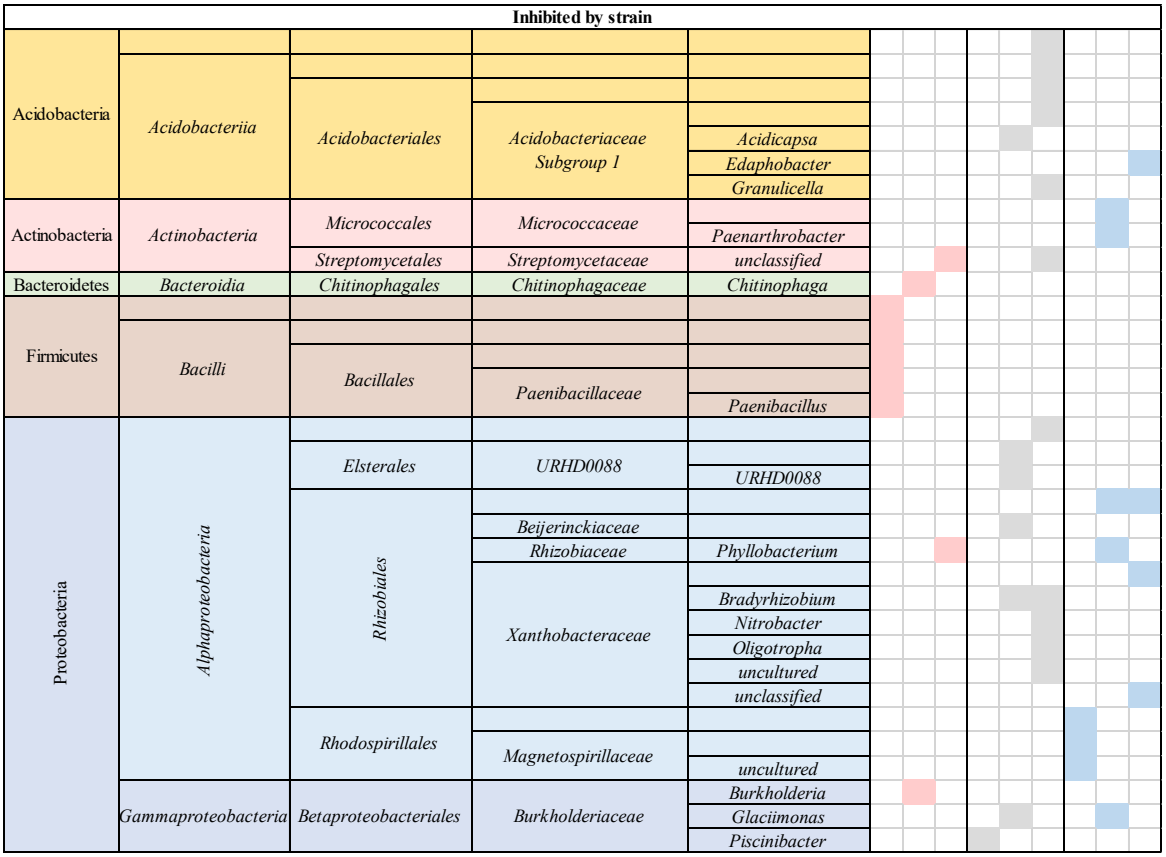


Figure 1-21. The most enriched taxa in soil control (blue) and plates interacting with SA (red), ST (green), TR (not identified). A) taxonomic cladogram; B) Linear discriminant analysis (LDA) effect size

Table 1-5. Taxa of cultivable soil bacteria enriched and inhibited by the presence of the co-culture strain SA, ST, TR after 14, 21 and 28 days of incubation.

Taxon					SA			ST			TR		
Phylum	*Class*	*Order*	*Family*	*Genus*	14	21	28	14	21	28	14	21	28
Enriched in interacting plates													
Acidobacteria	*Acidobacteriia*	*Acidobacteriales*	*Acidobacteriaceae Subgroup 1*	*Acidipila*									
Actinobacteria	*Actinobacteria*												
		Catenulisporales											
			Catenulisporaceae										
				Catenulispora									
		Micrococcales	*Microbacteriaceae*										
			Micrococcaceae	*Micrococcus*									
		Propionibacteriales											
			Nocardioidaceae										
		Streptomycetales											
			Streptomycetaceae										
				Streptacidiphilus									
Bacteroidetes	*Bacteroidia*	*Chitinophagales*											
			Chitinophagaceae										
				uncultured									
Proteobacteria	*Alphaproteobacteria*												
		Acetobacterales	*Acetobacteraceae*	*Acidicaldus*									
		Azospirillales											
			Azospirillaceae										
				unclassified									
				Azospirillum									
		Dongiales											
			Dongiaceae										
				Dongia									
		Elsterales											
			Elsteraceae										
				Aliidongia									
			URHD0088										
				URHD0088									
		Rhizobiales	*Beijerinckiaceae*	*unclassified*									
			Rhizobiaceae	*Phyllobacterium*									
			Xanthobacteraceae	*Nitrobacter*									
				Oligotropha									
				Xanthobacteraceae									
		Sphingomonadales											
			Sphingomonadaceae										
				Sphingomonas									
	Gammaproteobacteria	*Betaproteobacteriales*	*Betaproteobacteriales unclassified*	*Betaproteobacteriales unclassified*									
			Burkholderiaceae	*Pandoraea*									
				Ralstonia									
				uncultured									
				Undibacterium									
				Variovorax									
			Nitrosomonadaceae	*MND1*									
			Rhodocyclaceae										
				Rhodocyclaceae gen. unclassified									
			SC_I_84										
				SC_I_84									
		Gammaproteobacteria Incertae Sedis											
			Unknown Family										
				Acidibacter									
		Pseudomonadales	*Moraxellaceae*										
				Acinetobacter									
			Pseudomonadaceae										
				Pseudomonas									
		Salinisphaerales											
			Solimonadaceae										
				Nevskia									
		Xanthomonadales											
			Rhodanobacteraceae										
				Rhodanobacter									

Table 1-5 (part 2)



Yet, there were no obvious relations between number of differentially abundant LC-MS/MS features and OTUs. No compound among differentially abundant was known or connected to known compounds in molecular network derived in GNPS analysis.

Qualitative characterization of metabolite pool

The number of compounds common for all plates decreased in time from 534 LC-MS/MS features after 14 days of incubation, 514 after 21 days, to 489 after 28 days of incubation. After 14 days of incubation, the highest number of unique compounds (104) was detected in PC and CC plates with ST, decreasing to 48 and 52 after 21 and 28 days respectively. After 21 and 28 days of incubation, the highest number of unique compounds was detected for CC of SA (122 and 150 respectively). At each time point, for SA, in both PC and CC number of unique compounds was similar, but less than in CC. For TR, PC had higher number of unique compounds than CC or combination of PC and CC (Fig. 1-22).

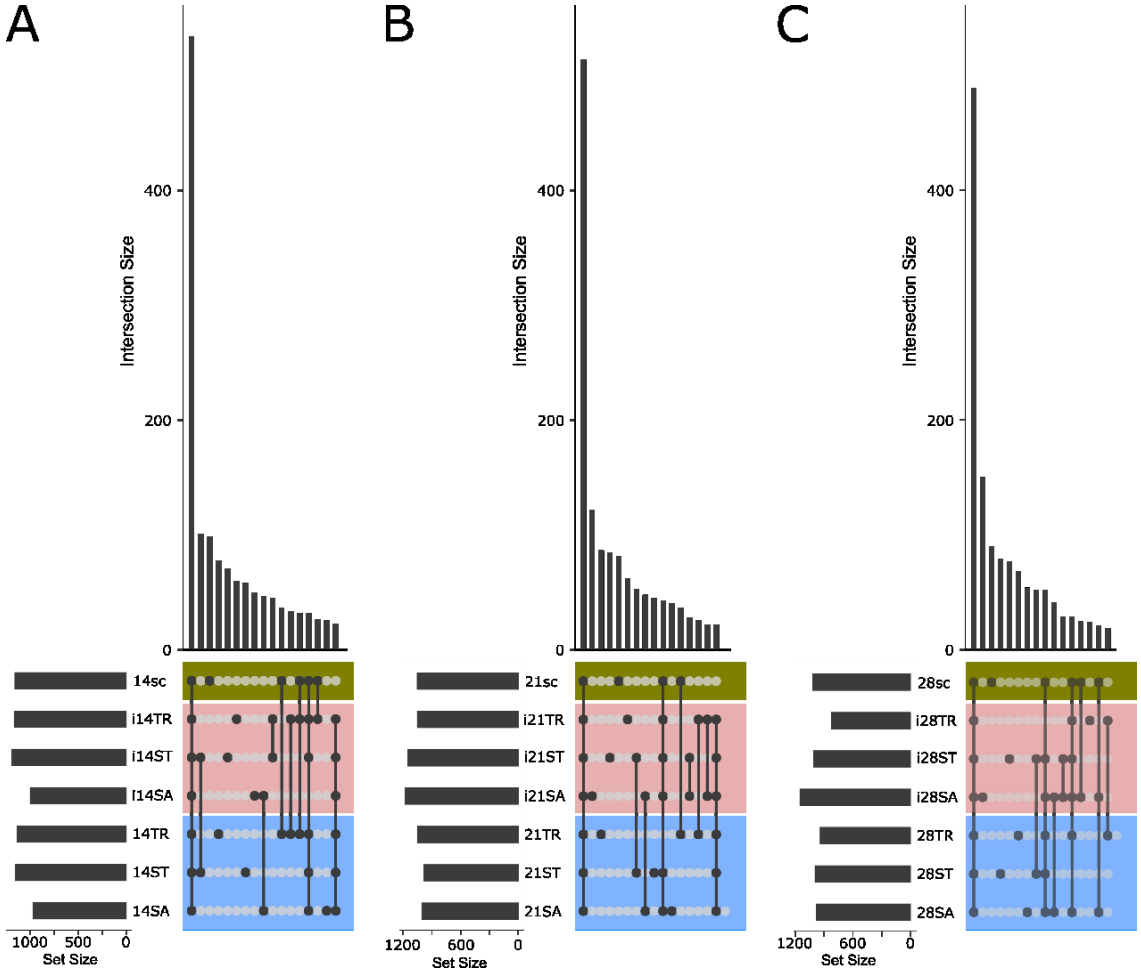


Figure 1-22. UpSet plots to summarize LC/MS-MS features detected in inter-zones of SC (green), PC (blue) and CC(pink) plates after 14, 21, 28 days of incubation. In each panel, the bottom left horizontal bar graph labeled Set Size shows the total number of LC/MS-MS features per treatment. The top bar graph in each panel summarizes the number of features for each unique or overlapping combination. Only 15 the most numerous combinations are present in the graph.

Among all obtained MS/MS spectra only 63 were represented by GNPS library hits (with MZError < 5 ppm), and 41 were unique library hits (Table S2). Those compounds are summarized in Fig. 1-23.

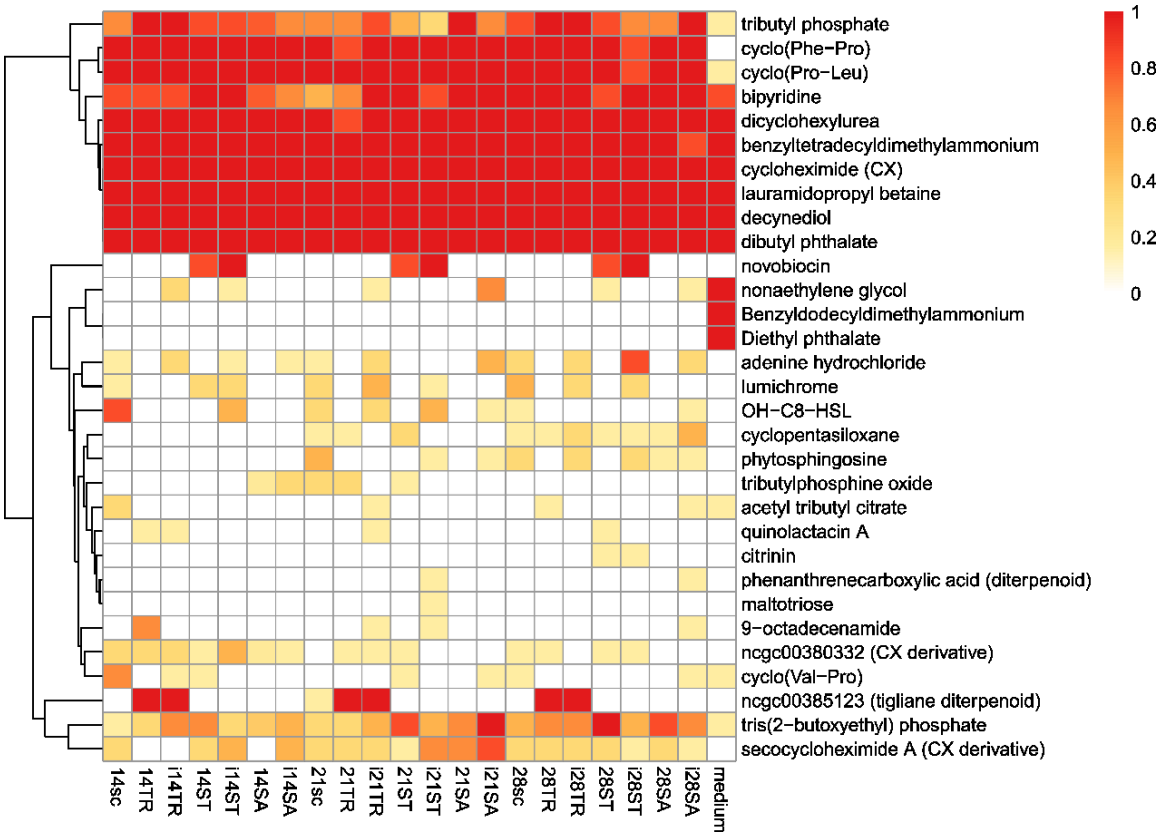


Figure 1-23. Heatmap of known compounds detected by the classical molecular network tool from the GNPS server in EtAc extracts of inter-zones of agar plates. Color scale reflects the proportion of plates within a treatment (n=6) with the respective compounds. Red color stands for the compound detected in all 6 plates (replicates) within the treatment, white - in none of the 6 plates. The length of incubation is described by days (14, 21, 28), and interacting strain (SA, ST, TR) or SC for soil control and interaction (i) on the bottom x axes. Hierarchical clustering is based on the Euclidean distance and complete linkage method.

Dicyclohexylurea, benzyltetradecyl-dimethylammonium, cycloheximide, lauramidopropyl betaine, decynediol, dibutyl phthalate were detected in all plates regardless the incubation time and strain present. Nonaethylene glycol, benzyldodecyl-dimethylammonium, and diethyl phthalate were detected in all plates with medium only, but not in cultivated plates with a few exceptions for nonaethylene glycol. Novobiocin was detected only in plates cultivated with ST and 7b,9-Dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5-oxo-1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-9ah-cyclopropa[3,4]benzo[1,2-e]azulen-9a-yl (NCGC00385123) acetate only in plates with TR.

N-3-hydroxyoctanoyl-L-Homoserine lactone (OH-C8-HSL) was detected in all plates with soil control after 14 days of incubation, but only in small number of plates after longer incubation. 2,5-Diketopiperazines (DKPs) such as cyclo(Phe- Pro) and cyclo(Leu-Pro) were detected in all plates, while cyclo(Val-Pro) - only in some plates with ST, and in about half of SC after 14 days of incubation. Lumichrome was detected at all incubation times in half of the SC and ST plates and in TR plates after 21 days. Citrinin was present only in 1 out of 6 plates with ST after 28 incubation days.

Molecular networks, which illustrate compounds sharing mass spectra, revealed multiple structurally related compounds specific for a particular treatment (Fig. 1-24).

Subnet 1 (Fig. 1-24) consists of three nodes, which show an unknown compound detected in all treatments and connected to nodes with assigned structure node 130397 (cyclamic acid) and node 199021 (omethoate) detected mostly in plates co-cultivated with SA (dark-red nodes). Subnet 2 consists of two nodes, which show an unknown compound connected to the node 362516 (Stachybotrysin B) detected mostly in PC and CC plates with TR (turquoise and blue respectively). Subnet 3 consists of a node of an unknown compound detected only in PC and CC plates with TR, an unknown ubiquitous node and node 87856 (trigonelle) detected mostly in PC plates with SA, TR and ST. Subnet 4 depicts a group of 15 compounds detected in CC plates and SC structurally related to heterocyclic compounds with multiple rings: node 369012 (4-(5,7-Dimethoxy-2-oxo-2H-chromen-6-yl)-3-hydroxy-2-methyl-2-butanyl β-D-glucopyranoside), node 374845 (1-[2-[4-(6-fluoro-1H-indol-3-yl)-3,6-dihydro-1(2H)-pyridinyl]ethyl]-5,6-dihydro-1H,4H-[1,2,5]thiadiazolo[4.3.2-ij]quinoline-2,2-dioxide), and node 366695 (olivil-4-O-glucoside). Subnet 5 consists of 6 unknown compounds structurally related to 3 known compounds - duplicated nodes 353622 and 353623 (tetrahydropalmatine) and node 345424 (dimefline), detected in PC and CC plates with SA. Subnet 6 shows 16 related compounds detected mostly in CC plates with SA and structurally close to sesquiterpene quinone (node 365831) and piperedin alkaloid (nodes 371676 and 371681) also detected in CC plates with ST. Subnet 7 consists of 7 compounds detected in CC and PC plates with ST related to the node with assigned structure node 361016 (otenzepad) and 360873 (schizokinen).

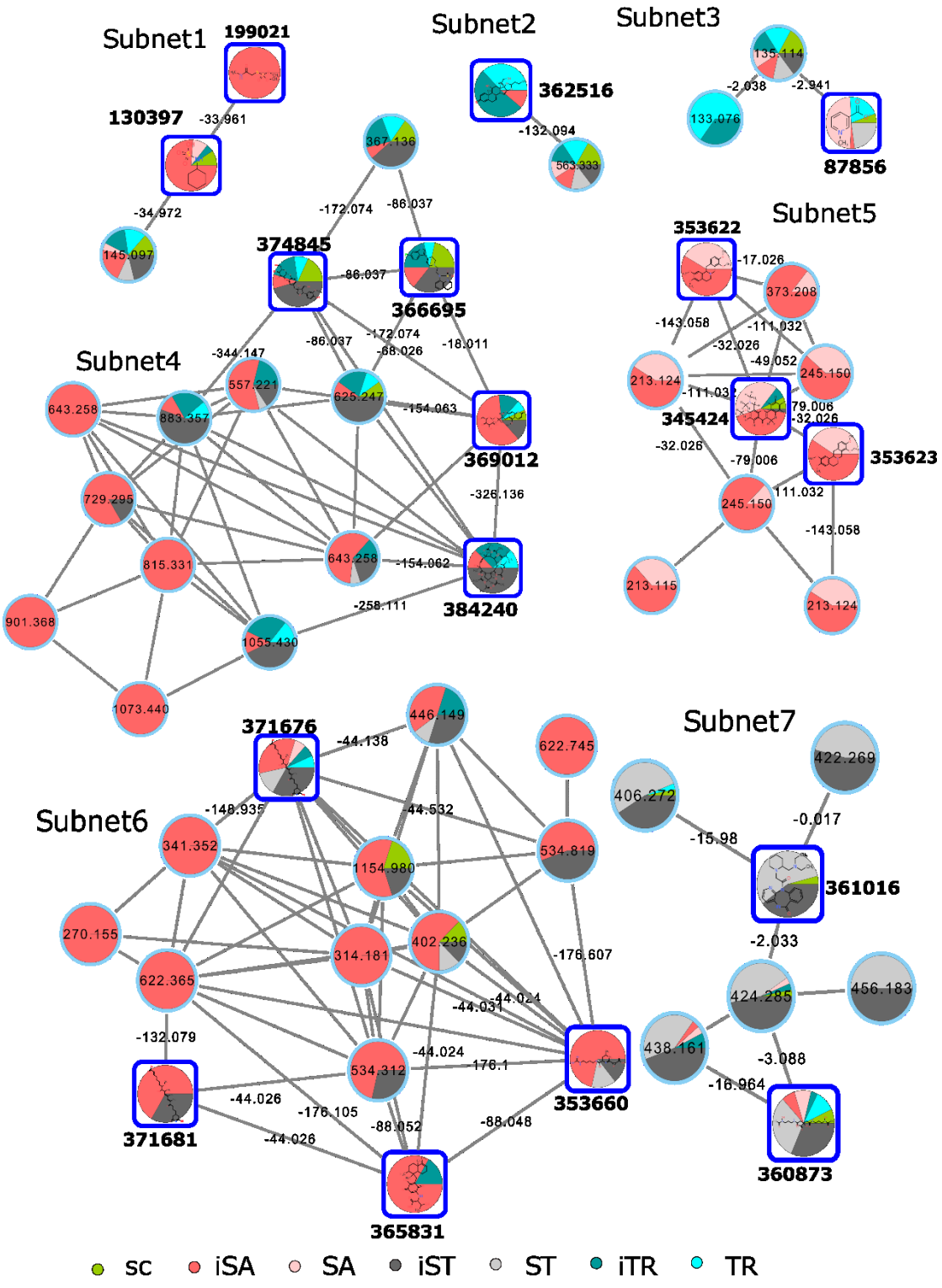


Figure 1-24. Subnetworks of structurally related compounds. The pie charts represent the share of treatments in which the node was detected.

Inhibitory activity

The antibiotic activity of the inter-zones occurred mostly only after 14 days of incubation, then diminished. After 14 days, antibiotic activity was observed in SC, iTR, TR, ST and iST inter-zones against *K. rhizophila*, and in TR, ST and iST inter-zones against *E. coli*. After 21 days, SC, ST and iST and after 28days only ST and iST showed antibiotic activity against *K. rhizophila.* SA did not show any antibiotic activity (Fig. 1-25).

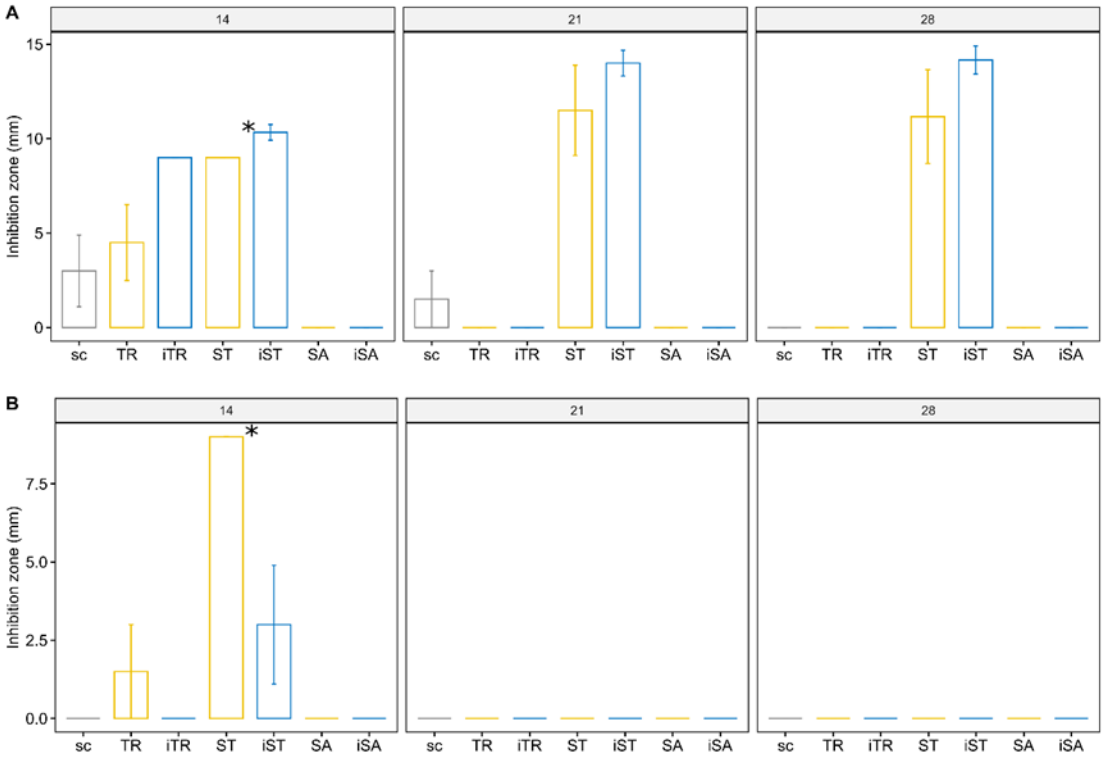


Figure 1-25. Agar diffusion method using agar plugs from inter-zone of plates against A) *Kocuria rhizophila* ATCC 9341 and B) *Escherichia coli* ATCC 10536. Barplot colors: in grey – soil control, yellow – PC plates, blue - CC plates. Labels represents strains: TR, SA, ST. Error bars represent SE (n=6)

Only the activity of ST was significantly higher in CC (Mdn=11 mm) than in PC (Mdn=9 mm) plates against *K.rhizophila* and lower in CC (Mdn=0) than in PC (Mdn=9) plates against *E.coli* (Wilcoxon test, p<.025; Table 1-6).

Despite the insignificant differences after 21 and 28 days of incubation, interacting plates with ST tended to have higher activity than non-interacting plates against *K.rhizophila* (Fig. 1-25, Table 1-6).

Table 1-6. The results of the two-sample Wilcoxon rank sum test for the inhibition activity of inter-zones coming from CC vs PC plates. *One-sample one-sided Wilcoxon test was performed to test if soil control activity was greater than zero.

Strain	Test strain	Days	Plate Type	Mean (mm)	SD (mm)	Mdn (mm)	W-statistic	p	Significance
ST	*K.rhizophila*	14	PC	9	0	9	30	<.025	*
			CC	10,3	1	11			
		21	PC	11,5	5,9	14	20,5	.735	
			CC	14	1,7	13			
		28	PC	11,2	6,1	13	22,5	.511	
			CC	14,2	1,8	14			
	E.coli	14	PC	9	0	9	6	<.025	*
			CC	3	4,7	0			
TR	*K.rhizophila*	14	PC	4,5	4,9	4,5	27	<.071	
			CC	9	0	9			
	E.coli	14	PC	1,5	3,7	0	15	.405	
			CC	0	0	0			
sc*	*K.rhizophila*	14	xxx	3	4,7	0	3	.173	
		21	xxx	1,5	3,7	0	1	.5	

Correlation between bacteria and individual compounds

OTU and metabolite diversity varied across the treatments (Fig. 1-26, Table 1-7). Generally, the OTUs clustered according to the incubation time (Fig. 1-26A), while the metabolites clustered by the co-cultured strain (Fig. 1-26B). Yet, while SA and ST samples formed distinct clusters of their metabolite profiles, TR samples were dispersed and clustered partly with soil control samples (SC). Bacterial communities from SC did not differ significantly from CC communities if incubation time and strain type were not considered. However, after taking into account the particular strain and time of incubation, up to 19% of the overall variability in OTUs was explained by the presence of the co-culture strain. Metabolite pools significantly differed between all pairwise comparisons of PC, CC and SC plates (PERMANOVA, Table 2). Bacterial diversity and metabolite pool were significantly correlated (Mantel statistic r=.13, p= .025).

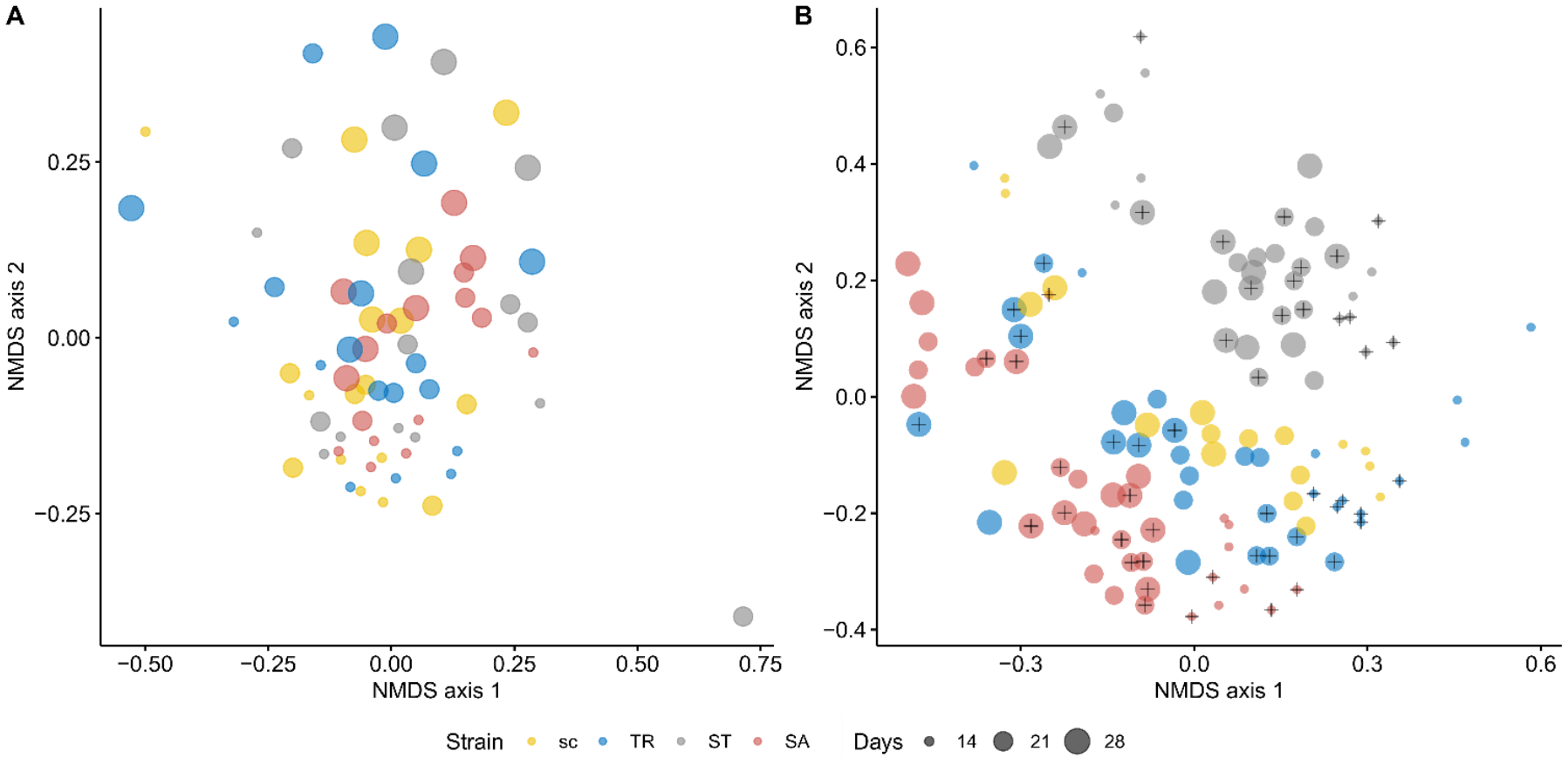


Figure 1-26. Non-metric multidimensional scaling (NMDS) of the Bray-Curtis distance depicting individual SC, CC and PC (with cross) plates with different strains (SA, ST, TR strains) after 14, 21, 28 days of incubation calculated from: A) relative abundances of OTUs of bacterial communities; B) LCMS compounds quantities (peak areas) extracted from inter-zone.

Table 1-7. PERMANOVA results of abundance data (OTUs and LCMS features) using the Bray-Curtis dissimilarity matrix with 1000 permutations. Tested factor is Type (PC,CC and SC plates), nested within Strain (SA,ST,TR, and SC) and Days (14, 21, and 28 days of incubation). P-values of the pairwise comparisons used Bonferroni adjustments for significant factor Type for LCMS features.

Variable	Factor	DF	F	R^2	P-value	Significance
OTU abundance	Type	1	1.5307	0.01981	0.09629	.
	Type:Strain	2	1.9537	0.05058	0.005599	**
	Type:Strain:Days	8	1.8521	0.19179	1.00E-04	***
LCMS features abundance	Type	2	11.9424	0.09166	1.00E-04	***
	Type:Strain	4	17.4509	0.26788	1.00E-04	***
	Type:Strain:Days	14	4.7065	0.25286	1.00E-04	***
Type (LCMS features, pairwise)	SC vs. PC	1	6.028989	0.080355	0.003	*
	SC vs. CC plate	1	4.312389	0.060472	0.003	*
	PC vs. CC plate	1	7.058912	0.064726	0.003	*

Positive correlations between OTUs and LC-MS/MS features dominated over negative ones in the network of high correlations (Spearman R>0.6, p<0.005) (Fig. 1-27). OTUs belonging to the same phylum tended to form positively-correlated clusters (assortativity degree =.58). The number of connections of OTUs to other OTUs and compounds varied across phyla (Fig. S1.1) (Kruskal-Wallis, chi-square (4, 155) = 24.21, p<.0001). Acidobacteria had the highest number of connections (*Mdn*=12), significantly higher than Actinobacteria (*Mdn*=2), Bacteroidetes (*Mdn*=2), and Firmicutes (*Mdn*=1), but not significantly different from Proteobacteria (*Mdn*=5) (Table S1-1). Median number of connections among all OTUs regardless phylum was 3, while 23 "hub" OTUs had more than 20 connections including: 2 OTUs of the genus *Occallatibacter* from the phylum Acidobacteria, 2 OTUs of the genus *Chitinophaga* of Bacteroidetes, and 19 OTUs of 10 Proteobacteria *Acidibacter, Allorhizobium, Azospirillaceae_unclassified, Bradyrhizobium, Nitrobacter, Oligotropha, Rhizobiaceae_unclassified, uncultured, URHD0088_ge, Xanthobacteraceae_unclassified.*

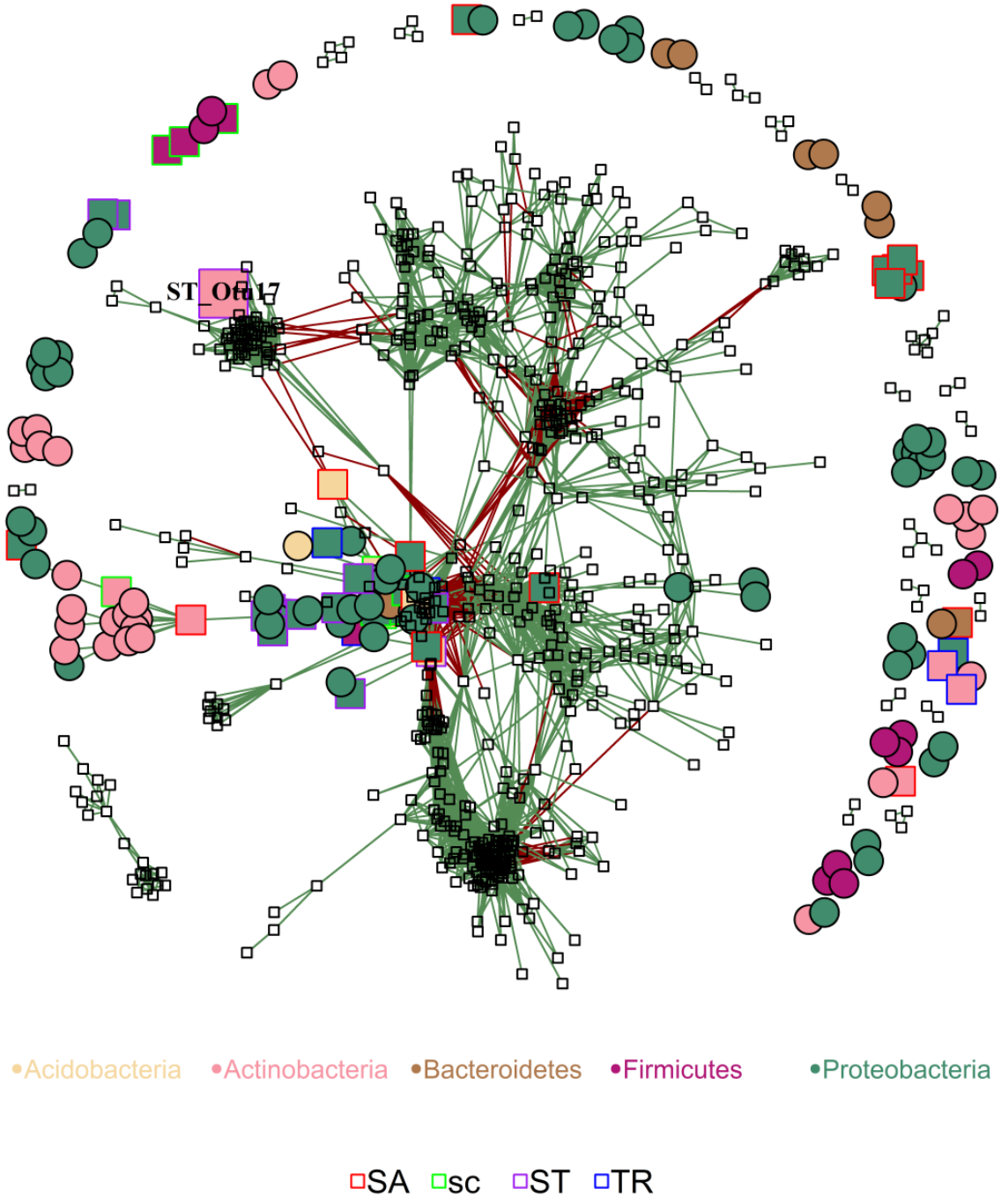


Figure 1-27. Network of Spearman correlations (R>0.6, p<0.005) between OTU abundancies (circles and big squares) and individual compounds (LCMS feature peak areas) (small white squares). Edge colors: green – positive, red – negative correlations. Node colors represent phyla. Large squares with different borderline colors represent OTUs enriched (LEfSe) in: green border – soil control (SC); red – in CC plates with SA strain; violet – with ST strain; blue – with TR strain.

Among OTUs phylogenetically closest to the interacting strains, only Otu17 (closest to ST strain) was present in the network and had multiple positive connections to the groups of compounds, which had direct and indirect (via other compounds) negative connections to OTUs enriched in the plates interacting with SA and belonging to Acidobacteria and Proteobacteria. OTUs representing other two strains SA and TR did not have high correlations (R>0.6) to any LC/MS-MS features nor related OTUs.

Discussion

Upper organic Ah and lower mineral G0 horizons of acid waterlogged gleysol differed in richness, diversity, evennest, as well as relative abundancies at different taxonomical levels. Ah horizon had higher richness, diversity and evennes than Go horizon. The higher bacterial diversity in the upper A horizons than in the lower B horizons was previousely observed for grassland soils and was positively correlated with the organic carbon content (C), the total nitrogen content (N), and the C:N ratio, which decreased with soil depth (Will et al., 2010). Similarly, upper Ah horizon of the soil from our study had higher C, N and C:N values than lower G0 horizon (Kopecky et al., 2011). Furthermore, diversity of actinobacteria community was also reported to be higher in upper than in lower horizon in the same soil (Kopecky et al., 2011). Another factor which may explain higher diversity in upper horizon is pH, which was positively correlated to bacterial richness and diversity in boreal forest soils (Dimitriu et al., 2010). Indeed, lower horizon of Trebon soil has pH 3.1-3,5, while upper horizon has higher pH 4.0-4.2 depending on season (Kopecky et al., 2011). Phyla Proteobacteria, Actinobacteria, Bacteroidetes were more abundant in Ah horizon, while Acidobacteria and Chloroflexi in G0 horizon, which may be explained by different pH and organic matter content in the horizons. On the one hand, lower pH in lower horizon may negatively influence Bacteroidetes and Proteobacteria and positively Acidobacteria (Nakayama et al., 2019), while higher higher content of organic matter in upper horizon may positively contribute to abundancies of *r*-strategists Bacteroidetes and Proteobacteria and negatively to *K*-strategists Acidobacteria (Philippot et al., 2010). Likewise, soil organic carbon, which decreases with the depth, was positively correlated with relative abundance of Actinobacteria and negatively to Chloroflexi in soils with different erosion depth (Zhang et al., 2022).

The most abundant soil genera, except for *Trebonia* described in our study and *Nitrobacter*, belong to unclassified genera of different phyla. This is consistent with the phenomen of the Great Plate Count Anomaly, where cultivation efforts lead only to isolation of less than 1% of total environmental richness (Epstein, 2013). Previousely Trebon Clade 16S rRNA sequences was described as dominant (76%) in actinobacteria clone library in the same soil (Kopecky et al., 2011). In our study, 16S rRNA sequences belonging to the genus *Trebonia* indeed were the most abundant among Actinobacteria,

consisting 25,7% in Ah and 53,5% in G0 horizons. Moreover, *Trebonia* was the most abundant genus in both horizons among all bacterial phyla. *Treboniaceae* may be adpated to low pH, as 16S rRNA sequences belonging to this taxon were found in multiple acidic environments (Rapoport et al., 2020). In addition, *Trebonia* was more abundant in lower horizon characterized by lower pH, lower content of organic matter and higher content of sand, than upper horizon. Similarly, Trebon Clade sequences represented 59% of all actinobacterial clones in forest acidic sandy soil (Kabelitz et al., 2009). Furthermore, the only described isolated strain of Trebonia was slowly-growing acidiphilic oligotroph (Rapoport et al., 2020).

The differences in relative abundancies of actinobacteria genera between horizons were mostly caused by much higher abundance of *Mycobacterium*, *Allostreptomyces* and *Streptomyces* in upper horizon and *Actinospica* in lower horizon. Similar differences were previousely observed in actinobacteria clone library (Kopecky et al., 2011). These differences were reflected at isolate level, when all strains belonging to *Streptomyces* were isolated from upper horizon and all strains belonging to *Actinospica* from lower horizon on VL55 medium. Interestingly, isolates belonging to *Actinospica* and *Catenulispora* had low 16S rRNA sequence similarity with described species and may represent novel species.Moreover, strains of *Actinospica* belonged to two phylogenetic clusters based on the long or short incubation time. The isolation of the putatively novel species within these genera may be explained by limited number of described isolates within *Catenulisporaceae* (donadio et al., 2005), use of not common medium for previousely uncultivable bacteria (Joseph et al., 2003), unusual acid soil source with the dominance of rare actinobacteria and different incubation time. For example, there is only three currently described species of *Actinospica* that were isolated after a long incubation period (Cavaletti et al., 2006; Golinska et al., 2015). While strains isolated on neutral media routinely used for isolation of actinobacteria (mineral agar Gauze 1, R2A, HV agar) belonged to previously described species, many strains of the common genus *Streptomyces* isolated on VL55 clustered separately from described species. The novelty of isolates may be explained by the acid pH used for isolation. In particular, two putatively novel species of acidotolerant streptomycetes isolated after a short period (8-17 days) of incubation clustered within a group of previousely described acidophilic *Streptomyces* species isolated from pine forest acidic soils in China on acidified medium with sucrose as carbon source (*Streptomyces guanduensis*, *Streptomyces paucisporeus*, *Streptomyces rubidus* and *Streptomyces yanglinensis*) (Xu et al., 2006). Similarly, among strains

belonging to *Streptacidiphilus*, some isolates putatively represented novel species. As many described *Streptacidiphilus* species were isolated on acidified starch casein agar medium from acidic forest soils (Kim et al., 2003; Cho et al., 2006, 2008; Golinska et al., 2013, 2016) and no connection to incubation time nor horizon was detected in our study, the isolation of putative novel *Streptacidiphilus* species within our study may be connected to the use of different medium based on xylan as a carbon source.

We isolated a representative of Trebon Clade and described a novel species *Trebonia kvetii*, genus *Trebonia* and family *Treboniaceae.* The strain was slowly-growing and oligotrophic, which may be an advantage in oligotrophic soil environment (Kielak et al., 2016). The genome analysis revealed the ability of the strain to utilize recalcitrant carbon sources, high number of genes for aromatic compounds degradation and genes for resistence to antibiotics and toxic compounds. This is in consistence with previousely observation that bacteria carrying aromatic degradation genes had more genes for antibiotic resistance than other bacteria (Xia et al., 2019). Similarly, phenol-degrading slowly-growing oligotrophic yeasts had higher tolerance towards heavy metals (Fernandez et al., 2017). Metagenomic analysis of microcosms also revealed connection between oligotrophy, K-strategy and higher abundance of genes related to resistance to antibiotics and toxic compounds and carbohydrate utilization assuming interference competition potential (Song et al., 2017). Analysis of BGCs for secondary metabolites revealed 11 BGCs with only 8 clusters with low (<18%) or no similarity to known BGCs. This shows great potential for the discovery of novel secondary metabolites produced by *Trebonia*, as expected for the representatives of novel lineages (Hoffman et al., 2018).

Given the separate phylogenetic position, three isolates, *Trebonia kvetii* 15TR583T, *Streptomyces sp.* 15TR67b, and *Streptacidifilus sp*. 15TR831 have been chosen for co-cultivation with all cultivable soil bacteria. In our study, bacterial community structure and metabolites were significantly affected by the presence of the co-cultured Actinobacteria strains. Moreover, bacterial community and metabolites were significantly correlated, which indicates that changes in bacterial community composition were associated with metabolite pool alterations. This is consistent with previous studies which also indicated a strong correlation between microbiome and metabolome in various environments. For example, phytoplankton community composition was shown to shape the bulk pool of bioavailable carbon of the surface ocean based on comparison of metabolites produced by different phytoplankton communities across environmental gradients (Heal et al., 2021). Host-specific microbial communities and metabolite profiles

associated with Scleractinian corals were correlated as well (Sogin et al., 2017). Finally, multiple studies on antimicrobial-producing inoculant bacteria demonstrated altered bacterial community composition after the introduction into the field. For instance, fermentation-promoting *Lactobacillus* strains inoculated into corn silage produced antimicrobials and other substances negatively correlated to reduced abundancies of non-desirable bacteria and positively correlated to other bacteria (Xu et al., 2019). Thus, specific metabolites have a definite effect on specific bacterial groups.

On the other hand, metabolites from the bacterial community influenced the metabolites produced by strains. That is supported by the observation of metabolites unique for plates with pure cultures of strains (PC), either those suppressed or stimulated by the interaction. Silencing of the production of some metabolites, specifically antibiotics, was reported for streptomycetes co-cultured with competitors (Westhoff et al., 2021). In our study, all three strains dynamically adapted their metabolome, silencing the synthesis of metabolites comparable in number to stimulated ones by co-culture. While we cannot be sure if stimulated metabolites were produced by strain or by co-cultured bacteria, we may suggest two mechanisms. First, cryptic BGCs specific for each interaction could be activated both in strains and co-cultured bacteria, as co-cultivation and social signals commonly trigger the synthesis of otherwise silent metabolites (Hoshino et al., 2019; Liu et al., 2020; Westhoff et al., 2021). Second, changed bacterial community and abundancies of respective bacterial groups would change produced metabolites proportion and pool, as species abundance predicts metabolic activity (Noecker et al., 2019)

The presence of interacting strains did not affect the relative abundance of bacterial phyla except for Acidobacteria inhibited by *Streptomyces,* nor the proportion of cultivable OTUs. However, differences between co-culture (CC) and soil control (SC) plates were detected at lower taxonomical levels, including the level of OTUs. The only influence at the phylum level can be explained by the inhibited genus *Granulicella* which dominates Acidobacteria. A similar negative correlation between *Streptomyces* and Acidobacteria was also found in lettuce root microbiome based on OTUs defined at 95%, 97%, and 100%, but not at 90% OTU cut-off levels (Cardinale et al., 2014), which as well suggests the correlation only on the level of genus (Rossi-Tamisier et al., 2015). The importance of comparison of bacterial communities at the level of lower taxonomic units was also demonstrated on bacteria from different iron mining areas, where bacterial

community structures differed significantly at genus and OTU level, but not by phyla (Hong et al., 2015).

Each strain affected the bacterial community and metabolite profile differently. While *Streptacidiphilus* had an overall stimulating effect over the other bacterial taxa, *Streptomyces* had an inhibitory effect. Furthermore, *Streptomyces* inhibited both gram-positive and gram-negative bacteria in agar diffusion tests, whereas *Streptacidiphilus* did not demonstrate any antimicrobial activity. One of the possible explanations for these differences might be the production of novobiocin detected in all plates with *Streptomyces*. Novobiocin, originally obtained from *Streptomyces niveus* (Harris et al., 1955; Tamura et al., 2008), is an antibiotic effective against Gram-positive bacteria but it also has a limited activity against Gram-negative bacteria (May et al., 2017). Moreover, in the correlation network multiple negative correlations coming from OTU17 corresponding to the 16S rRNA sequence of *Streptomyces* 15TR67 strain from our experiment were observed, which further demonstrates the inhibitory interactions. However, novobiocin alone cannot explain that in agar diffusion tests, *Streptomyces* inhibited Gram-positive *Kocuria rhizophila* of Actinobacteria family *Micrococcaceae* at all time points of incubation, but Gram-negative gammaproteobacteria *Escherichia coli* only after 14 days of incubation. Moreover, in co-cultivation with soil bacteria after 14 days of incubation the only significantly inhibited bacterium was as well gammaproteobacteria, *Piscinibacter*, while after 21 and 28 days the inhibited taxa comprised multiple Alphaproteobacteria, Acidobacteria and only one Gram-positive actinobacterium belonging to *unclassified Streptomycetaceae.* Therefore, different mechanisms of inhibition should exist, whether the production of multiple antimicrobials or more competitive resource utilization. Both interference and exploitative competition are possible as streptomycetes possess multiple biosynthetic gene clusters for secondary metabolites (Barka et al., 2015) and even one strain can produce multiple antimicrobials and multiple siderophores (Behie et al., 2017; Challis and Hopwood, 2017), some of which can act synergistically (Challis and Hopwood, 2017). Moreover, streptomycetes may have ecological advantages by ability to utilize recalcitrant nutrients by producing acid and alkaline phosphatases (Solans et al., 2019) and hydrolytic enzymes like cellulases, chitinases, proteases, and especially xylanases (Javed et al., 2021), as xylan is the main carbon source in VL55 medium (Sait et al., 2002). As with longer incubation time nutrients may be depleted, exploitative competition may explain the broad taxonomical spectrum of inhibited bacteria.

Interaction via metabolites significantly affected the antibiotic activity of *Streptomyces* strain in increasing inhibitory zones against gram-positive and decreasing against gram-negative test strains. The increase of antibiotic activity can be explained by the increase in antibiotic synthesis, in antibiotic activity by structure modification or by the production of novel more potent antibiotics. For example, co-culturing fungal endophyte *Paraconiothyrium* with different co-inhabiting fungi led in some interactions to as big as an eightfold increase in production of anticancer paclitaxel (Soliman&Raizada, 2013). Increased antibiotic activity against *Bacillus subtilis* produced in the co-cultures of *Rhizopus peka* and *Bacillus subtilis* (inhibition zone of 25 mm) was previously observed in comparison with that in each of the pure cultures (inhibition zones of 0-15 mm) and explained by enzyme from *B. subtilis* in producing the active antibiotic from *R. peka* (Fukuda et al., 2008). Finally, *Candida albicans* produce volatile antifungal farnesol, but at the presence of skin commensal fungi *Trichophytum rubrum* – more potent dihydrofarnesol (Moody, 2014). Decreased antibiotic activity in co-culture can be explained by antibiotic inactivation by modification or silencing of its production. It was shown that in complex bacterial communities, collective antimicrobial resistance can take place by cooperative inactivation of antibiotics (Vega&Gore, 2014), e.g., by releasing the free beta-lactamase enzyme into their environment (Brook, 2009). Silencing of antibiotic production was demonstrated for one-third of *Streptomyces* isolates in the presence of antagonistic competitor against other *Streptomyces* strains (Westhof et al., 2021). Furthermore, metabolic response was always interaction-specific (Westhof et al., 2021). Therefore, *Streptomyces* strain from our study might produce different antibiotics affecting gram-positive and gram-negative bacteria differently, and interaction outcome might be modified by the presence of co-inhabiting bacteria.

Some structurally related compounds with putative antimicrobial activities were detected in CC, but not PC plates with *Streptomyces*, including different alkaloids and compounds with coumarin rings (Roberts, 2013; Tataringa and Zbancioc, 2019; Yan et al., 2021). Yet, the detected compounds were also detected in CC plates with *Streptacidiphilus*, which did not show inhibitory phenotype in our study. Moreover, the group of compounds structurally related to bioactive compound otenzepad or siderophore schizokenin, were detected both in PC and CC plates with *Streptomyces*. Thus, other not detected or structurally unknown molecules should be responsible for inhibitory phenotype of the tested *Streptomyces*.

Streptacidiphilus enriched the proportion of several Actinobacteria and Proteobacteria orders, while inhibiting only a limited number of genera at each time point. Namely, it had a stimulating effect on Actinobacteria *Catenulisporales*, *Propionibacteriales* and *Streptomycetales*, on *Alphaproteobacteria* including orders *Azospirales*, *Dongiales*, *Elsterales*, *Sphingomonadales* and several genera of different families of *Rhizobiales*, on gammaproteobacterial orders *Salinisphaerales* and *Xanthomonadales*, family *Rhodocyclaceae* and several genera of *Burkholderiaceae* family. The broad stimulation effect was also reflected at high number of compounds detected only in CC plates with *Streptacidiphilus,* including many structurally related compounds with possible biological activities. For example, omethoate has pesticidal and acaricidal activity and increases cancer cells proliferation (Huo et al., 2019). Tetrahidropalmatine is a plant alkaloid which have analgesic and sedative effect for human (Wang&Mantsh, 2012). Cyclamic acid is artificial sweetener produced from cyclohexylamine obtained by the reduction of aniline (Bizzari et al., 1996), toxic compound first derived from indigo plant leaves (Rappoport, 2007). Piperedin alkaloids are known mainly as acutely toxic teratogenic compounds from toxic plants (Green et al., 2012) and may have antifungal activity (Yan et al., 2021). Sesquiterpene quinones have anti-tumor, anti-HIV, and anti-inflammatory activities and have been isolated from sponges (Capon, 1995). Interestingly, the detected compounds are somehow related to plants and not reported for antibacterial activity. Although little is known about plant-associated *Streptacidiphilus*, several studies indicated that this genus is enriched in healthy plant rhizosphere of banana (Fu et al., 2019), ratooning sugarcane (Gao et al., 2019), cadmium hyperaccumulator *Sedum plumbizincicola* (Hou et al., 2021), as well as present as endophyte of native herbaceous plants in Korea (Kim et al., 2012). Thus, *Streptacidiphilus* might be important for some plants and responsible for the production or the stimulation of production by co-inhabiting bacteria of plant-associated bioactive metabolites, which needs further investigations.

There are several possible mechanisms, which can promote the growth and cultivability of bacteria: metabolic cross-feeding, degradation of bacteriocins and antimicrobials preventing the growth, changes in physical parameters of environment (e.g. pH), production of signal molecules and growth factors, like Rpf lysozyme-like protein, during the growth of helper bacterium (Vartoukian et al., 2010). Similarly, peptidoglycan from *Bacillus cereus* stimulated the growth of rhizospheric Bacteroidetes *Cytophaga* and *Flavobacterium* producing extracellular peptidoglycan-hydrolyzing

activity (Peterson et al., 2006). However, the stimulating phenotype of *Streptacidiphilus* cannot be explained from the detected compounds. Physical parameters, including pH should have been stable, as VL55 medium uses a buffer system (Sait et al., 2002).

The differences in responses to the co-culture of *Streptomyces* and *Streptacidiphilus* are intriguing. Although a great degree of strain-level variations may be expected (Belknap et al., 2020), both genera belong to the same family *Streptomycetaceae* and have high a number of the biosynthetic gene clusters (BGCs) for the secondary metabolites with possible antibacterial and antifungal activities (Malik et al., 2020). Yet, despite the phylogenetic relatedness with *Streptomyces*, the genus *Streptacidiphilus* is under-explored with no natural products described so far, except for atypical polyketide spirotetronate, which, however, did not show any biological activity (Elsayed et al., 2020). In our experiment, both *Streptacidiphilus* and *Streptomyces* inhibited only *unclassified Streptomycetaceae* after 28 days of incubation. The inhibition of closely related actinobacteria was reported before for *Pseudonocardia* and for *Streptomyces* as niche defense mechanism (Behie et al., 2017), especially against bacteria with genetic relatedness or overlap in BGCs (Westhof et al., 2021).

In contrast to *Streptomyces* and *Streptacidiphilus* strains, *Trebonia* had the lowest number of changed compounds and OTUs at each time point nor inhibited as many bacterial taxa, as other strains. The metabolite pool of plates with *Trebonia* was also the most similar to soil control. Again, small metabolite pool changes may be correlated to small changes in bacterial community. Furthermore, *Trebonia* had the highest number of compounds unique for PC plates, thereby the production of which have been inhibited by the interaction with other bacteria. We also confirmed that *Treboniales* and, what is more, *Trebonia* genus was the most abundant in the soil among all genera of all phyla, as was found in Kopecky et al., 2011. One possible explanation of small changes is that bacterial community may be "adapted" to metabolites produced by *Trebonia* in real environment, as well as Trebonia adapted metabolite production based on signals from co-cultured cells from the same environment. Likewise, in study of *Streptomyces* against a reporter strain of *Escherichia coli*, the presence of *Streptomyces* strains co-isolated from the same soil in most cases suppressed otherwise the inhibitory interactions enabling competitors coexist as neighbors (Abrudan et al., 2015). In acidic soil, the dominance of *Trebonia* may be explained by the higher-substrate affinity, efficiency of substrate incorporation into biomass and high tolerance to inhibitory chemicals of slowly-growing oligotrophs, which outcompete „fast-growing" bacteria in oligotrophic environments such as bulk soil

(Yin et al., 2022). Likewise, slowly-growing oligotrophic Acidobacteria are abundant in acid soils and peatlands (Dedysh, 2018). Moreover, *Trebonia* possesses multiple genes for chitinases, endoglucanases, endoxylanases precursors (Rapoport et al., 2020), which may give the genus advantages in this type of environment with recalcitrant carbon. A genomic trade-off between the high capacity for resource exploitation versus antibiotic production was demonstrated for *Streptomyces* (Schlatter&Kinkel, 2015). Similarly, *Trebonia* might have invested into exploitative strategy rather than into inhibitory phenotype, which is supported by low number of inhibited taxa.

As previousely mentioned, the genome of *Trebonia kvetii* contains multiple BGCs not similar to known ones including genes for terpene biosynthesis. Indeed, we detected 7b,9-Dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5-oxo-1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-9ah-cyclopropa[3,4]benzo[1,2-e]azulen-9a-yl, tigliane diterpenoid, produced by *Trebonia* strain regardless the interactions with other bacteria. Tigliane diterpenoids are phorbol-like terpenoids derived from plants and exhibit tumor-promoting, inflammatory, antitumor and antivirus activities (Wang et al., 2015). Apart from tigliane, one compound detected in CC and PC plates with *Trebonia* was annotated as Stachybotrysin B. Stachybotrysin B is antiviral from the fungus *Stachybotrys*, structurally containing naphtalene and benzofuran rings (Zhao et al., 2017). These complex compounds may stimulate the growth of specific bacteria with degradation capacity. For example, increased relative abundances of *Pandoraea* and *Pseudomonas* genera were detected a in CC plates with *Trebonia*. Both taxa were reported as xenobiotic and recalcitrant compounds degrading bacteria (Peeters et al., 2019) and was detected among antibiotic-degrading bacterial genera in sludge (Wang et al., 2015; Yang et al., 2020) and enriched in media with bisphenol (Wang et al., 2019) and lignocellulosic biomass (Bandounas et al., 2011).

In the present study, many metabolites even in SC plates diffused freely into the medium and were not only detected in inter-zone but exerted an inhibitory effect on test strains in inhibition testing. Yet, many metabolites might have been produced to the medium but not diffused further to the inter-zone due to the spatially random distribution of colonies of different taxa on the plate surface. Further to that, the great complexity of various interconnected interactions, like QS and QSI signals, affecting antibiotics and siderophore production (Polkade et al.,2016), as well as extracellular QS (Czajkowski, 2009) and antibiotic degrading enzymes (Nicoloff&Andersen, 2015) might further increase the variability in the resulted metabolome. For example, lumichrome was

detected at all incubation times only in half of the SC and CC replicate plates with *Streptomyces* and *Trebonia*, but not with *Streptacidiphilus*. Lumichrome can be synthesized by many bacteria and plants act as plant auxin and as QS signal-mimic for *LasR* type QS system of bacteria (Rajamani et al., 2008), thereby possibly affecting further bacteria in co-cultured system. Therefore, it should be synthesized by some bacteria from soil suspension zone, whether lacking from or not diffused into inter-zones of respective plates or degraded. For instance, the symbiotic bacterial consortium of *Microbacterium paraoxydans* and *Nocardioides nitrophenolicus* can degrade lumichrome (Kanazawa et al., 2020). This can be related to the lack of lumichrome in plates with *Streptacidiphilus*, which stimulated both *Nocardioidaceae* and *Microbacteriaceae.*

Interestingly, some of the detected compounds were observed in cell-to-cell, inter-species or inter-kingdom signaling. For example, cyclodipeptides (CDPs) also detected in our study, were reported to inhibit QS signaling (Holden et al., 2002). In particular, cyclo(Leu-Pro) (cLP) and cyclo(Phe- Pro) (cPP) were detected in all plates with growing bacteria, while cyclo(Pro-Val) (cPL) again only in occasional CC and SC plates and some PC with *Streptomyces*. In addition, cLP and cPP were detected in antiviral active culture filtrates of several genera of lactic acid bacteria (Kwak et al., 2013). Therefore, it is possible that cLP and cPP may serve as common antiviral agent produced by bacteria, rather than having inter-bacterial signaling function.

We also detected QS signal molecule N-(3-hydroxy-octanoyl)-homoserine lactone (OH-C8-HSL) in all SC plates without interacting strains after 14 days of incubation. OH-C8-HSL was described as a QS signal in *Burkholderia, Agrobacterium tumifaciens* (Duerkop et al., 2008), *Xanthomonadaceae*, *Rhodobacteraceae,* and *Hyphomonadaceae* (Zhang et al., 2019), *Vibrio fisheri* (Girard et al., 2019), *P.fluorescens* (Khan et al., 2005). However, none of these producers were detected as inhibited by co-cultured strains, but metabolic profiles changed. Furthermore, *Streptacidiphilus* inhibited only gram-positive *Paenibacillus*, whereas AHL-based QS is typical for gram-negative bacteria (Polkade et al., 2016). Thus, we suggest that actinobacteria strain decreased OH-C8-HSL concentration rather than the proportion of the producing organism. The possible explanation is the production of quorum-quenching enzymes by co-cultured strains or enriched bacteria, such as AHL-lactonases or AHL-acylases (Dong and Zhang, 2005).

Thus, the study demonstrated that each selected strain significantly influenced other members of the bacterial community, shaped metabolite pool and manipulated

signal molecules. Moreover, the diffused metabolites might reach physiologically relevant concentrations and affect mutual interactions with the whole bacterial community. More specifically, *Streptacidiphilus sp.* 15TR831 stimulated many phylogenetically distant bacterial taxa and may be further studied as a biological control agent. In contrast, *Streptomyces sp.* 15TR67b inhibited multiple taxa and possibly produced antibiotics with inhibitory activity for competitive suppression of other bacteria. Finally, *Trebonia* produced unusual metabolites, although having a minimal effect on other cultivable bacteria, when assessed *in vitro* on a solid agar media.

Part II. Characterization of phytopathogenic and biocontrol actinobacteria from the tuberosphere of potatoes coming from different geographical regions

Phytopathogenic actinobacteria causing potato common scab (CS)

Hypothesis : Thaxtomin-bearing Pathogenicity Island (PAI) is not correlated to phylogeny by 16S rRN but to the site of the strain origin as a result of horizontal gene transfer (HGT).

Goal: To characterize PAI types of phytopathogenic actinobacteria in relation to their origin and taxonomic placement

Tasks:

1. Isolate actinobacteria from the tuberosphere of potatoes coming from different geographical locations
2. Determine potentially phytopathogenic isolates based on the positive PCR of the *txtAB* gene fragment representing the potential for thaxtomin production
3. Assess PAI types of pathogenic isolates based on the PCR amplification of other virulence genes

Biocontrol actinobacteria reducing potato common scab

Hypothesis 1: The most successful biocontrol strains have strong inhibitory activity against phytopathogen and high growth which can be tested *in vitro*.

Hypothesis 2: Biocontrol strains affect soil microbial community towards selection of suppressive taxa

Goal: To develop approach for a selection of effective biocontrol strains and demonstrate their effect on soil microbial communities

Tasks:

1. Test actinobacteria for inhibitory activity against phytopathogenic strains *Streptomyces scabiei* and *S.acidiscabies*
2. Measure growth of antagonistic strains in vermiculite
3. Select the strongest antagonists with high growth rates to assess the ability to reduce CS severity in pot assay and affect the indigenous microbial community

Materials and Methods

Isolation of actinobacteria from potato tuberosphere

Actinobacteria were isolated from common scab lesions of freshly collected potato samples from different agricultural fields in the Czech Republic (Velhartice, Havlickova Borova, Slavkov, Trutnov) using plate serial dilution on Gauze's agar 1 (Gauze et al., 1983) and R2A medium in 2014. Actinobacteria from 40 localities in South America (natural sites in continental Chile and Chiloe island, and agricultural fields in Argentina) were isolated from air-dried potato-peel samples on R2A medium. The cultivation media were supplemented with cycloheximide (300 mg/L), planted with 100 mL of a solution of vortexed potato peels in sterile deionized water in serial dilutions of 10^{-3}, 10^{-4}, 10^{-5} and incubated at 28°C for 1 week.

Analysis of PAI

The procedures for DNA extraction and sequence analysis of the 16S rRNA gene were the same as described in Part I (section 1.1.1). The isolate was designatedto be phytopathogenic based on the positive PCR amplification of a fragment of *txtAB* (*txtA* and *txtB*) genes, which are pathogenicity determinants in the main scab-causing *Streptomyces* species (Flores-Gonzalez et al., 2008). Phytopathogenic isolates were further subjected to amplification of other genes typical for pathogenicity islands (Table 2-1). The temperature of annealing was adjusted based on the gradient PCR with *S.turgidiscabies* DSM 41838 and *S.scabiei* DSM 41658.

The phylogenetic trees based on 16S rRNA were annotated based on metadata (location, CS symptoms) and resulted presence/absence PCR products of the virulence genes using *iTOL* (Letunik&Bork, 2021) and *ggtree* package (Yu, 2020) in *R* software (R Core Team, 2020). The amplified fragments of the complementary virulence genes were sequenced (Sanger, Macrogen) and after quality control and trimming, the sequences were aligned for comparison using *mafft* (Katoh&Standley, 2013) and blasted against NCBI database. Pairwise distances between aligned DNA sequences were calculated using *mothur v.1.48.0* (Schloss et al., 2009). Visualization and statistics calculation was performed in *R 4.0.3* (R Core Team, 2020).

Table 2-1. Primers used for the amplification of virulence genes from the pathogenicity island bearing genes for the thaxtomin production

PCR primers	Sequence	T ann. (ref)	T ann. (adj)	target	product length (bp)	reference
Stx1a	GTGGACCGTGGAGCATCT	60	58	*txtA – txtB*	400	Flores-Gonzalez et al. 2008
Stx1b	CAGTTCGGCGTAACTCAGC					
txtBC1_F	CCTTCACACCCTGRACAT	58	55	*txtB – txtC*	1400-1500	Kopecky, unpublished
txtBC1_R	AGTTCGGTGAAGTTGGGY					
Nf	ATGAGCGCGAACGGAAGCCCCGGA	60	58	*Nec1*	700	Bukhalid et al. 1998
Nr	GCAGGTCGTCACGAAGGATCG					
Tom3	GAGGCGTTGGTGGAGTTCTA	55	58	*TomA*	392	Wanner, 2006
Tom4	TTGGGGTTGTACTCCTCGTC					
P11_f	ATAGACGATGACGAGCCCTG	55	55	*fas5*	1338	Aittamaa et al., 2010
P11_r	GCTCTGGTGAGGTGTTCTCC					
P19_f	GTAGGTGGGACCAGGTGAGA	55	58	*fas6*	576	Aittamaa et al., 2010
P19_r	GGGGCTGACGGAACTACAC					

Selection and assessment of biocontrol activities of antagonistic actinobacteria (Publication IV)

Methods for *in vitro* inhibition and growth testing, pot experiment and microbial communities assessment are described in Publication IV (Patrmanova et al., 2021).

Genome assembly and annotation of successful biocontrol strain Streptomyces sp. 09ZI22

Streptomyces sp. 09ZI22 identified as a potential biocontrol agent against common scab of potato in series of pot experiments (Patrmanova et al., 2022) was grown on GYM agar medium at 28°C for three days. Genomic DNA was isolated by phenol-chloroform extraction after mechanical disruption of cells frozen in liquid nitrogen (Lee et al., 2003). DNA was sequenced by Illumina MiSeq sequencer (DNA Services Facility, Research Resources Center, University of Illinois) with 245-fold genome coverage. Quality of paired-end 2x250 bp reads was assessed using FastQC v. 0.11.9 (Andrews, 2010). Genome was assembled using GenomeFinisher v. 1.4. with NCBI Blast 2.5.0. GFinisher refines and finalizes prokaryotic genomes assemblies using the bias of GC Skew to identify assembly errors and organizes the contigs/scaffolds with genomes references (Guizelini et al., 2016). As a reference genome, the complete genome of *Streptomyces fulvissimus* DSM 40593 chromosome, 7,905,758 bp long (accession

number CP005080.1) was used. A target *de novo* assembly was performed in Patric 3.6.9. web server (Davis et al., 2020) using TrimGalore 0.6.5 for trimming reads before assembly (Krueger et al., 2015), Unicycler v. 0.4.8 for assembly (Wick et al., 2017) and Pilon 1.23 for polishing with default settings (Walker et al., 2014). An alternative *de novo* assembly was derived from the trimmed by *fastp* 0.20.1 (Chen et al., 2018) paired-end Illumina MiSeq reads using SPAdes 3.13.0 with default parameters (Bankevich et al., 2012). Quality of genome assemblies was assessed using QUAST v. 5.1.0 (Gurevich et al., 2013). Genome was annotated using RAST tool kit (RASTtk) in Patric 3.6.9. web server (Davis et al., 2020) and NCBI Prokaryotic Genome Annotation Pipeline (PGAP) 5.2 in NCBI (Tatusova et al., 2016). Essential genes were identified using The Antibiotic Resistant Target Seeker (ARTS) comparative pipeline and TIGRfam Equivologs implemented in ARTS server (Alanjary et al., 2017). Secondary metabolite gene clusters were predicted using Antibiotics & Secondary Metabolite Analysis SHell (antiSMASH) (Blin et al., 2021). The evidence of inter-genus horizontal transfer was inferred based on phylogenetic incongruence of essential genes also in ARTS server. Briefly, core genes from phyla specific complete reference genomes are aligned using MAFFT-linsi method. Protein alignments and nucleotide sequences are run with pal2nal (Suyama et al., 2006) to produce codon alignments, trimmed with *trimal* (Capella-Gutiérrez et al., 2009). Final evolutionary trees are inferred in RaxML using General Time Reversible (GTR) substitution model and GAMMA distribution of site rate variation (Stamatakis et al., 2015). 16S rRNA sequence was extracted from GenBank annotation file and searched for the similarity using BLASTn against NCBI database. Plasmids were assembled from WGS data using plasmidSPAdes v. 3.13.0 (Antipov et al., 2016) in Patric 3.6.9. web server. BLASTn search was performed on the resulted contigs against NCBI database sequences to check the similarity with publicly available plasmid nucleotide sequences.

Results (phytopathogenic actinobacteria)

In a total, 275 actinobacteria strains isolated from potatoes originating in the Czech Republic and 240 strains from potatoes coming from South America were chosen for the DNA extraction and tested for the presence of *txtAB* region.

Most of the actinobacterial isolates belonged to the genus *Streptomyces*: 75% strains from the Czech Republic, 92% strains from Argentina and Chile, and 95% of isolates from Chiloe island. Other genera of actinobacteria (called rare actinobacteria) were isolated from Chiloe island) (*Nocardia*), Argentina (*Amycolatopsis, Nocardia*), and Chile (*Brevibacterium, Cutibacterium, Micromonospora, Nocardia*), while from the Czech Republic 15 other genera than streptomycetes belonged to *Arthrobacter, Catellatospora, Glycomyces, Krasilnikoviella, Kribbella, Lentzea, Microbacterium, Micromonospora, Nocardioides, Nonomuraea, Paenarthrobacter, Promicromonospora, Pseudarthrobacter, Saccharothrix, Streptosporangium* (Fig.2.1).

Out of those, 20% of isolates (105 out of 515 strains) had *txtAB* region: 15% (40/275) of strains from the Czech Republic, 27% (20/73) of strains from Argentina, 28% (22/80) of strains from Chile, and 27% (23/87) of strains from Chiloe island. All *txtAB*-positive isolates belonged to the genus *Streptomyces* (Table 2.1, Fig. 2.2).

On average, half of the *txtAB*-positive isolates clustered close to or had the highest sequence similarity based on the 16S rRNA gene with streptomycetes previousely described as scab-causing in the literature (later referred as known scab-causing species) (Fig. 2.2, Table 2.1, 2.2). While 14 and 35% of phytopathogenic isolates from Chile and Chiloe island respectively had the closest BLAST match with only species *S. caviscabies*, 65 and 70% of *txtAB*-positive strains from Argentina and Czech Republic respectively had the closest BLAST matches with 7 known scab-causing species. The majority (58%) of phytopathogenic isolates from the Czech Republic have been identified as *S.europaeiscabiei*, while the diversity of known scab-causing strains from Argentina were more equally represented by *S.europaeiscabiei* (20%), *S.stelliscabies* (15%)„ *S.turgidiscabies* (10%), *S.caviscabies* (10%), *S.acidiscabies* (5%), *S. scabiei* (5%)„ (Table 2.2).

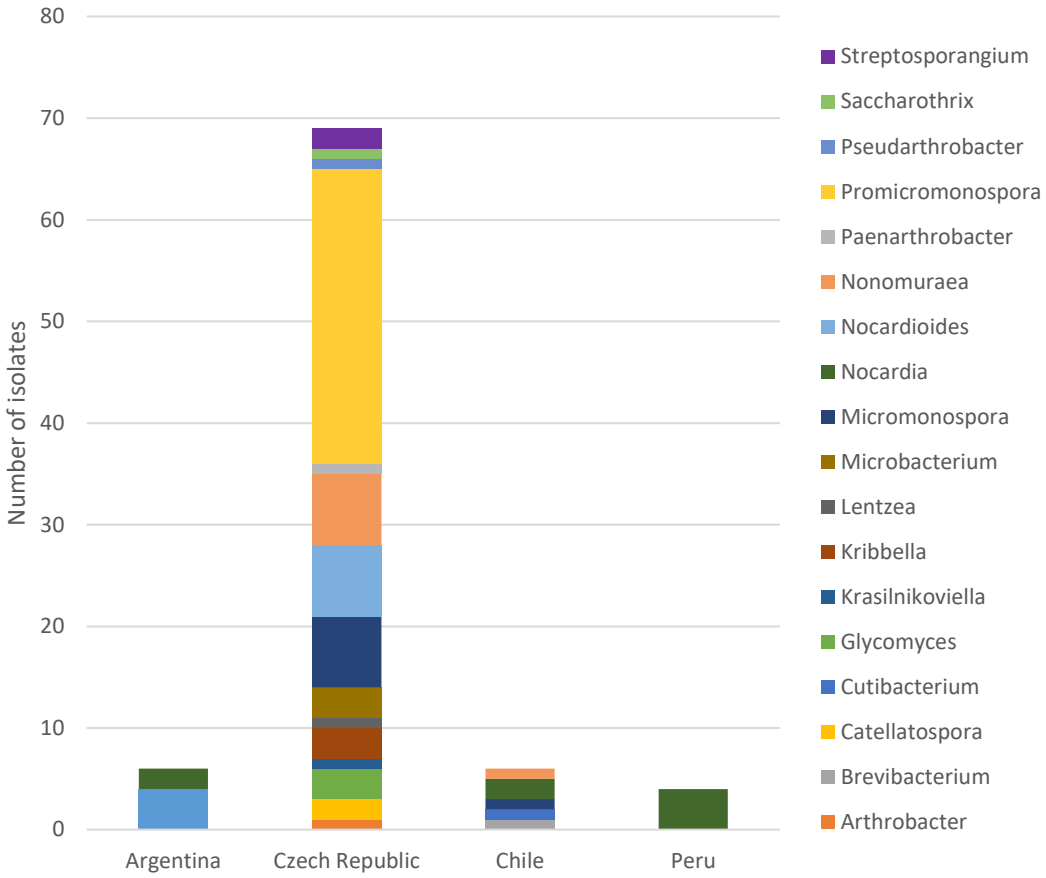


Fig.2.1. Isolates belonging to rare actinobacteria

Based on the presence of different virulence genes, 10 types of PAI compositions were detected. Primers P11 for the *fas5* gene gave different products of variable lengths and were omitted from the analysis. Within all possible compositions, only compositions with amplified *txtBC* region together with all other virulence genes including *fas6* were not detected among the phytopathogenic isolates (Table 2.3). Isolates clustered based on the presence or absence of txtBC in two large clusters (Fig.2.3). A cluster of isolates with the detected *txtBC* region has been mostly (76%) identified within known scab-causing streptomycetes. A cluster of isolates with no amplified *txtBC* had a subcluster of isolates lacking other genes and represented mostly (68%) by known phytopathogens and subclusters with different gene combinations represented only partly (22%) by known scab-causing streptomycetes (Fig.2.3).

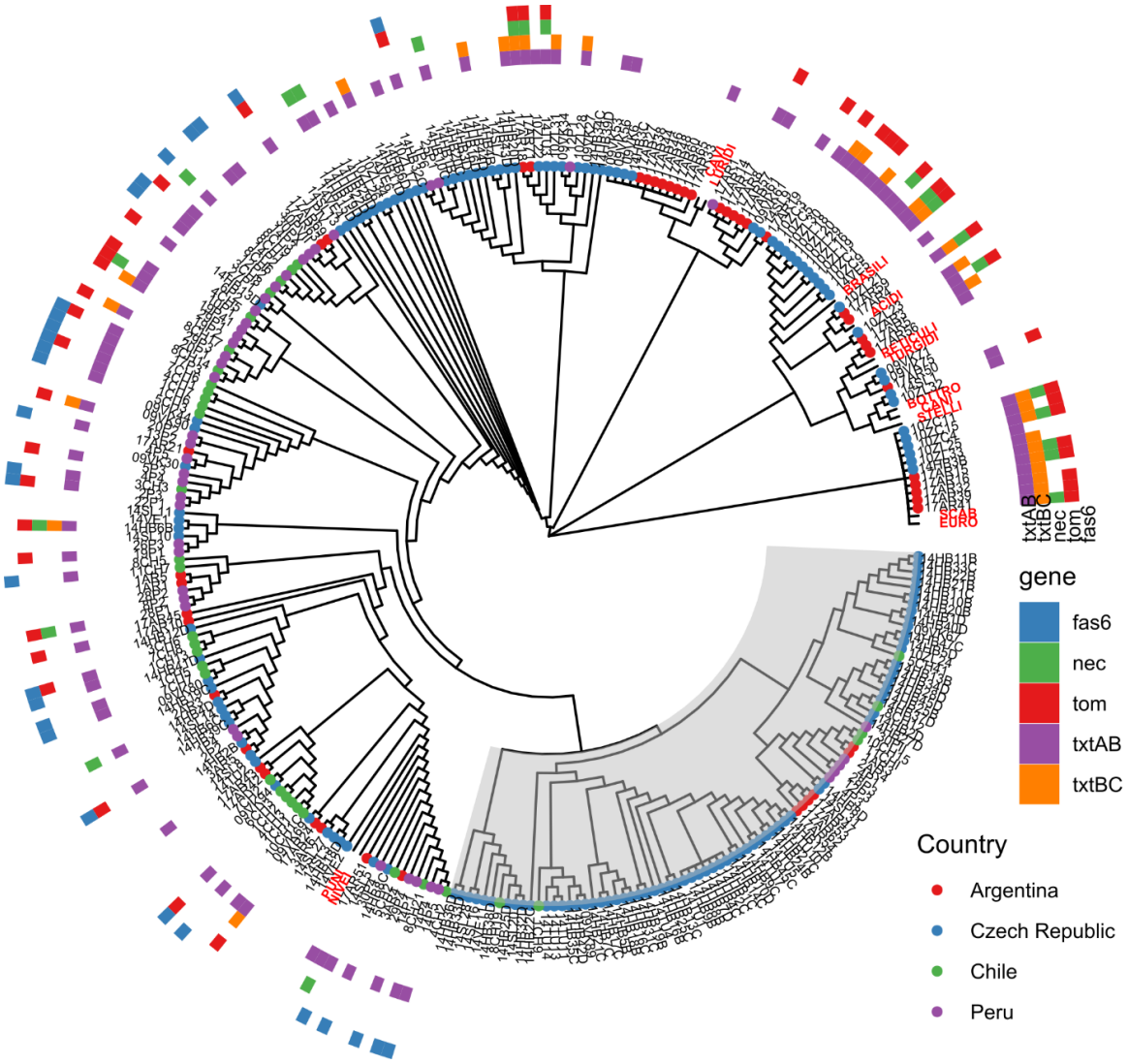


Fig.2.2. Phylogenetic tree of actinobacterial strains isolated from tuberosphere of potato originating from different countries (different tree tips colors). This cladogram was inferred using the maximum-likelihood method (FastTree) under the GTR+GAMMA model based on the partial 16SrRNA gene alignment. Outer circles indicate the presence of the appropriate genes from the thaxtomin-bearing pathogenicity islands. Non-streptomycetes are highlighted in grey. Known scab-causing streptomycetes are labeled in red: ACIDI - *S.acidiscabies* ATCC 49003T (D63865.1); SCAB - *S.scabiei* ATCC 49173T (NR_025865.2); BOTTRO - *S.bottropensis* NBRC 13023 (AB184262); BRASILI - *S.brasiliscabiei* IBSBF 2867 (KY783755); CANI - *S.caniscabiei* NE06-02F (MT360639); CAVI - *S.caviscabies* strain 8 (KU743151); EURO - *S.europaeiscabiei* KACC 20186T (NR_042790.1); NIVEI - *S.niveiscabiei* KACC 20254T (NR_037095.1); PUNI - *S.puniciscabiei* LMG 21391T (NR_025156.1); RETICULI - *S.reticuliscabiei* CFBP 4531T (NR_025293.1); STELLI - *S.stelliscabiei* CFBP 4521T (NR_025294.1); TURGIDI - *S.turgidiscabies* ATCC 700248T (NR_040828.2)

Table 2.1. List of *txtAB*-positive actinobacteria isolates

N	Strains	gene				Country	NCBI Blast closest match	Accession	Identity, %	Alignment length, bp
		txtBC	*nec*	*tom*	*fas6*					
1	17AR10	0	0	1	0	Argentina	*Streptomyces cyaneus*	NR_114820.1	100.000	1342
2	17AR15	1	1	1	0	Argentina	*S.stelliscabiei*	NR_116532.1	100.000	1347
3	17AR16	1	0	0	0	Argentina	*S.stelliscabiei*	NR_116532.1	99.926	1351
4	17AR17	0	0	1	0	Argentina	*S.cyaneus*	NR_114820.1	100.000	1346
5	17AR18	1	1	1	0	Argentina	*S.scabiei*	NR_025865.2	98.838	1119
6	17AR21	0	0	0	1	Argentina	*S.caviscabies*	NR_115449.1	99.446	1084
7	17AR24	1	0	1	0	Argentina	*S.graminifolii*	NR_134196.1	100.000	1333
8	17AR3	0	1	1	0	Argentina	*S.europaeiscabiei*	NR_116533.1	100.000	1326
9	17AR32	1	0	1	0	Argentina	*S.europaeiscabiei*	NR_116533.1	100.000	1347
10	17AR33	0	0	0	0	Argentina	*S.caviscabies*	NR_115449.1	99.926	1346
11	17AR39	1	0	1	0	Argentina	*S.europaeiscabiei*	NR_116533.1	100.000	1347
12	17AR4	1	1	1	0	Argentina	*S.acidiscabies*	NR_025866.2	100.000	1323
13	17AR41	1	1	1	0	Argentina	*S.stelliscabiei*	NR_116532.1	99.926	1347
14	17AR5	1	0	0	0	Argentina	*S.turgidiscabies*	NR_040828.2	99.844	1279
15	17AR56	0	0	0	0	Argentina	*S.turgidiscabies*	NR_040828.2	99.923	1307
16	17AR71	1	1	1	0	Argentina	*S.europaeiscabiei*	NR_042790.1	99.436	1063
17	17AR8	0	0	0	0	Argentina	*S.cyaneus*	NR_114820.1	100.000	1346
18	1AR1	0	0	0	1	Argentina	*S.cangkringensis*	NR_117959.1	99.632	1086
19	1AR5	0	0	0	1	Argentina	*S.aldersoniae*	NR_116223.1	99.077	1084
20	2AR3	0	0	0	1	Argentina	*S.canus*	NR_043347.1	99.812	1062
21	09VK53	0	0	0	0	Czechia	*S.caviscabies*	NR_115449.1	100.000	1346
22	09VK56	0	0	0	0	Czechia	*S.caviscabies*	NR_115449.1	100.000	1346
23	09VK69	0	0	0	0	Czechia	*S.lincolnensis*	NR_119101.1	98.323	1312
24	10ZC1	0	0	0	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.752	1210

25	10ZC11	1	1	1	0	Czechia	*S.europaeiscabiei*	NR_116533.1	100.000	1347
26	10ZC15	1	0	1	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.926	1347
27	10ZC3	0	0	1	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.918	1216
28	10ZC4	1	1	1	0	Czechia	*S.europaeiscabiei*	NR_116533.1	100.000	1347
29	10ZC5	1	0	0	0	Czechia	*S.scabiei*	NR_025865.2	99.917	1204
30	10ZC9	1	1	1	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.926	1347
31	10ZL16	0	0	0	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.852	1347
32	10ZL17	0	0	0	0	Czechia	*S.europaeiscabiei*	NR_042790.1	99.719	1066
33	10ZL18	0	0	1	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.627	1342
34	10ZL19	0	1	1	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.852	1347
35	10ZL21	0	0	0	0	Czechia	*S.rishiriensis*	NR_112392.1	99.768	1294
36	10ZL22	0	0	0	0	Czechia	*S.europaeiscabiei*	NR_042790.1	99.631	1085
37	10ZL23	1	0	0	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.925	1340
38	10ZL25	0	0	0	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.926	1347
39	10ZL26	0	0	0	0	Czechia	*S.europaeiscabiei*	NR_116533.1	98.827	1194
40	10ZL28	1	0	0	0	Czechia	*S.europaeiscabiei*	NR_042790.1	99.539	1085
41	10ZL29	1	0	0	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.835	1211
42	10ZL30	1	0	1	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.918	1215
43	10ZL31	1	0	0	0	Czechia	*S.europaeiscabiei*	NR_042790.1	99.618	1048
44	10ZL32	0	0	0	0	Czechia	*S.bottropensis*	NR_115571.2	100.000	1216
45	10ZL33	1	0	0	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.926	1347
46	14HB19D	0	1	0	0	Czechia	*S.fructofermentans*	NR_043344.1	98.773	1222
47	14HB1B	0	1	0	0	Czechia	*S.avidinii*	NR_115452.1	95.978	1069
48	14HB20D	1	0	0	0	Czechia	*S.europaeiscabiei*	NR_116533.1	98.883	1253
49	14HB22D	0	1	0	0	Czechia	*S.fructofermentans*	NR_043344.1	98.934	1219
50	14HB3B	1	1	1	0	Czechia	*S.europaeiscabiei*	NR_116533.1	100.000	1347
51	14HB44D	1	0	0	0	Czechia	*S.cyaneofuscatus*	NR_115383.1	98.473	1244

52	14HB4D	0	1	0	0	Czechia	*S.galilaeus*	NR_040857.1	97.720	1316
53	14HB6b	1	1	1	0	Czechia	*S.finlayi*	NR_043354.1	99.851	1338
54	14SL1	0	0	1	0	Czechia	*S.bottropensis*	NR_115571.2	99.923	1295
55	14SL5	0	1	0	0	Czechia	*S.collinus*	NR_041063.1	99.830	1177
56	14SL9	0	0	1	0	Czechia	*S.argenteolus*	NR_112300.1	99.926	1346
57	14TU2	0	0	0	0	Czechia	*S.rishiriensis*	NR_112392.1	99.699	1330
58	14TU3	0	1	1	0	Czechia	*S.rishiriensis*	NR_112392.1	98.566	1116
59	14VE5	1	1	1	0	Czechia	*S.europaeiscabiei*	NR_116533.1	99.925	1341
60	14VE6	1	0	0	0	Czechia	*S.europaeiscabiei*	NR_042790.1	99.524	1050
61	11CH7	0	0	0	1	Chile	*S.drozdowiczii*	NR_041424.1	98.988	1087
62	17AR51	0	0	0	0	Chile	*S.coelicoflavus*	NR_115371.1	100.000	1338
63	1CH1	0	0	0	1	Chile	*S.olivochromogenes*	NR_112483.1	99.124	1027
64	1CH13	0	0	0	1	Chile	*S.niveus*	NR_115784.1	98.427	1208
65	1CH2	0	0	1	1	Chile	*S.olivochromogenes*	NR_112483.1	99.229	1038
66	1CH5	0	0	0	1	Chile	*S.camponoticapitis*	NR_152020.1	99.522	1045
67	1CH6	0	0	0	1	Chile	*S.niveus*	NR_115784.1	99.326	1039
68	1CH7	0	0	1	1	Chile	*S.niveus*	NR_115784.1	98.532	1226
69	3CH3	0	0	0	1	Chile	*S.caviscabies*	NR_115449.1	99.531	1066
70	3CH8	0	0	0	1	Chile	*S.caviscabies*	NR_115449.1	98.085	1201
71	4CH1	0	0	0	1	Chile	*S.caviscabies*	NR_115449.1	99.904	1042
72	4CH2	0	0	0	1	Chile	*S.anthocyanicus*	NR_115390.1	99.437	1066
73	4CH3	0	0	0	1	Chile	*S.olivochromogenes*	NR_112483.1	99.447	1085
74	5CH2	0	0	0	1	Chile	*S.violaceolatus*	NR_112370.1	99.816	1085
75	5CH3	0	0	0	1	Chile	*S.niveus*	NR_115784.1	98.346	1209
76	6CH3	0	0	0	1	Chile	*S.olivochromogenes*	NR_112483.1	99.807	1037
77	7CH6	0	0	0	1	Chile	*S.niveus*	NR_115784.1	99.173	1209
78	7CH7	0	0	0	1	Chile	*S.camponoticapitis*	NR_152020.1	99.171	1086

79	7CH8	0	0	1	1	Chile	*S.niveus*	NR_115784.1	99.004	1205
80	8CH1	0	0	0	1	Chile	*S.anthocyanicus*	NR_115390.1	99.127	1146
81	8CH5	0	0	0	1	Chile	*S.drozdowiczii*	NR_041424.1	99.252	1069
82	8CH6	0	0	0	1	Chile	*S.sampsonii*	NR_025870.2	98.584	1059
83	12P2	0	0	1	0	Chiloe	*S.sampsonii*	NR_025870.2	98.986	1085
84	14P1	0	0	0	1	Chiloe	*S.anthocyanicus*	NR_115390.1	99.262	1084
85	14P2	0	1	0	1	Chiloe	*S.anthocyanicus*	NR_115390.1	99.448	1087
86	14P3	0	0	0	1	Chiloe	*S.anthocyanicus*	NR_115390.1	98.597	1212
87	14P4	0	0	0	1	Chiloe	*S.violaceolatus*	NR_112370.1	99.264	1087
88	14P5	0	0	0	1	Chiloe	*S.anthocyanicus*	NR_115390.1	99.530	1064
89	17P3	0	0	1	1	Chiloe	*S.sanglieri*	NR_041417.1	97.601	1209
90	19P1	0	0	1	0	Chiloe	*S.olivoviridis*	NR_112325.1	99.353	1082
91	19P2	1	0	1	0	Chiloe	*S.caviscabies*	NR_115449.1	99.904	1040
92	19P41	0	0	1	0	Chiloe	*S.caviscabies*	NR_115449.1	99.631	1085
93	1P4	0	0	1	1	Chiloe	*S.galilaeus*	NR_040857.1	99.087	1205
94	1P6	0	0	1	1	Chiloe	*S.caviscabies*	NR_115449.1	99.327	1188
95	1PIV	0	0	1	1	Chiloe	*S.sampsonii*	NR_025870.2	99.327	1189
96	20P1	1	0	1	0	Chiloe	*S.caviscabies*	NR_115449.1	99.355	1085
97	20P2	1	0	1	0	Chiloe	*S.cyaneus*	NR_114820.1	99.446	1084
98	27P1	0	0	0	1	Chiloe	*S.atratus*	NR_112503.1	99.355	1086
99	28P1	0	1	1	0	Chiloe	*S.microflavus*	NR_103947.1	99.352	1081
100	29P1	0	1	1	0	Chiloe	*S.caviscabies*	NR_115449.1	99.904	1040
101	2P3	0	0	1	1	Chiloe	*S.caviscabies*	NR_115449.1	99.262	1084
102	30P4	0	0	0	1	Chiloe	*S.albogriseolus*	NR_042760.1	99.215	1147
103	30PX	0	0	0	1	Chiloe	*S.sampsonii*	NR_025870.2	98.233	1132
104	5P3	0	0	1	0	Chiloe	*S.caviscabies*	NR_115449.1	99.448	1086
105	6P2	0	0	1	0	Chiloe	*S.caviscabies*	NR_115449.1	99.167	1081

Table 2.2.Share of *txtAB*-positive isolates (%) identified as known scab-causing streptomycetes based on 16S rRNA sequence similarity > 99%

NCBI BLAST match	Argentina	Czechia	Chile	Peru	Total
Streptomyces europaeiscabiei	20	58	0	0	26
Streptomyces caviscabies	10	5	14	35	14
Streptomyces stelliscabiei	15	0	0	0	3
Streptomyces bottropensis	0	5	0	0	2
Streptomyces scabiei	5	3	0	0	2
Streptomyces turgidiscabies	10	0	0	0	2
Streptomyces acidiscabies	5	0	0	0	1
Total	65	70	14	35	50

Some PAI types were detected only in isolates from the Czech Republic and Argentina: composition 1 (*txtAB*), 2 (*txtAB*+*txtBC*), 10 (*txtAB*+*txtBC*+*tom*+*nec*), while composition 7 (*txtAB*+*tom*+*fas6*) were detected only in isolates from Chile and Chiloe island. All isolates from Chile had *fas6* gene.

The PAI type was significantly related to the origin of the phytopathogen (Chi-squared (27, 105) = 96.942, p-value = p < .001). Phytopathogens coming from Chile were more likely to have PAI composition 4 (with only *fas* as an additional virulence factor). Samples from Argentina were likely to have composition 10 (with *tom* and *nec*). Phytopatogens from Chiloe island were likely to have compositions 3 and 7 (both with *tom*) and unlikely compositions 1, 2, 10 (without additional genes or with *txtBC* detected). Finally, phytopathogenic streptomycetes from the Czech Republic were likely to have compositions 1, 2 and 5 and unlikely 4 and 7 (having *fas*) (Fig.2.4).

Pairwise distances based on the 16S rRNA gene differed between isolates with different PAI compositions (Kruskal-Wallis chi-squared = 1410.1, df = 53, p-value <.001). Pairwise 16S rRNA distances were significantly lower than average distances between phytopathogenic strains both having PAI compositions 2 or 10 (pairwise Wilcox-test, p-adjusted (Benjamini-Hochberg) <.001, large effect size >.6) (Fig.2.5).

Table 2.3. Number of *txtAB*-positive isolates from the Czech Republic (CR), Argentina (AR), Chile (CH), and Chiloe island (CI) with all possible PAI compositions

N	PAI composition	gene					Number of isolates			
		txtAB	***txtBC***	***tom***	***fas6***	***nec***	***CR***	***AR***	***CH***	***CI***
1	*txtAB* +	+	-	-	-	-	13	3	0	0
2	*txtAB* + *txtBC* +	+	+	-	-	-	8	2	0	0
3	*txtAB* + *tom* +	+	-	+	-	-	4	2	0	5
4	*txtAB* + *fas6* +	+	-	-	+	-	0	4	18	7
5	*txtAB* + *nec* +	+	-	-	-	+	5	0	0	0
6	*txtAB* + *txtBC* + *tom* +	+	+	+	-	-	2	3	0	3
7	*txtAB* + *tom* + *fas6* +	+	-	+	+	-	0	0	3	5
8	*txtAB* + *tom* + *nec* +	+	-	+	-	+	2	1	0	2
9	*txtAB* + *fas6* + *nec* +	+	-	-	+	+	0	0	0	1
10	*txtAB* + *txtBC* + *tom* + *nec* +	+	+	+	-	+	6	5	0	0
11	*txtAB* + *txtBC* + *nec* +	+	+	-	-	+	0	0	0	0
12	*txtAB* + *txtBC* + *fas6* +	+	+	-	+	-	0	0	0	0
13	*txtAB* + *txtBC* + *tom* + *fas6* +	+	+	+	+	-	0	0	0	0
14	*txtAB* + *txtBC* + *fas6* + *nec* +	+	+	-	+	+	0	0	0	0
15	*txtAB* + *txtBC* + *tom* + *fas6* +	+	+	+	+	-	0	0	0	0
16	*txtAB* + *txtBC* + *tom* + *fas6* + *nec* +	+	+	+	+	+	0	0	0	0

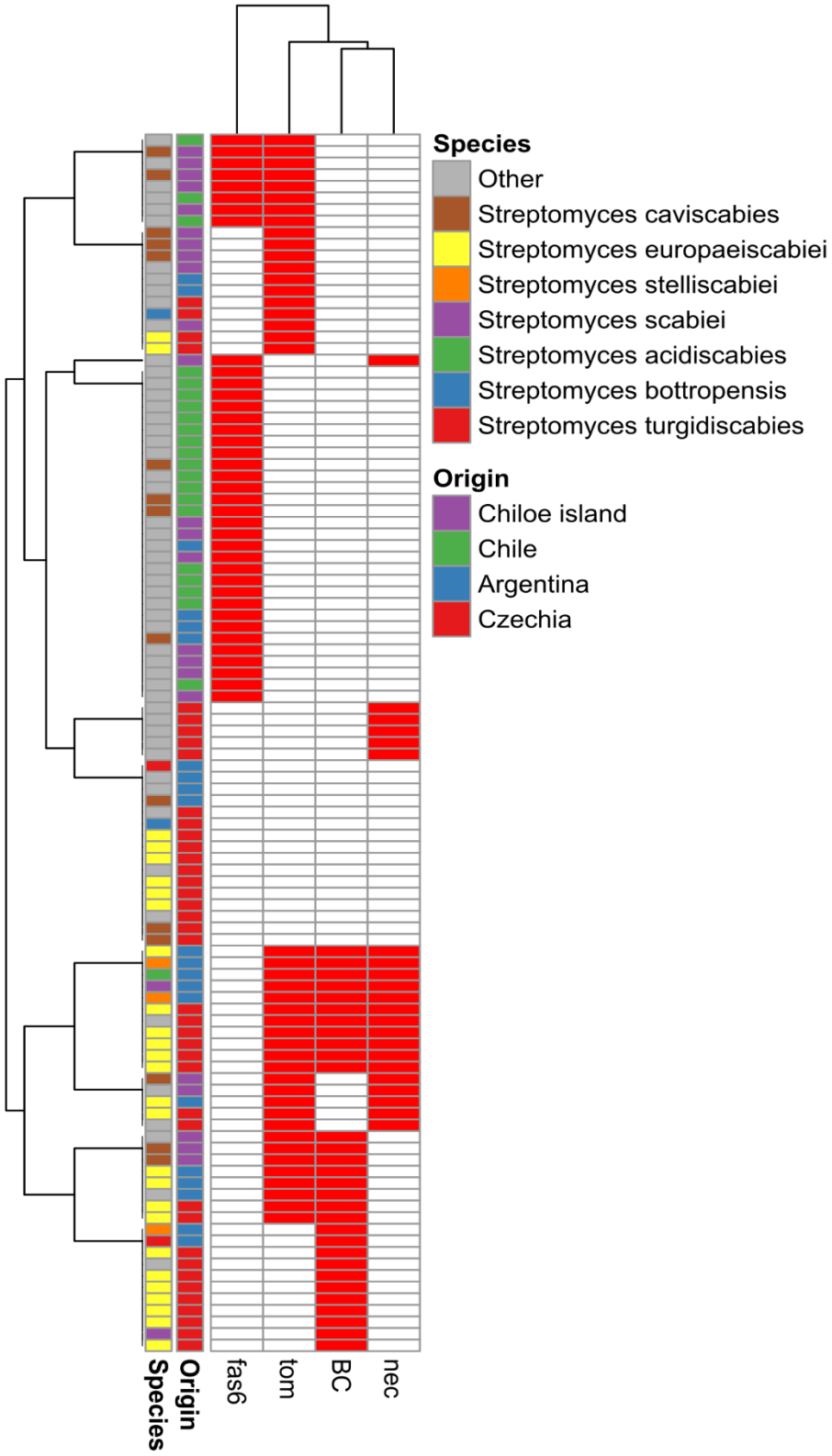


Fig.2.3. Hierarchical clustering of *txtAB*-positive isolates based on the presence/absence of genes within thaxtomin-conferring PAI. Isolates are identified as known scab-causing species based on 16S rRNA sequence similarity (closest NCBI BLAST match).

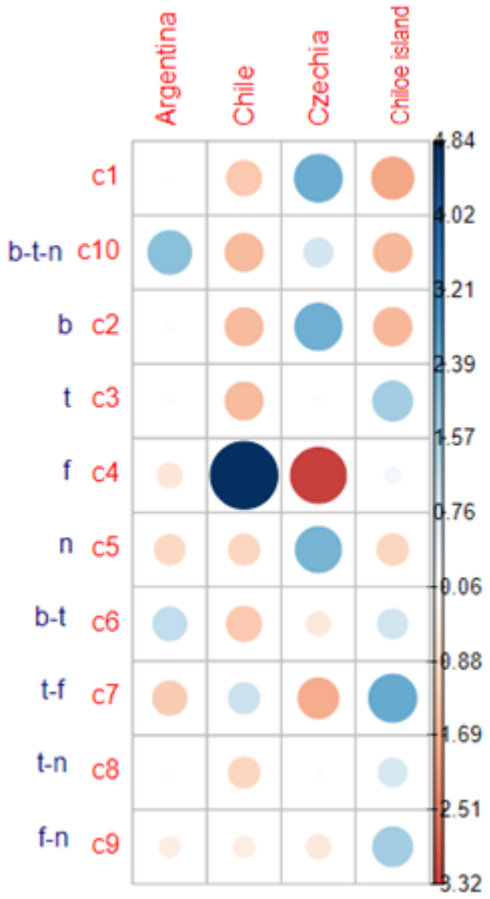


Fig.2.4. Standardized Pearson residuals contributing to the total Chi-square score. Positive associations between Country and PAI type (c1-c10) are blue, negative associations in red. The size of the circle is proportional to the amount of the contribution. Additional genes are next to the PAI type in blue: b - *txtBC*, t- *tom*, f - *fas6*, n – *nec*.

Pairwise distances based on the 16S rRNA gene between isolates belonging to the genus *Streptomyces* both coming from the Czech Republic (within distances) were lower (0,038±0,019) than between an isolate from the Czech Republic and another isolate from another country (between distances) (0,042±0,021) (Wilcox test, p<.001). Similarly, within distances were lower (0,035±0,017 and 0,035±0,021) than between distances (0,042±0,021 and 0,041±0,021) for Argentina and Chiloe island respectively (Wilcox test, p<.001). However, within distances (0,047±0,035) for isolates from Chile did not differ from between distances significantly (0,041±0,02) (Wilcox, p=.203).

Most of the *txtAB* sequences were identical with the exception of single nucleotide mutations for a part of isolates. Isolates identified as *S.turgidiscabies* based on 16S rRNA gene sequence had multiple sites with single nucleotide mutations (Fig.2.4). Most of the *txtAB* sequences coming from the Czech Republic and Argentina, except for strains

17AR5 and 17AR56 belonging to *S.turgidiscabies*, had similar low pairwise distances regardless of phylogenetic distances (Fig.2.5).

There were 3 distinct groups of isolates characterized by the different deletions of variable length in intergenetic region *txtBC* (Fig.2.6). Isolates, which had amplified *txtBC* region, and coming from the same country had similar *txtBC* forward sequences regardless of phylogenetic distance. The same was valid when comparing isolates coming from the Czech Republic and Argentina. The only 2 isolates having *txtBC* sequences and coming from Chiloe island had higher distances both phylogenetically and based on *txtBC* sequences (Fig.2.7).

No correlations between phylogenetic distances, origin and distances based on *fas6*, *tom* nor *nec* genes have been observed (Fig.S2.1-3).

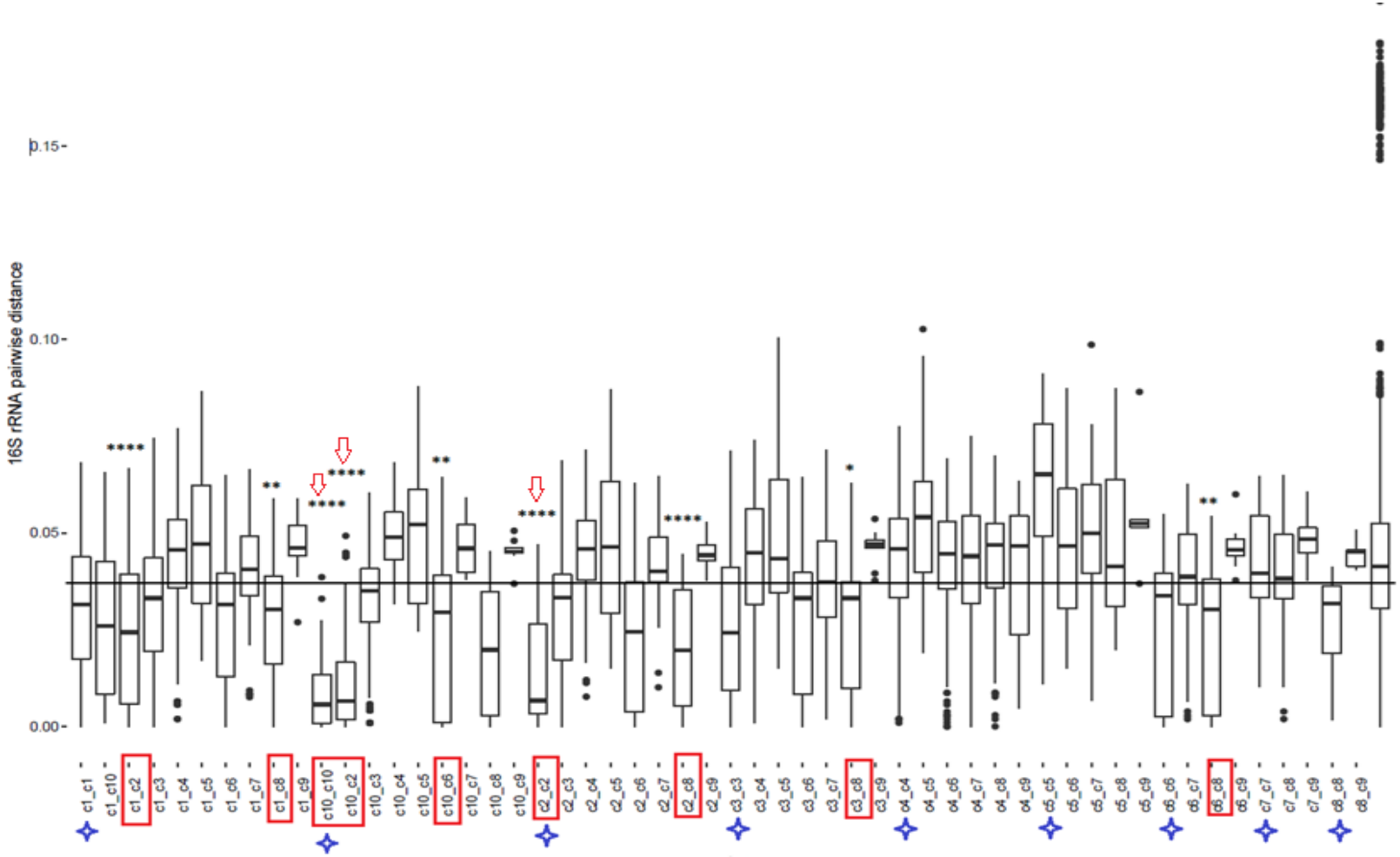


Fig.2.5. Pairwise phylogenetic distances between streptomycetes with different PAI compositions (c1-c10). The last boxplot shows data for *txtAB*-isolates. Pairs with 16S rRNA distances lower than average are highlighted in red rectangles. Stars indicate pairwise distances of strains within the same PAI composition. Red arrows indicate strains with large effect size of differences.

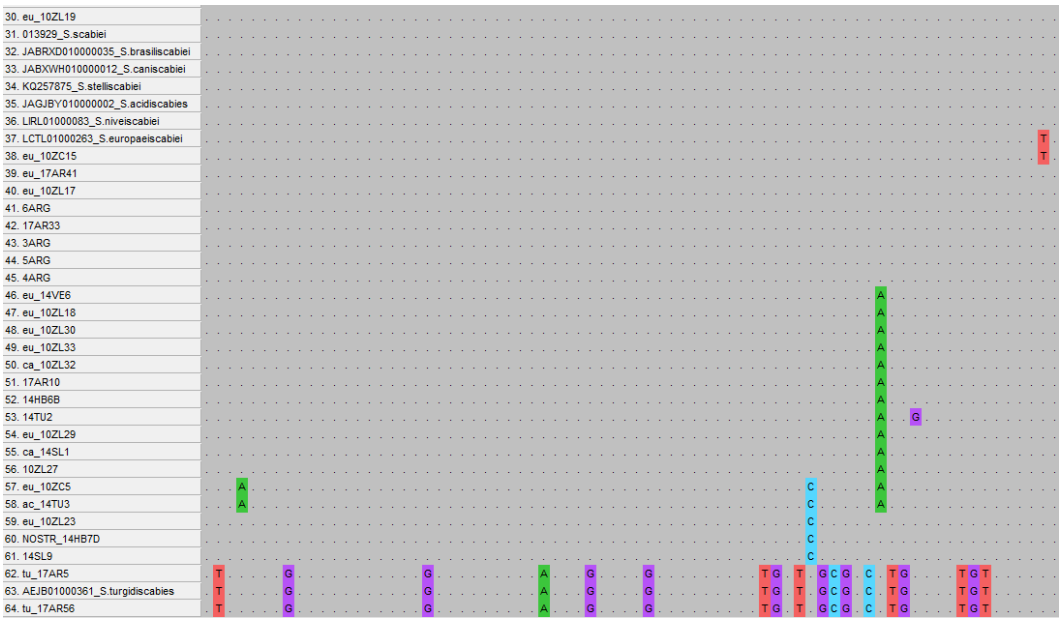


Fig.2.4. Variable sites from *txtAB* sequence alignment. Same nucleotides are presented as dots. Isolates with the same sequences are not present.

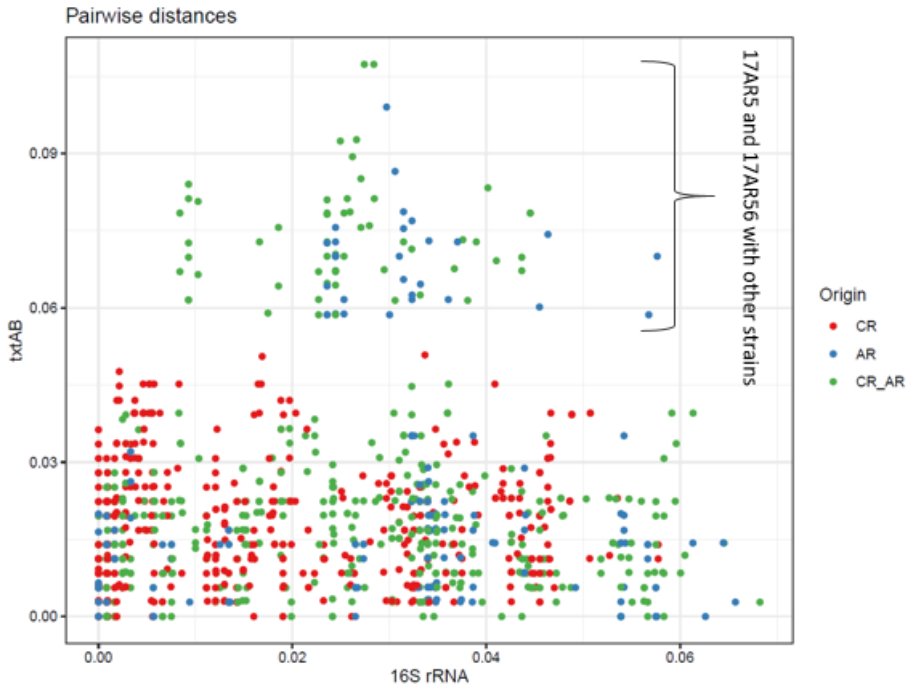


Fig.2.5. Pairwise distances based on sequence dissimilarities of 16S rRNA and *txtAB* genes of pair of isolates both coming from the Czech Republic (CR) or both from Argentina (AR) or one from Argentina and the second one from Czech Republic (CR_AR).

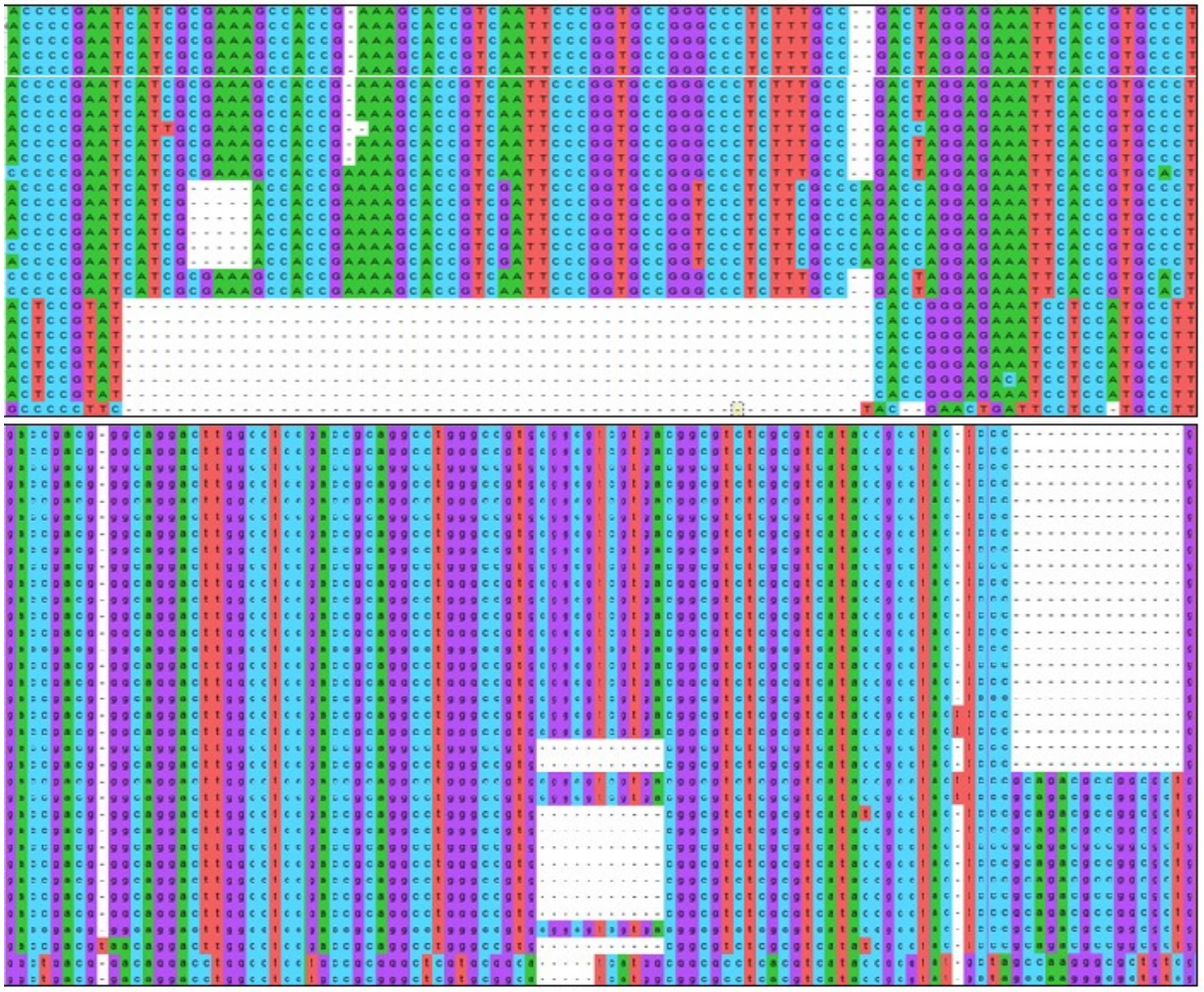


Fig.2.6. Sequence alignment of chosen isolates of the variable part of the intergenetic region *txtBC* sequenced in forward (upper) and reverse (lower) directions

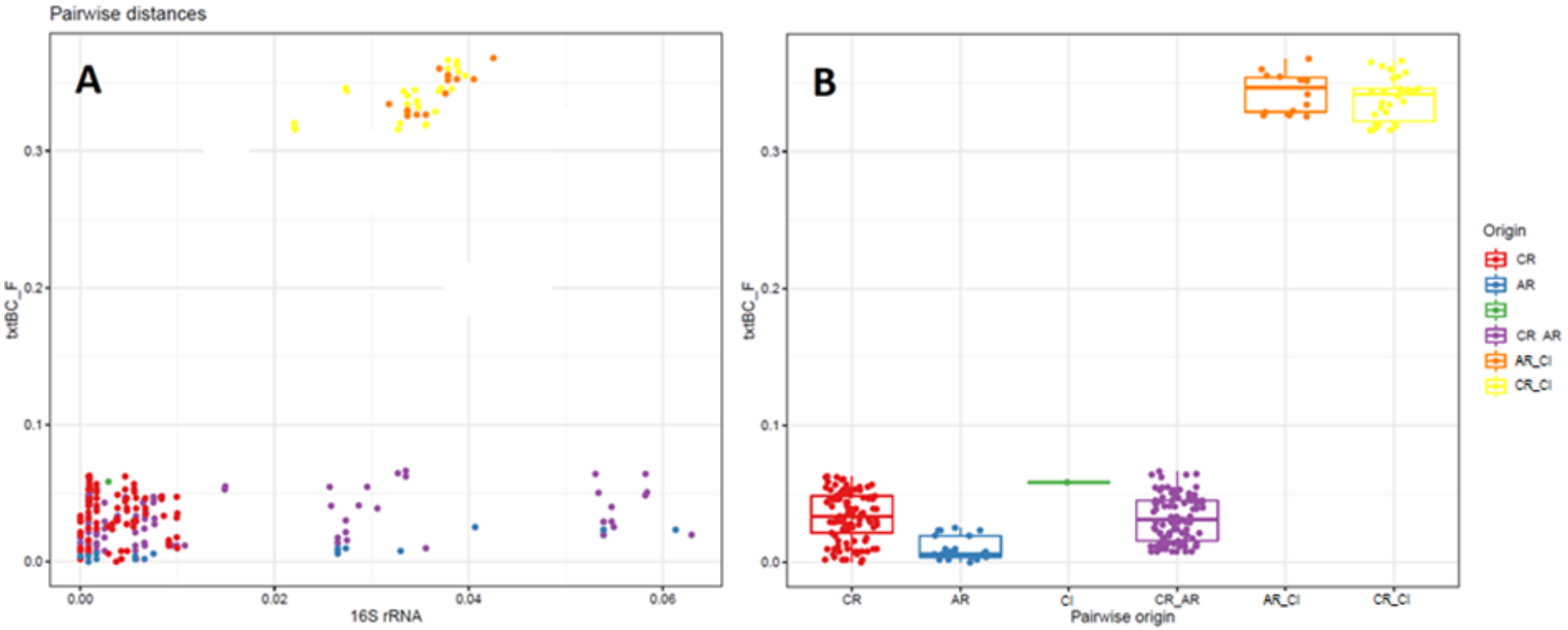


Fig.2.7. Pairwise distances based on sequence dissimilarities of 16S rRNA and *txtBC* genes of pair of isolates originating from different countries. CR – distances between two isolates coming from the Czech Republic. CR_CI – the distance between one isolate from the Czech Republic and another from Chiloe island.

Discussion (phytopathogenic actinobacteria)

Most of the actinobacteria isolates from potato tuberosphere originating in different countries were represented by the genus *Streptomyces*. However, there were higher numbers of rare actinobacteria from the tuberosphere of potatoes coming from the Czech Republic than from Latin America. This can be explained by the different pretreatment conditions, which are crucial for the isolation of different taxonomical groups of actinobacteria (Tiwari&Gupta, 2013), as samples from the Czech Republic were freshly collected, while potato peels from South America were stored air-dried. A quarter of actinobacteria isolates were determined as phytopathogenic, all belonging to the genus *Streptomyces* as expected because all currently described scab-causing actinobacteria also belong to that genus. Based on the 16S rDNA sequence similarity, most of the phytopathogenic isolates from the Czech Republic were closely related to *S. europaeiscabiei*, which was firstly isolated from potatoes in France (Bouchek-Mechiche et al., 2000) and reported to dominate scab-causing streptomycetes in Norway (Dees et al., 2013). However, many isolates with the detected genes for thaxtomin production did not cluster with the known scab-causing streptomycetes, in particular, those coming from Chile and Chiloe island. Moreover, the majority of these strains did not have *txtBC* detected, although based on the thaxtomin BGC all *txtAB*-positive isolates should have intergenic *txtBC* region amplified (Loria et al., 2008). Therefore, thaxtomin BGC may be incomplete and non-functional. On the other hand, some of these *txtAB+/txtBC-* strains had additional virulence genes which may contribute to the pathogenic phenotype. Yet, again the presence of *tom* and *nec1* genes were detected previously in non-pathogenic *txtAB*-negative rhizosphere isolates (Wanner, 2009). Nevertheless, such non-pathogenic rhizospheric bacteria may serve as a source or reservoir for some or all of these pathogenicity-associated genes (Wanner, 2009). In another study, scab-causing streptomycetes from Uruguay were tested for pathogenicity on tuber slices and were also phylogenetically diverse, in many cases not clustering with the known scab-causing species (Lapaz et al., 2017). In Chile and Chiloe island, only 14 strains and 35% of phytopathogenic isolates, respectively, had the closest match with the scab-causing streptomycete *S. caviscabies*. *S. caviscabies* was firstly isolated from deep-pitted potato lesions in Canada (Goyer et al., 1996). Interestingly, *S. caviscabies* was detected among antagonistic bacteria naturally occurring in disease-suppressive soils (Adesina et al., 2007). Moreover, *S. caviscabies* was described as a non-pathogenic wheat root

endophyte, as no thaxtomin was detected in its culture after 10 days of incubation, while the presence of *txtA* and *txtB* genes was tested in the study (Coombs&Franco, 2003). Yet, the maximum production of the thaxtomin is expected after 4-5 days of incubation with a rapid decrease thereafter (St-Onge et al., 2010). Therefore, the ability of *S. caviscabies* to produce thaxtomin is controversial, as in the case with *txtBC*-negative isolates described above. Given the high proportion of uncertainty regarding the pathogenicity in streptomycetes with detected *txtAB* sequence together with the possible inability of *S.caviscabies* to produce thaxtomin, *txtAB*-positive strains from Chile and Chiloe island may not be phytopathogenic. Therefore, the next recommended step is to test the ability to produce thaxtomin by „suspicious"isolates *in vitro* and in bioassay.

We detected 10 out of 16 possible PAI types regarding the presence and absence of *fas6*, *nec1*, *tom* and *txtBC* genes. Similarly, wide variability of PAI types has been observed within *S. turgidiscabies* strains isolated from Finland (Aittamaa et al., 2010) and scab-causing isolates from Uruguay (Lapaz et al., 2017). Thus it seems, thaxtomin PAI has a mosaic structure and virulence factors may be transferred independently (Aittamaa et al., 2010).

Sequences of *txtBC* region differed between strains by large deletions. Such variability may be expected by its intergenic nature. Moreover, chaperone encoded by the *txtH* located in the thaxtomin BGC between *txtB* and *txtC* and required for the proper folding of thaxtomin NRPS domains (Li et al., 2019) may be substituted by chaperones from other distant NRPS pathways with low amino acid similarity to *txtH*-encoded protein (Li et al., 2020).

Many isolates lacking *txtBC* had *fas6* detected. The *fas6* gene encodes a lysine decarboxylase homolog, which is the virulence determinant in *Rhodococcus fascians* (Joshi&Loria., 2007) located on a 200-kb conjugative linear plasmid within a *fas* operon responsible for the cytokinin biosynthesis and subsequent leafy gall symptoms on plants (Crespi et al., 1994). As genes from the *fas* operon are flanked by IS110 family transposable element and were detected in well-conserved thaxtomin PAI only in *S. turgidiscabies*, they could have been transferred by transposition only after the PAI acquisition by *S. turgidiscabies* (Joshi&Loria, 2007). Interestingly, the only two isolates with the closest sequence similarity to *S. turgidiscabies* based on 16S rRNA did not have *fas6*, although among all scab-causing streptomycetes only *S. turgidiscabies* has been

reported to bear the *fas* operon in the thaxtomin PAI (Zhang et al., 2016). Moreover, there were many isolates with *fas6* but no other virulence factors all originating in South America and all of them had the closest 16S rRNA sequence similarity matches with *S. caviscabies* or non-pathogenic streptomycetes. Thus, it is probable that the isolates may be non-pathogenic plant-associated bacteria, as the ability to synthesize cytokinins have been observed in many plant-associated and plant growth-promoting bacteria (Glick, 2012) and the pathogenicity of *S. caviscabies* is under question (Coombs&Franco, 2003). Furthermore, there are many examples of the same genes playing important roles both in symbiotic and phytopathogenic bacteria and transferred horizontally whether from a pathogen to a symbiont or *vice versa* (Hentschel&Hacker, 2000). As potato and possibly also potato pathogens were introduced to Europe from South America (Torrance&Taliansky, 2020), south American isolates may represent a valuable source to study the evolution of phytopathogenicity of the tuberosphere-associated bacteria.

Both phylogenetic and geographical origins significantly affected PAI types in terms of their composition and in some cases also sequence similarities between the studied virulence genes. For example, the most differing *txtAB* and *txtBC* gene sequences were detected between the isolates and database sequences belonging to *S. turgidiscabies*, apart from other *txtAB*-positive species. Except for Chile, *txtAB*-positive isolates coming from the same country tended to be phylogenetically (based on 16S rRNA) closer to each other in comparison with phylogenetic distances to isolates from other countries. Similarly, different scab-causing species were found in a patchy pattern on both local fields and larger geographic scales, although several different pathogenic and non-pathogenic streptomycetes could be isolated from the same lesion (Wanner, 2009). Interestingly, even with higher phylogenetic diversity, isolates from Chile tended to have the same PAI composition 4 (*fas6*), which may suggest a more recent HGT occurring between those strains.

Surprisingly, several PAI composition types were found only for isolates from the Czech Republic and Argentina. In addition, the isolates from Argentina had similar *txtBC* sequences regardless of the phylogenetic distances of isolates coming both from Argentina and the Czech Republic, indicating possible HGT of thaxtomin BGC between streptomycetes in Argentina and a possible transfer of pathogens with the same thaxtomin BGC to Europe or vice versa. Overall, on the one hand, we observed similarities between Argentina and the Czech Republic with more known scab-causing species, similar PAI

compositions, and *txtBC* sequences. On the other hand, *txtAB*-positive isolates from Chile and Chiloe island were dominated by other than described scab-causing streptomycetes, having distinct PAI composition and *txtBC* sequences distant from isolates coming from Argentina and the Czech Republic. Such contrasting differences may be connected to the origin of potato samples. Namely, samples from Argentina and the Czech Republic have been mostly collected in commercial agricultural fields, while samples from Chile and Chiloe island mostly represented local native cultivars (Kopecky, pers.comm.), which have a wide variability of health-promoting micronutrient and antioxidant levels (Andre et al., 2007) and possibly also possess different cultivar-specific microbiomes (Manter et al., 2010).

Results and Discussion (biocontrol actinobacteria)

In **Publication III** (Kopecky et al., 2021), we reviewed the environmentally-friendly ways for the control of common scab (CS) disease using additions of micronutrients and modification of microbial communities. Briefly, the control should be based on the balanced nutrition together with the promotion of suppressive microbial communities with high mineral solubilization capabilities, plant-growth promotion or antagonism and competitiveness to the pathogen. Accordingly, there are several approaches to promote soil suppressive communities, whether by organic and mineral amendments or by direct application of biocontrol strains.

In **Publication IV** (Patrmanova et al., 2022) we focused on an application of antagonistic strains into soil to decrease CS severity. The **aim** was **to develop a selection approach for suitable biocontrol agents** based on their *in vitro* properties: strong inhibition of scab-causing streptomycetes on plates and high growth rate in vermiculite. In brief, 56 tuberospheric actinobacteria were tested: 30 strains had strong inhibitory activity, 14 of them had high growth rate. Consequently, three isolates and a mixture of six isolates with strong inhibitory activity and high growth rates were selected for the pot experiment to assess their biocontrol activity. The most effective strain (09ZI22), which significantly reduced the severity of the common scab in the pot, had the highest number of CFU/g in vermiculite cultivation out of all antagonistic isolates and strongly inhibited both *S. scabiei* and *S. acidiscabies.* Microbial community of soil inoculated by the strain 09ZI22 differed significantly and were farther separated from soil control without treatment than communities after treatments with other strains. Moreover, the application of the strain 09ZI22 increased many bacterial taxa, previously associated with the plant-growth promotion and the suppression of several soil-borne diseases, including CS. Thus, we concluded that inhibitory activities of the inoculated strain and its interaction with indigenous bacteria contributed to CS suppression.

Genome analysis of strain 09ZI22, the most successful CS biocontrol strain

The complete 16S rRNA sequence extracted from the whole genome was 100% similar to *Streptomyces fulvissimus* DSM 40593 (NR_103947.1) and accordingly, the genome-based phylogenetic position supports the relatedness of the strain to *Streptomyces fulvissimus* (Fig. 2.7).

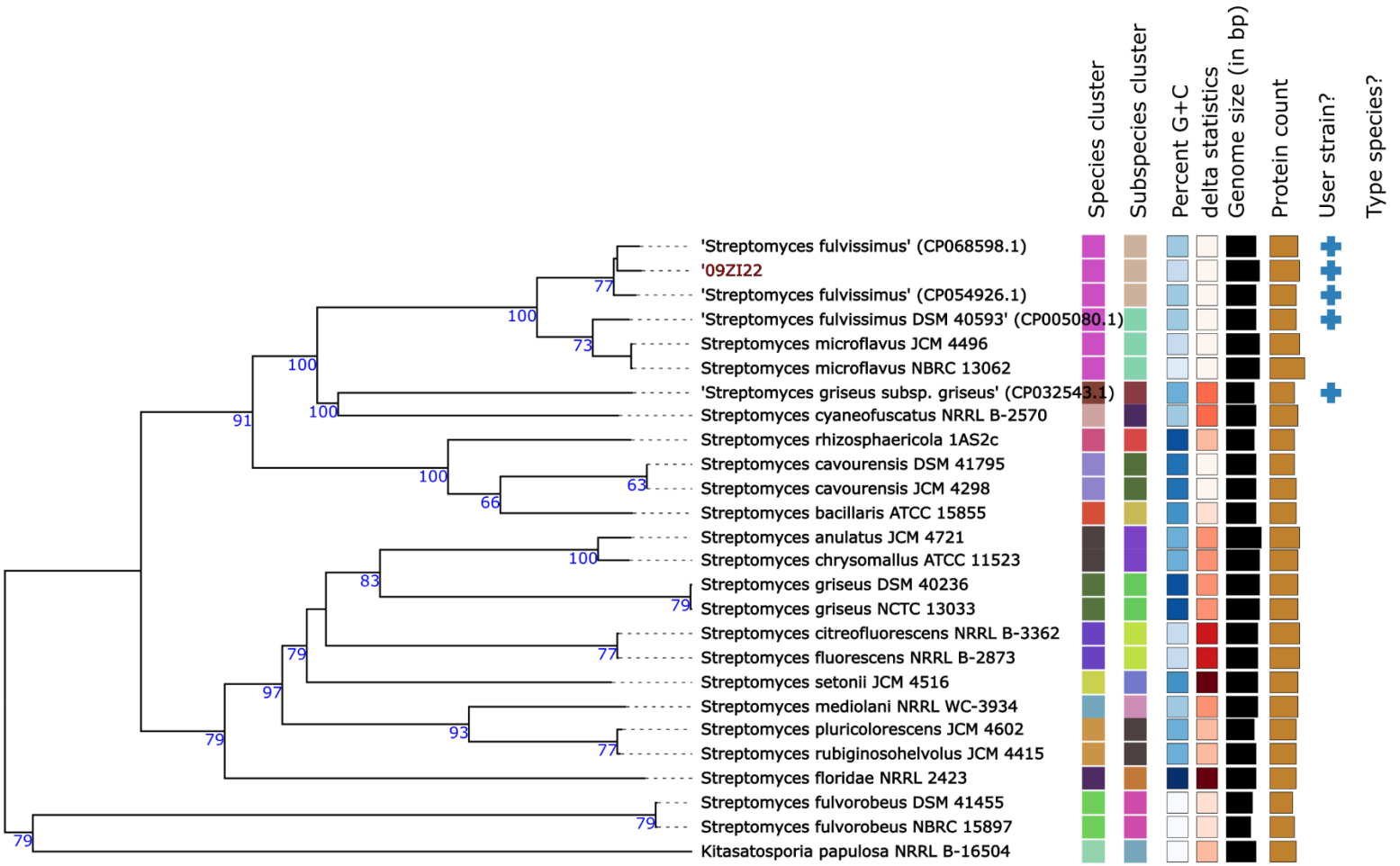


Fig.2.7. GBDP (Genome Blast Distance Phylogeny) based on genome data of the 20 closest type strain genomes from TYGS server. Branch support was inferred from 100 pseudo-bootstrap replicates each.

Genome was assembled using three methods, two *de novo* and one reference-based method. Genome statistics of the genome assemblies are presented in Table 2.4. After reference-based refinement with *GFinisher*, there were 27 contigs, an estimated genome length of 8,044,614 bp, and an average G+C content of 71.49%. The N50 length, which is defined as the shortest sequence length at 50% of the genome, is 424,475 bp. The L50 count, which is defined as the smallest number of contigs whose length sum produces N50, is 8.

According to PGAP annotation, the genome has 7,108 total genes, 7,031 protein-coding sequences (CDS), 6,875 coding genes, 70 transfer RNA (tRNA) genes, 4 ribosomal RNA (rRNA) genes (2 complete 5S, 1 complete 16S and 1 complete 23S rRNA), and 156 pseudogenes. RAST annotated 3948 proteins with the functional assignment and 3405 proteins without functional assignment. ARTS pipeline detected 591 core genes and 36 biosynthetic gene clusters for secondary metabolites (BGC) hits identified with antiSMASH (Table 2.5). A circular graphical display of the distribution of the genome annotations is provided in Fig. 2.8. Seven out of nine NRPS modules are associated with siderophore biosynthesis. 56 genes for Antimicrobial Resistance (AMR) were detected against PATRIC AMR database.

The ARTS pipeline outputted 591 core genes for phylum Actinobacteria with 68 genes detected as HGT transferred. Most of the HGT assigned genes (45) were affiliated with various species within the same genus *Streptomyces*. Interestingly, most frequent non-streptomycete genus, from which HGT might occur, was *Frankia* - 11 genes shared a common node with the strain. None of the other actinobacterial genera, shared more than 1 gene with the strain. Interestingly, 4 genes might have been acquired from *S. scabiei* (Table 2.6).

There were 68 contigs assembled with *plasmidSPAdes* with a total length of 73,516 bp with only 12 protein-encoding genes with the functional assignment and 74 hypothetical proteins. No BGS, no AMR, no core genes were detected. BLAST search detected some parts of the contigs being similar to known plasmid sequences, e.g. contig1 with length 33,425 bp was 83.74% identical to 158,122 bp length complete plasmid of *Streptomyces venezuelae* NRRL B-65442 (CP018075.1) and 90.03% identical to 608,416 bp length plasmid of *Streptomyces fulvissimus* strain NA06532 (CP054927.1).

Table 2.4. Genome statistics of genome assemblies of the strain

Genome statistics	Target assembly (Patric pipeline)	Alternative assembly (SPAdes)	Reference-based assembly (GFinisher)	Reference (CP005080)
NG50	424,475	567,955	795,285	
NG90	119,795	103,491	273,992	
NA50	-	-	-	
NA90	-	-	-	
NGA50	-	-	-	
NGA90	-	-	-	
LG50	6	5	4	
LG90	19	18	11	
LA50	-	-	-	
LA90	-	-	-	
LGA50	-	-	-	
LGA90	-	-	-	
Mismatches				
# N's per 100 kbp	0	11,28	0	
# N's	0	970	0	
Statistics without reference				
# contigs	82	185	26	1
# contigs (>= 0 bp)	89	4355	26	
# contigs (>= 1000 bp)	69	90	26	
# contigs (>= 5000 bp)	49	51	26	
# contigs (>= 10000 bp)	43	45	24	
# contigs (>= 25000 bp)	36	34	20	
# contigs (>= 50000 bp)	28	28	17	
Largest contig	1,158,672	1,035,969	1,352,575	
Total length	8,485,872	8,599,718	8,044,614	7,905,758
Total length (>= 0 bp)	8,488,669	9,726,876	8,044,614	
Total length (>= 1000 bp)	8,475,682	8,538,855	8,044,614	
Total length (>= 5000 bp)	8,429,134	8,471,093	8,044,614	
Total length (>= 10000 bp)	8,385,739	8,423,079	8,029,906	
Total length (>= 25000 bp)	8,264,946	8,238,263	7,959,637	
Total length (>= 50000 bp)	7,977,557	8,033,326	7,838,056	
N50	400,814	523,463	599,835	
N90	88,376	76,834	173,59	
L50	7	6	5	
L90	24	24	12	
GC (%)	71,31	71,29	71,49	71,48

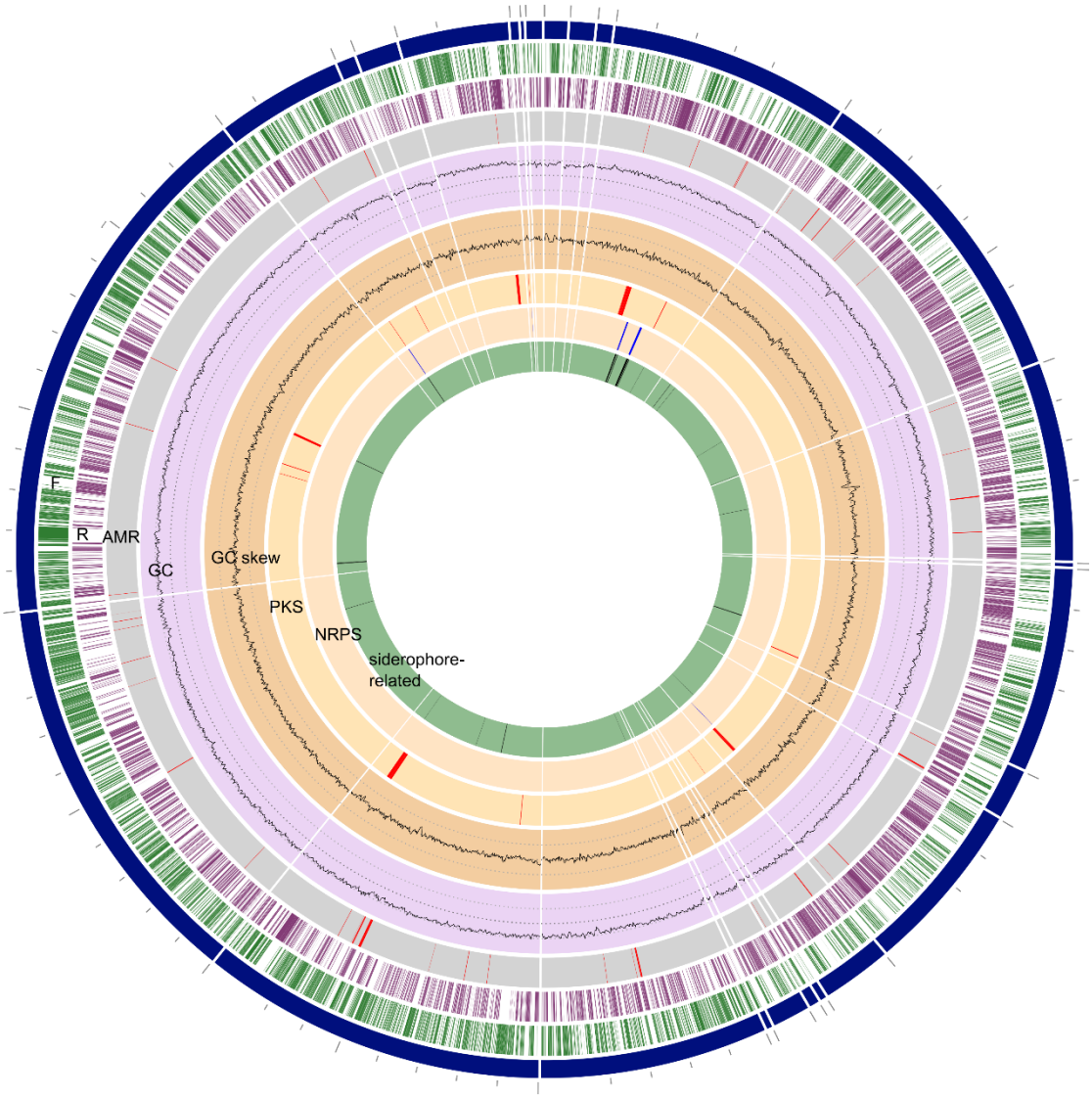


Figure 2.8. A circular graphical display of the distribution of the genome feautures of biocontrol strain 09ZI22. From outer to inner rings: the contigs (blue), CDS on the forward strand (green), CDS on the reverse strand (violet), CDS with homology to known antimicrobial resistance genes (AMR) (red on grey), GC content, GC skew, CDS with homology to known polyketide-synthases (PKS) (red on orange), non-ribosomal peptide-synthesase (NRPS) (blue on orange) and CDS related to siderophore synthesis and transport (blue on green)

Table 2.5. Biosynthetic gene clusters (BGC) for secondary metabolites (SM) hits identified with antiSMASH in genome of biocontrol strain 09ZI22 (JAHWTL000000000.1)

N	Region	Type	Location	Most similar known cluster	SM class	Similarity
1	Scaffold 4.1	ectoine	25,09-35,491			
2	Scaffold 4.2	butyrolactone	127,119-138,003	coelimycin P1	PKS I	16%
3	Scaffold 4.3	terpene	167,156-189,369	geosmin	Terpene	100%
4	Scaffold 4.4	transAT-PKS,PKS-like,T1PKS,NRPS	197,291-304,136	streptobactin	NRP	88%
5	Scaffold 4.5	NRPS	335,189-383,423	coelichelin	NRP	81%
6	Scaffold 4.6	NRPS,T1PKS	393,095-443,638	arsono-polyketide	Polyketide	45%
7	Scaffold 4.7	T3PKS	453,183-494,301	herboxidiene	Polyketide	6%
8	Scaffold 6.1	ectoine	68,895-79,293	ectoine	Other	100%
9	Scaffold 8.1	lanthipeptide-class-ii,lanthipeptide-class-iii	283,597-314,874			
10	Scaffold 8.2	siderophore	368,604-380,382	desferrioxamin B	Other	100%
11	Scaffold 8.3	NRPS-like	457,893-501,726	bottromycin A2	RiPP:Bottromycin	39%
12	Scaffold 10.1	thiopeptide,LAP	104,593-139,144			
13	Scaffold 10.2	NRPS	336,962-390,797	phosphonoglycans	Saccharide	3%
14	Scaffold 16.1	betalactone	54,54-82,213	divergolide	PKS I	6%
15	Scaffold 18.1	lanthipeptide-class-iii	217,684-240,347	AmfS	RiPP:Lanthipeptide	100%
16	Scaffold 18.2	terpene	580,722-610,227	murayaquinone	Polyketide	10%
17	Scaffold 19.1	siderophore	58,794-73,499	ficellomycin	NRP	3%
18	Scaffold 19.2	butyrolactone	227,716-238,669			
19	Scaffold 19.3	RiPP-like	400,497-408,944			
20	Scaffold 19.4	terpene	424,953-444,505	2-methylisoborneol	Terpene	100%
21	Scaffold 19.5	NRPS-like,NRPS	542,261-588,628	salinomycin	PKS I	14%
22	Scaffold 19.6	NRPS-like,arylpolyene	645,834-687,079	formicamycins A-M	Polyketide	11%
23	Scaffold 19.7	NRPS	695,213-742,981	nonactin	Polyketide	71%
24	Scaffold 19.8	terpene	1,010,422-1,036,996	hopene	Terpene	69%

25	Scaffold 19.9	linaridin	1,058,548-1,079,156	pentostatine / vidarabine	Other	9%
26	Scaffold 19.10	T1PKS,NRPS,RiPP-like	1,116,194-1,169,169	SGR PTMs	NRP + Polyketide	100%
27	Scaffold 20.1	NRPS,PKS-like	36-111,01	decaplanin	NRP:Glycopeptide	7%
28	Scaffold 22.1	T3PKS	31,013-72,05	arsono-polyketide	Polyketide	16%
29	Scaffold 23.1	melanin	1,597-12,064	melanin	Other	100%
30	Scaffold 23.2	T3PKS	44,592-85,644	herboxidiene	Polyketide	9%
31	Scaffold 23.3	terpene	169,417-195,011	isorenieratene	Terpene	100%
32	Scaffold 23.4	T1PKS,NRPS,thiopeptide,LAP	208,089-260,676	lactazole	RiPP:Thiopeptide	33%

Table 2. Horizontally transferred genes detected by ARTS server to the biocontrol strain 09ZI22 from the plant-beneficial *Frankia* and phytopathogenic *S.scabiei*

Associated with:	Protein family	Product	Function	Duplication
Frankia	SHMT	serine hydroxymethyltransferase	biosynthesis of nucleotides, protein, and methyl group	3
	TIGR00180	ParB/RepB/Spo0J family partition protein	chromosomal and plasmid partition proteins	2
	TIGR00232	transketolase	energy metabolism, Pentose phosphate pathway]	2
	TIGR00369	thioesterase	enterobactin biosynthesis etc	
	TIGR00500	methionine aminopeptidase	to produce the mature form of cytosolic proteins by removing the N-terminal methionine	
	TIGR01048	diaminopimelate decarboxylase	lysin biosynthesis	2
	TIGR01296	aspartate-semialdehyde dehydrogenase (peptidoglycan organisms)	amino acid biosynthesis	2
	TIGR01764	excise	from temperate phage, plasmids, and transposons, as well as DNA binding domains of other proteins, such as a DNA modification methylase.	
	TIGR03083	uncharacterized Actinobacterial protein		
	TIGR03086	uncharacterized Actinobacterial protein		
	TIGR03181	pyruvate dehydrogenase E1 component, alpha subunit	carbohydrate metabolism	2
S.scabiei	TIGR00009	ribosomal protein L28	protein synthesis	2
	TIGR00273	iron-sulfur cluster-binding protein	binds two 4fe-4S iron-sulfur clusters	
	TIGR01031	ribosomal protein L32	protein synthesis	2
	TIGR02227	signal peptidase I	protein secretion	5

The length of the reference genome is 7,905,758 bp, while the length of the 09ZI22 strain assembled based on the reference genome is 8,044,614 bp, which is 138,856 bp longer. Besides, *de novo* assemblies detect much longer sizes in comparison with the reference, 580,114 bp and 693,960 bp longer for Unicycler and SPAdes assemblers, respectively. GC content differs between *de novo* and reference-based assemblies. Essential genes with evidence of inter-genus horizontal transfer were detected. Moreover, *PlasmidSPAdes* assembled potential plasmid contigs. Thus, we may expect plasmids to be present in the strain and play role in horizontal gene transfer (HGT).

PlasmidSPAdes algorithm is based on the computation of the read coverage from the WGS and was designed primarily for circular plasmids (Antipov et al. 2016), it may not work perfectly for the linear plasmids of streptomycetes. Besides, we did not recover any complete plasmidic DNA sequence, although BLAST results support the plasmidic origin of the assembled contigs. Therefore, for future research, we recommend using special protocols for plasmid isolations (such as pulse electrophoresis) or/and using SMRT long-read sequencing for the detailed insight of the *S. fluvissimus* genome.

The strain genome bears a large number of biosynthetic gene clusters (BGCs; 36), which is comparable to many streptomyces (Lee et al. 2020). Among detected BGCs, there are several clusters for the possible synthesis of antibiotics (e.g. decaplanin) and ribosomally synthesized and post-translationally modified peptides (RiPPs) (e.g. bottromycin). Some RiPPs are known to be bacteriocins, which act as antibiotics against closely related bacteria (Soltani et al., 2021). As the strain 09ZI22 protects the potato against CS, the production of bacteriocins may be one of the possible mechanisms for inhibition of the *S. scabiei* pathogenic activity. Interestingly, bottromycin has been discovered from *S.bottropensis* (Waiswisz et al., 1957), recently described as another causative agent of CS (Sarwar et al., 2018). Surprisingly, *S.scabiei* also produces bottromycin (Liu et al., 2021).

Detected multiple siderophore-related and AMR genes suggest high competing potential of the strain in natural environments. Siderophores were reported to promote growth of plants and enhance their protection against phytopatogens, so the production of siderophores might be crucial in biological control (Ahmed and Holmström 2014). Moreover, four genes were detected as potentially HGT-related to *S. scabiei* (with one related to iron-sulfur binding) and may interfere/enhance competition. 11 TIGRfam gene families may be derived from *Frankia*, a known actinobacterial symbiont (Benson and Silvester 1993). Many of them are associated with amino acid metabolism, two may be

plasmid-associated. *Frankia* was the only non-streptomycete with more than one detected horizontally transferred gene, thus we may suggest this transfer is non-random and might reflect adaptation to plant-associated habitats. Duplication of these genes further supports its HGT nature.

Thus, efficient biocontrol strain has strong antagonistic potential against phytopathogen whether via antibiotics or siderophores, mechanisms supporting PGPR bacteria *in situ*, high growth rate and adaptation to compete in the same plant-associated environment.

Conclusions

In this thesis we attempted to assess relationships between actinobacteria, their secondary metabolites and interactions with other bacteria in soil and plant environments.

In the first part we concentrated on actinobacteria from acid soil dominated by a previously uncultured actinobacterial lineage and isolated a collection of rare actinobacteria, including dominating slowly-growing novel family of *Treboniaceae*. The isolation from unexplored environment, use of specific oligotrophic media with acidic pH and long incubation time lead to isolation of potentially novel actinobacterial species of different genera. We demonstrated the substantial differences in bacterial communities between soil horizons both at the cultivation-independent and the level of isolates. In some cases, even with short incubation period, potential new species of *Streptomyces* were isolated exclusively from either the organic or mineral soil horizon, pointing to the importance of soil horizons when describing soil bacterial diversity or isolating novel bacteria.

We designed a co-culturing experiment where the influence of an interacting actinobacterium (*Trebonia*, *Streptomyces* or *Straptacidiphilus*) on total culturable soil bacteria and diffusible metabolite pool was observed. We demonstrated that metabolite pool was correlated with bacterial community and all three actinobacteria strains specifically affected the other bacteria in the community. *Streptomyces* overall inhibited the other cultivable soil bacteria and produced antibiotics determined both by inhibition tests and by MS-MS spectra match with novobiocin. Oppositely, *Streptacidiphilus*, despite belonging to the same family *Streptomycetaceae*, had an intensive stimulating effect on various distant bacterial taxa and produced bioactive metabolites, but it did not inhibit test bacteria. *Streptacidiphilus* has been reported as plant-associated, in the future we suggest testing the strain for its biocontrol activity by modifying and stimulating soil bacterial community towards more diverse and healthy state of soil.

Both the genome analysis and analysis of molecular networks based on MS-MS spectra revealed the ability of *Trebonia* to produce and degrade unusual complex aromatic metabolites. Moreover, *Trebonia* dominated among all bacterial genera in soil and had multiple resistance genes to antibiotics and heavy metals. Yet, in co-culture, slowly growing *Trebonia* shut down the production of many metabolites in the presence of other soil bacteria on the plate and affected the relative abundancies of only limited number of taxa. We suggest that the dominance of Trebonia Clade in the acid soils is rather explained

by its resistance and ability to utilize complex carbon than by the interference competition, which could be a successful strategy for more rapidly-growing actinobacteria, such as *Streptomyces*.

Nonewithstanding that all three actinobacteria strains affected metabolite pool and bacterial community differently, all were able to degrade QS molecule(s) produced by gram-negative bacteria, which could be a common feature of actinobacteria and need to be further tested by a targeted approach. Moreover, we detected other known signal molecules freely diffusible in the medium and degraded only in the presence of a particular strain probably by stimulating soil bacteria capable to degrade such compounds only in consortia. Interactions with total culturable bacteria affected inhibitory activity of *Streptomyces* on test cultures. This should be considered when applying antimicrobials *in situ* and management practices should be concentrated on approaches targeting overall microbial community rather than individual strains.

In the second part of the thesis, we explored both phytopathogenic and biocontrol actinobacteria connectedto the common scab of potato (CS). We analyzed pathogenicity islands (PAI) of phytopathogenic streptomycetes and showed that both phylogeny and geographical origin affect the compositional type and sequence similarities of specific virulence genes. Commercial agricultural practices including tuber importing may, in some cases, promote the spread of particular PAI types even between geographically distant countries and phylogenetically distant species confirming a horizontal gene transfer. Streptomycetes coming from the tuberopsphere of potato from native cultivars from South America, mostly belonged to various species of *Streptomyces* previously unknown to be pathogenic or even reported as plant beneficial. The further effort should be concentrated on *in vitro* pathogenicity tests and probable re-estimation of currently used *txtAB* marker as an indicator of phytopathogenicity.

Finally, we developed an approach to select biocontrol strains based on the ability to inhibit scab-causing streptomycetes and grow successfully in vermiculite. In the first part of the thesis, we already observed in natural soils that strains with high growth rate on the plate have a higher effect upon the cultivable bacterial community. Similarly, the most successful biocontrol strain had also the highest growth rate on plates in comparison with the other strains and significantly influenced microbial community *in situ* towards plant-growth promoting state. The genome analysis with high number of biosynthetic clusters for the production of multiple siderophores and antimicrobials and high number of negative strong correlations in co-occurrence network of soil bacterial OTUs suggested

that the biocontrol streptomycete is strong interference and explotaitive competitor. Future research could reveal if a similar approach could be applied to other plant systems and phytopathogens.

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List of Author's Publications

I. Madrova, P., Vetrovsky, T., Omelka, M., Grunt, M., Smutna, Y., **Rapoport, D**., ... & Sagova-Mareckova, M. (2018). A short-term response of soil microbial communities to cadmium and organic substrate amendment in long-term contaminated soil by toxic elements. *Frontiers in microbiology, 9*, 2807.

II. **Rapoport, D.,** Sagova-Mareckova, M., Sedláček, I., Provaznik, J., Králová, S., Pavlinic, D., ... & Kopecky, J. (2020). *Trebonia kvetii gen. nov., sp. nov*., an acidophilic actinobacterium, and proposal of the new actinobacterial family *Treboniaceae fam. nov*. *International Journal of Systematic and Evolutionary Microbiology, 70*(9), 5106-5114.

III. Kopecky, J., **Rapoport, D**., Sarikhani, E., Stovicek, A., Patrmanova, T., & Sagova-Mareckova, M. (2021). Micronutrients and soil microorganisms in the suppression of potato common scab. *Agronomy, 11*(2), 383.

IV. Patrmanova, T., Krizkova, I., **Rapoport**, D., Kopecky, J., Hrychova, S., & Sagova-Mareckova, M. (2022). Inoculations of soil by antagonistic strains modify tuberosphere bacterial communities and suppress common scab of potatoes. *Applied Soil Ecology*, 176, 104491.

V. Patrmanova, T., Krizkova, I., **Rapoport, D**., Kopecky, J., Hrychova, S., & Sagova-Mareckova, M. (submitted, under review). Data on the genome assembly and annotation of *Streptomyces sp. 09ZI22*. *Data in Brief*

VI. Samoilova, E., Tejnecký, Vá., Kopecky, J., Drabek, O., Stovicek, A., Vokurková, P., **Rapoport, D.,** Němeček, K., Buresova-Faitova, A., Chotěborský, R., Patrmanová, T., Hromasová, M., Sagova-Mareckova, M. (submitted, under review). The structure of microbial communities in redoximorphic microsites of Gleysol. *European Journal of Soil Science*

Author's contribution

I. Soil DNA extraction
II. Strains isolation, cultivation, DNA extraction, PCR, genome assembly and analysis, drafting and writing manuscript, submitting
III. Drafting and writing manuscript chapters on micronutrients, organic amendments, rhizosphere microorganisms, and inoculation, preparing tables
IV. Strains isolation, cultivation, manuscript editing
V. Genome assembly and analysis, manuscript preparation
VI. Sequence processing, manuscript editing

On behalf of all the co-authors, I declare the participation of Daria Rapoport in research and publication writing as described above.

RNDr. Marketa Sagova-Mareckova, Ph.D.

Supplementary Material

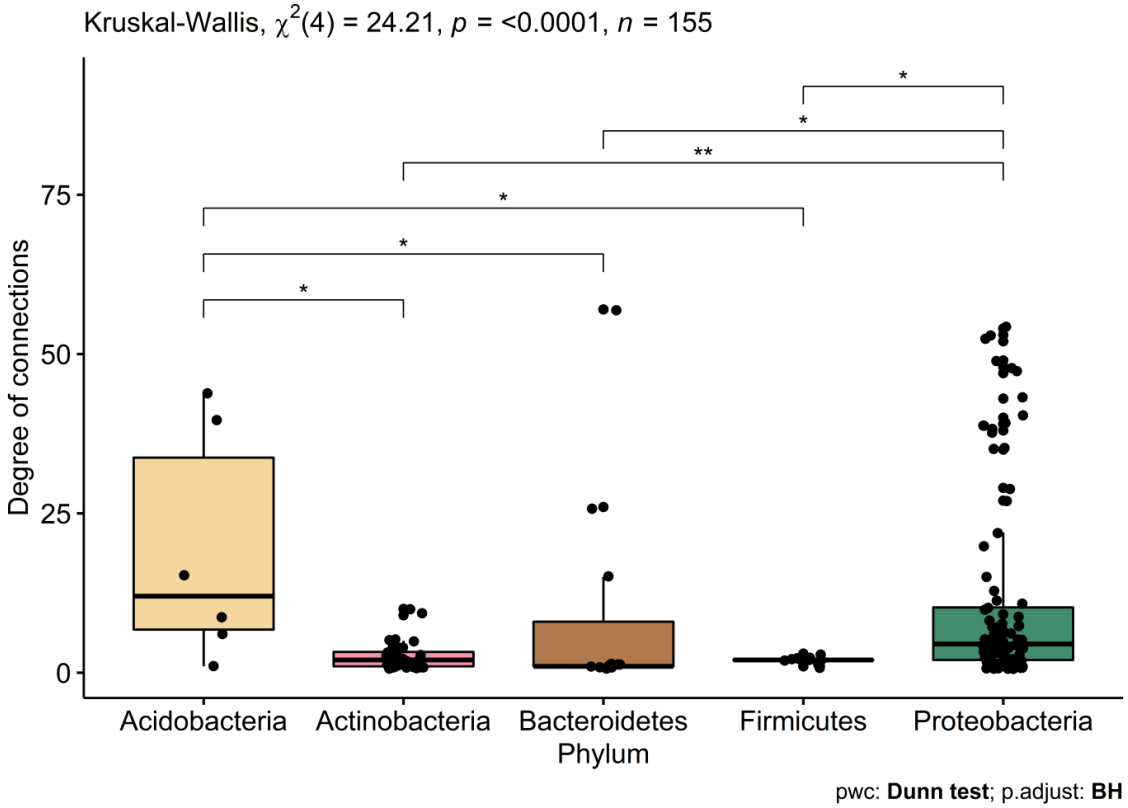

Figure S1-1. Comparison of numbers of connections between OTUs belonging to different phyla based on network in Fig 9. Asterisks represent p-values of post-hoc pairwise Dunn test: * ($p<0.05$); ** ($p<0.01$).

Table S1-1. Results of the multiple comparisons performed on the values of degree (numbers) of connections between OTUs. Cells show the z statistics of the Dunn tests (below diagonal) and corresponding p-values after Benjamini-Hochberg correction (above diagonal)

	Acidobacteria	**Actinobacteria**	**Bacteroidetes**	**Firmicutes**	**Proteobacteria**
Acidobacteria		0.0098*	0.0102*	0.0067*	0.1369
Actinobacteria	2.885254		0.4773	0.5099	0.0018*
Bacteroidetes	2.568355	0.056944		0.4956	0.0152*
Firmicutes	2.783447	0.232796	0.135745		0.0081*
Proteobacteria	1.305838	-3.573402	-2.361155	-2.816429	

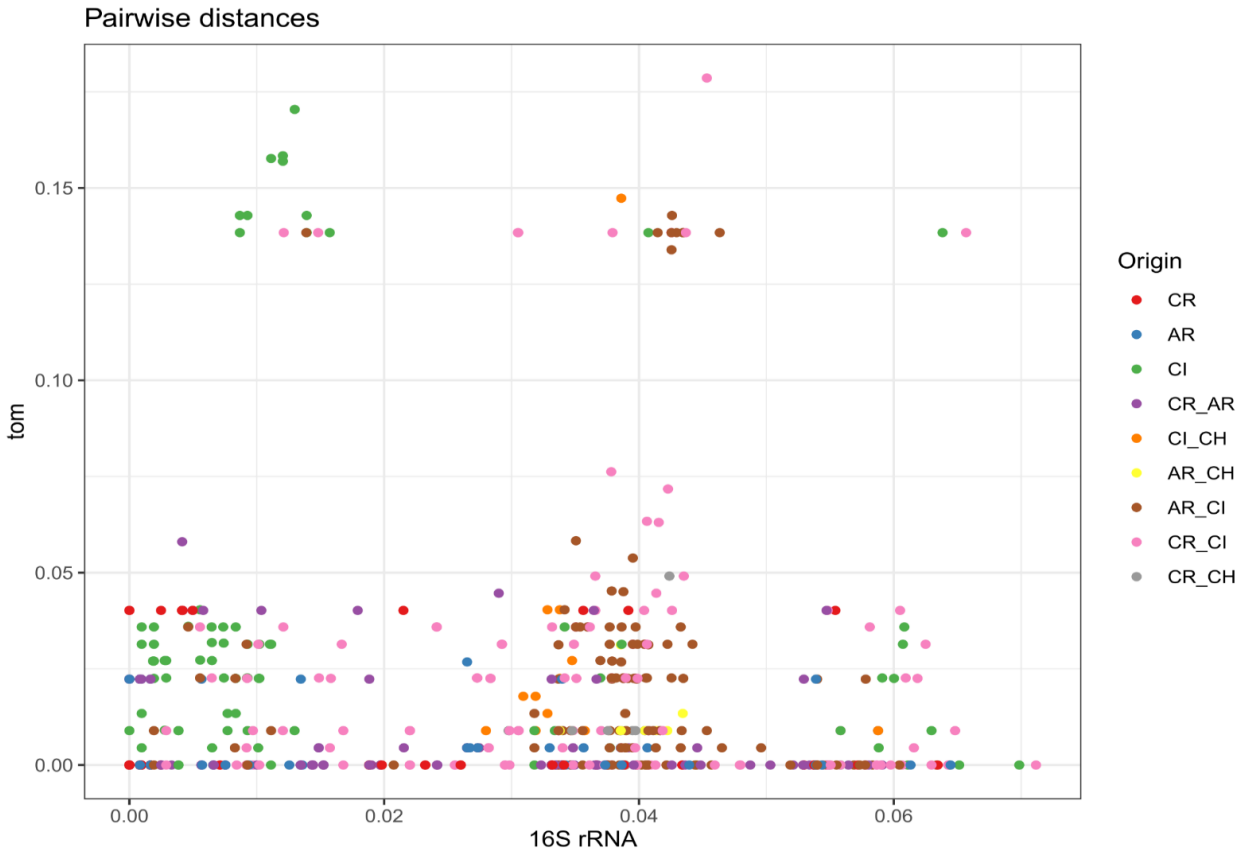

Fig.S2.1. Pairwise distances based on sequence dissimilarities of 16S rRNA and *tom* genes of isolate pairs originating from different countries. For example, CR – distances between two isolates coming from the Czech Republic. CR_CI – the distance between one isolate from the Czech Republic and another from Chiloe island

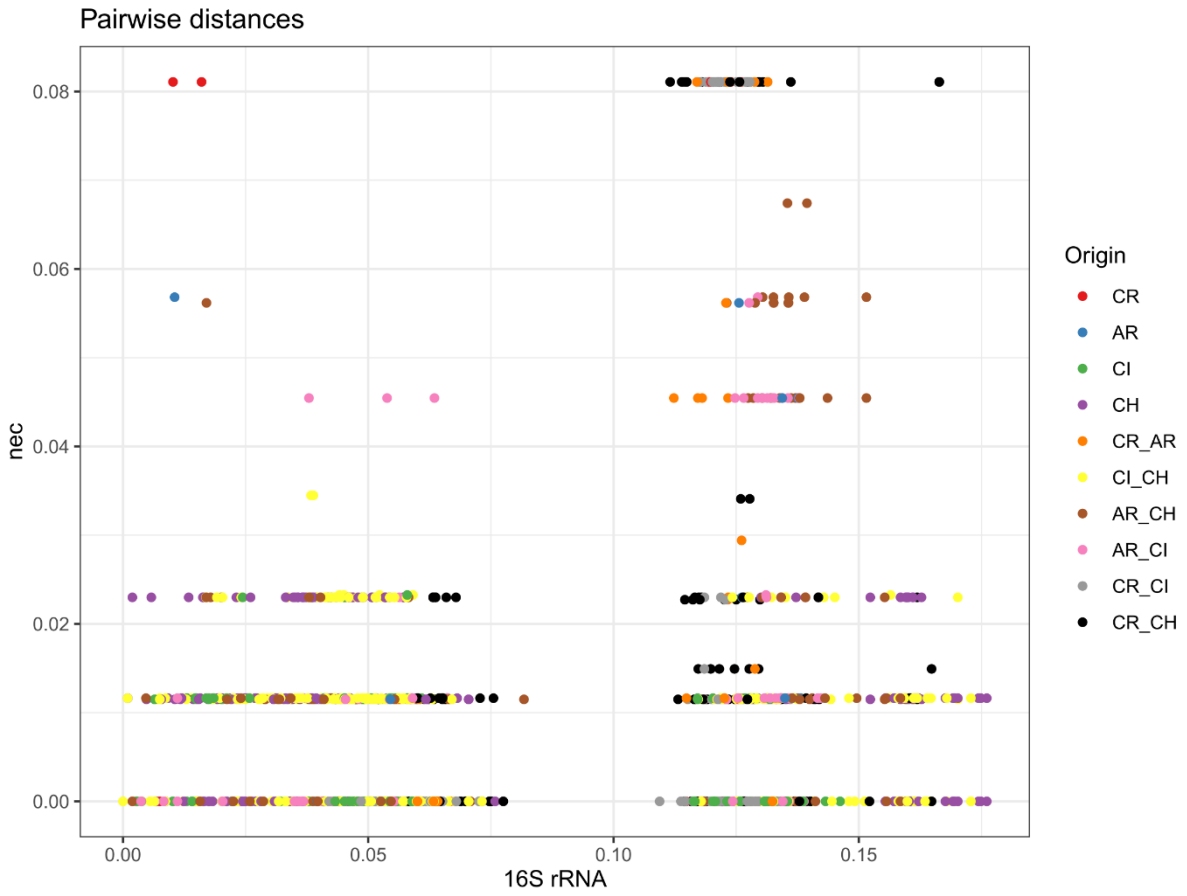

Fig.S2.2. Pairwise distances based on sequence dissimilarities of 16S rRNA and *fas6* genes of isolate pairs originating from different countries. For example, CR – distances between two isolates coming from the Czech Republic. CR_CI – the distance between one isolate from the Czech Republic and another from Chiloe island

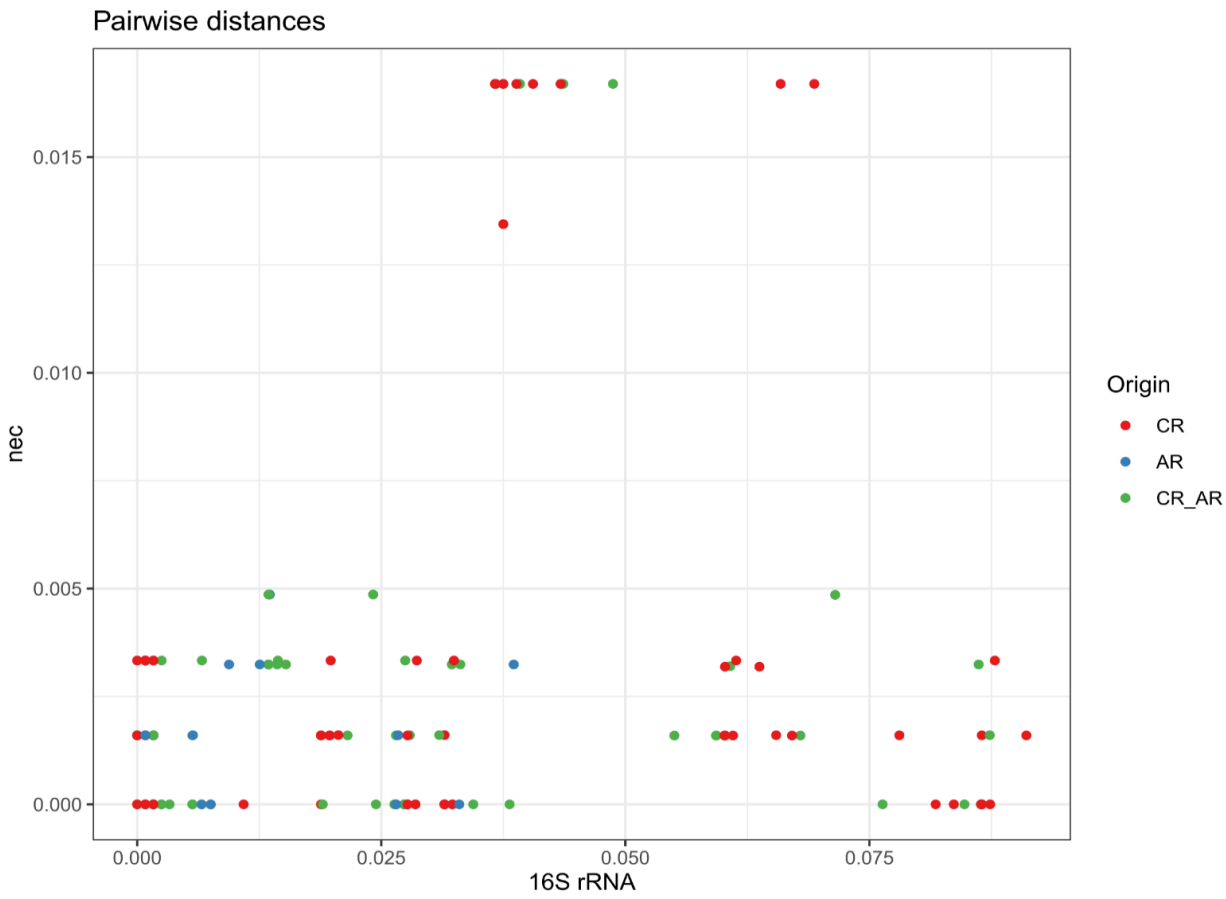

Fig.S2.3. Pairwise distances based on sequence dissimilarities of 16S rRNA and *nec* genes of isolate pairs originating from different countries. For example, CR – distances between two isolates coming from the Czech Republic. CR_CI – the distance between one isolate from the Czech Republic and another from Chiloe island.