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Bachelor Thesis

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‘Determination of the Presence of Pathogenic Island in Causative Agents of Potato Common Scab’

Bachelor Thesis

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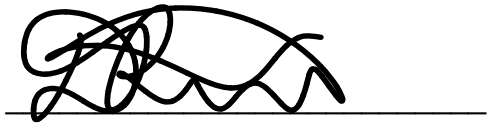
Declaration

I hereby declare that I have authored this bachelor's thesis carrying the name "Determination of the Presence of Pathogenic Island in Causative Agents of Potato Common Scab" independently under the guidance of my supervisor. Furthermore, I confirm that I have used only professional literature and other information sources that have been indicated in the thesis and listed in the bibliography at the end of the thesis. As the author of the bachelor's thesis, I further state that I have not infringed the copyrights of third parties in connection with its creation.

Prague, May 1st, 2021. 

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Determination of Pathogenicity and the Presence of Pathogenic Island in Causative Agents of Potato Common Scab

Summary:

Common scab is one of the most widespread and known potato diseases, which is caused by actinobacterial pathogen belonging to several species of the genus *Streptomyces*. In the current work, the presence of genes coding for the common scab pathogenicity determinant, dipeptide thaxtomin, along with other virulence factors frequently present in thaxtomin-pathogenicity island were determined. Various bacterial strains were isolated from tuberosphere of potatoes with different common scab severity levels and geographically distinct localities. There were 134 strains isolated from several potato cultivars grown at several locations in the Czech Republic and South America. The strains were isolated and cultivated on R2A agar medium and then the same liquid medium. Their DNA was extracted, and PCR was performed for genes *16S, stx1 a/b, TxtBC1_F/R*, *Tom 3/4*, *P19 f/r* and *Nec f/r*. The results were analyzed using sequencing and a phylogenetic tree was created to demonstrate relatedness between the strains. The results supported our hypothesizes that the presence of potato common scab depends also on the environmental conditions such as the location of origin or a local pool of pathogenic bacteria; and that sites would differ in the dominant pathogenic island type. Moreover, it was found that genes for thaxtomin may be horizontally transferred not only within the *Streptomyces* genus, but among other distantly related actinobacterial genera such as *Nocardia*, *Nonomureae, Promicromonospora,* and *Micromonospora,* and the genes can even be found in gram-negative phylum *Proteobacteria* in genera *Phyllobacterium, Pseudomonas, Azamonas,* and others. Lastly, it was recorded *that Nec* genes are involved in pathogenicity and might be linked to the thaxtomin A biosynthetic genes.

Keywords: potato common scab, *Streptomyces spp., Actinobacteria,* pathogenicity, pathogenic islands

Table of Content:

1. Introduction

Solanum tuberosum L. is one of the leading major crops in the entire world. It grows in a vast variety of climates, performing perfect yields in temperatures between 0°C and 30°C. Its' production is important as a quality nutritional food source containing 20 amino acids, including a high concentration of essential amino acids such as isoleucine, leucine, lysine, methionine, phenylalanine, threonine, histidine, and valine (Bartova V., 2015; B.P. Hughes, 1958). Byproducts of potato produce or specific cultivars are used in other areas of agriculture such as starch, alcohol, or feed production.

Common scab is a disease very prominent in potato plants, which causes many yield quantity and quality issues, delayed emergency of the tuber as well as soil contamination. It degrades the appearance of the potato tubers, which decreases market value as well as makes the peel and infected crates unusable for further processing. Moreover, scabs release dormant bodies into the soil, where they wait for favorable conditions and a suitable host to infect. Despite extensive research on the topic, to this day there is no cure for the disease or a resistant cultivar available. In 2002 economic losses from potato common scab in Canada were estimated to 17,3 million Canadian dollars (Hill J., 2005). In the same year in Australia, losses were estimated at around 3.66 million Australian dollars per acre, which is over 4% of the industry value (Wilson CR., 2004).

The main causative agent is believed to be *Actinobacteria*, more specifically its' *Streptomyces spp.* To combat the issue it is important to determine the presence of pathogenic island and pathogenicity in the causative agents of the common scab.

More research on the bacteria species and its' interaction with potato cultivars in various locations will help understand the primal mechanisms by which the pathogen infects the plant. This research is another step towards more sustainable and less wasteful agriculture.

2. Objectives of the work

The main objective of the experiment was to determine different pathogenic island types and their relation to the taxonomy, source, and pathogenic potential of *Actinobacteria* in potato isolates, which come from different fields in the Czech Republic and South America infected with common scab.

Pathogenic island types are described in terms of the presence/absence of the genes coding for the main pathogenicity determinant of the common scab thaxtomin (using primers *stx1 a/b* and *TxtBC1_F/R*) and other virulence genes such as tomatinase, fascinase, and necrotizing protein (*Tom 3/4*, *P19 f/r* and *Nec f/r*, respectively). The main goal is to analyze the final data from PCR sequencing of all the primers and sum up the pathogenicity and patterns.

The first hypothesis is that the presence of potato common scab depends not only on the presence of the pathogen at the location but also on a potato cultivar, soil type, and geographical location. The second hypothesis is that the thaxtomin gene will have a different evolutionary tree than the 16S rRNA gene due to horizontal gene transfer since they didn't evolve together but were acquired from the environment or different strains, therefore sites would differ in the dominant pathogenic island type.

3. Literature Overview

3.1 Potato common scab

3.1.1 *Solanum tuberosum L.* and potato common scab

The potato tuber as an agricultural crop remains one of the most important and imperative commodities around the world, taking fourth place in agriculture worldwide, being a staple produce not only as a food source but also used in starch, alcohol, and feed productions (Labruyère, R. E., 1971). Despite the high economical and agricultural importance of potatoes (*Solanum tuberosum L.),* it is subject to many diseases, which can be caused by fungi, bacteria or viruses. Among one of those, the most famous is potato common scab disease, which has first been noted in John Claudius Loudon's Encyclopedia of Agriculture in 1825 and has been an important disease of potatoes worldwide ever since (Slack S.A., 1991). More specifically, it has been rated among the top five diseases in potato seeds produced in the USA.

Common scab has distinctive visual and quality parameter characteristics that appear on the surface of potatoes as dark or brown lesions with corky texture and can go deep beneath the tuber surface as raised or deep-pitted lesions. The soluble brown pigment is melanin produced by pathogenic strains (R. Loria et al. 1997). Depending on how deep and intense the disease progresses and how much the specific plant is affected, it can decrease the nutritional and economic value of the final produce – usually part of the tuber is still edible, but the damaged appearance greatly reduces the consumption rate and price point (Hill J., 2005). Another crucial consequence is wasted product, which makes this agriculture production less economically and environmentally sustainable.

3.1.2 Resistant methods against common scab

Despite the intense research on how to prevent the occurrence of common scab in *Solanum tuberosum L.,* there is yet to be found a reliable disease-resistant or tolerant cultivar or developed an effective research-based measure against the pathogen distribution (Merete W., 2012). Some alternative ways to partially combat common scab is to control soil moisture and pH. One of the strategies is to maintain high soil moisture during the early stage of the tuber development (Powelson M.L.,1993). Furthermore, it has also been noted that there has been observed different level of pathogenicity of individual potato cultivars in different locations (Wanner L.A., .2006). This could be due to differences in climate, location of specific micro-flora, or differences in the pathogen population.

3.2 *Actinobacteria* and potato common scab

3.2.1 Actinobacteria and its taxonomy

Actinobacteria is a Gram-positive bacterial phylum, and it is one of the largest bacteria phyla known to this day, which was present on Earth as far as 2.7 billion years ago (Law, J.W.-F., 2020). In their genome actinobacteria has a high content of guanine plus cytosine (G+C), which makes them more stable (in comparison to bacteria with higher adenine plus thymine (A+T) content), due to more hydrogen bonding and therefore higher resistance to denaturation (Essaid Ait Barka, 2015). Content of C+G in DNA ranges from 51% to 70% in *Streptomyces* (Marco Ventura, 2007). The phylum includes 6 classes: *Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria,* and *Thermoleophilia.* There are over 110 currently recognized genera, such *as Streptomyces, Nocardia, Micromonospora, Frankia, Propionbacterium, Mycobacterium, Gordania*, and many more (Nouioui, I., 2018). The largest class *Actinobacteria* has 15 orders: Actinomycetales, Actinopolysporales, Bifidobacteriales,

Catenulisporales, Corynebacteriales, Glycomycetales, Jiangellales, Kineosporiales, Micrococcales, Micromonosporales, Propionibacteriales, Pseudonocardiales, Streptomycetales, Streptosporangiales, and Frankiales (Battistuzzi, F.U., 2004).

Actinobacteria are mostly found in soil (95% of those are *Streptomyces spp*) and the minority is also found in aquatic ecosystems in optimal temperatures from 25°C to 30°C (Essaid Ait Barka, 2015). They have significant global functions such as producers of enzymes, being important agents of carbon and nitrogen cycles, as well as being the source of production of many antibiotics and chemotherapeutic drugs, for example, doxorubicin from *Streptomyces peucetius* and bleomycin from *Streptomyces verticillus* (Omura, S. 2008).

3.2.2 Horizontal Gene Transfer

Horizontal (or lateral) gene transfer is a source of new genes and functions to the recipient of the transferred genetic material, it is a mechanism, which allows the development of new evolutionary traits (Boto, L., 2010). The concept of horizontal gene transfer between different organisms was introduced at the beginning of the 1990s as a way to compare a completely different set of genes (Hilario E., 1993). “Today it is estimated that between 1.6 and 32.6 percent of the genes of each microbial genome have been acquired by horizontal gene transfer” (Koonin, E.V., 2001). Lateral gene transfer results both from the successful transfer of genetic material (that's accompanied by processes such as transduction, conjugation, or transfection, and ulterior recombination) and from the survival of the transferred genetic material throughout the generations.

3.2.3 Streptomyces spp. as causative agents of potato common scab

There are many known and unknown factors about how bacteria cause potato common scab. It always depends on certain environmental and agricultural conditions such as the soil, cultivar, seed quality, and presence of other pathogens in the area. However, it is known that common scab is caused by Gram-positive bacteria of the

genus *Streptomyces* that are soil-borne or transmitted by seed tubers (Slack SA (1991). The genus belongs to the phylum Actinobacteria and is one of the most studied out of all on this group. For example, “many economical and medicinal values, such as two-thirds of antibiotics, are developed from Actinomycetes worldwide, and about 80% of antibiotics are developed from *Streptomyces spp*” (Wang. D, 2017). A variety of *Streptomyces spp* also acts as sources of insecticides, bioinsecticides, and bioherbicides such as Mildiomycin, Herbimycin, and more (Essaid Ait Barka, 2015). Despite that, *Strepromyces spp* can act as pathogens themselves, which often results in a health decrease of the plant. Many species of *Streptomyces*, specifically *Streptomyces scabies, Streptomyces turgidiscabies, Streptomyces europeascabies, and Streptomyces acidiscabies* make the most common appearance with the potato common scab (Sohaib I., MDPI, 2020). *Streptomyces scabies* is the oldest out of those pathogens and is present worldwide, while *Streptomyces turgidiscabies* and *Streptomyces acidiscabies* were originally in Japan and in the United States (Essaid Ait Barka, 2015).

The main issue is that there is very little known about the mechanisms, by which the bacteria finds the host, colonizes it, and is able to overcome the defense mechanisms of the host. The study by Wang A. and Lazarovits G. found the relationship between population density of pathogenic Streptomyces in the rhizosphere and common scab occurrence being r(person’s correlation coefficient)=1.00 (2005). That number is much higher than was found in the previous studies: r=0.55 by Ryan and Kinkel (1997). It shows that the bacteria multiply the quickest in the rhizosphere and then follow the growth of the tuber, while its population declines the further it is from the source (Lynch and Whipps, 1991; Wang A. and Lazarovits G. 2004).

3.2.4 Pathogenicity and pathogenic islands of *Streptomyces spp.*

Streptomyces spp. are prokaryotic bacteria pathogens often confused with fungi because they produce spores to aid their spread, as well as their distinguishing ability to produce nonfragmenting substrate mycelium, which colonizes organic matter in the soil

(R. Loria et al., 1997). *Streptomyces scabies* is the most resistant out of all the other *Streptomyces species*, and can survive in various pH and temperatures, while other species such as *Streptomyces acidiscabies* are not able to survive in the soil for long periods of time (Manzer, F. E. et al., 1977). Therefore, cultivars resistant to *Streptomyces scabies* show the most resistance to other *Streptomyces species.* What S*treptomyces spp.* causing common scab share in common is having phytotoxin thaxtomin in their pathogenic islands (Lawrence, C.H., et al., 1990). Both *Streptomyces scabies* and *Streptomyces acidiscabies* produce thaxtomin A, while *Streptomyces ipomoeae* produces thaxtomin C (King, R.R., 1995). However, other independent virulence factors (e.g. nec1, tomA, fas operon) may or may not be present on a large mobile pathogenicity island together with a biosynthetic gene cluster for thaxtomin production in different combinations (Loria et al.2006; Lerat et al., 2009). Some of these virulence factors were demonstrated to have necrotizing or colonizing potential, while the role of others remains unclear (Loria et al.2006). Pathogenic island types based on the presence/absence of different virulence factors and their organization were reported to differ between different streptomycetes species (Li et al., 2019). Even in the same species of scab-causing *S.turgidiscabies* isolated from potatoes originated in Finland, four types of PAI were observed (Aittamaa et al., 2010).

3.2.5 Thaxtomin's role in potato common scab development

The main pathogenicity determinant of common scab symptoms is a phytotoxin thaxtomin produced by all known common scab pathogens: "Thaxtomin A is a potent phytotoxin that serves as the principle pathogenicity determinant of the common scab pathogen, *Streptomyces scabiei*, and is also a promising natural herbicide for agricultural applications" (Yuting Li, 2020). Thaxtomins are cyclic dipeptides (2,5-diketopiperazines) containing 4-nitrotryptophan and phenylalanine residues. "Thaxtomin A (1) (the major metabolite) and thaxtomin B(4) were first identified as secondary metabolites inS.scabiesin-fected potato slices" (King R.R., 1989). "Four other thaxtomins (compounds2, 3, 5 and 6) were later isolated from similar sources" (King

R.R., 1992). Thaxtomin A, being the more prominent one is synthesized by two peptide synthase genes: txtA and txtB (Healy F.G., 2000).

Its' role was discovered by measuring the ability to produce Thaxtomin A by various *Streptomyces scabiei* isolates (Loria R., 1995). This is backed up by the study of Healy F.G.: "Targeted gene disruption of the thaxtomin synthase genes provided definitive evidence that thaxtomin production is required for the infection of intact host tuber tissue by Streptomyces acidiscabies" (2000). This means that if the pathogenic causative agent has low thaxtomin production, it's not able to inflict permanent or serious damage with common scab such as a bacteria with a high thaxtomin production level would be able to. A summery or what is known about the interaction of common scab and *Streptomyces spp.* is shown in Figure 1: The biosynthetic pathway for the pathogenicity determinant thaxtomin and the gene cluster encoding thaxtomin biosynthesis have been fully elucidated (Bignell et al. 2010), and numerous details of the regulation of this gene cluster have been described. Definitive verification of pathogenicity determinants is still missing for common scab in developing potatoes, as is information on plant-derived factors involved in the interaction between pathogenic *Streptomyces* and potato tubers, indicated in the red boxes in Figure 1. Potato cultivars, which exhibit a high degree of tolerance to common scab should be given more attention in order to elucidate the mechanism behind resistance, such as tryptophan production or excretion into the rhizosphere. In addition, deep-sequencing technologies are now being used to study potato microbial communities of endophytes, mycorrhizae, rhizosphere, and soil in order to gain a more complete picture of the effects of microbial communities on plant health and common scab disease (Merete W., 2012).

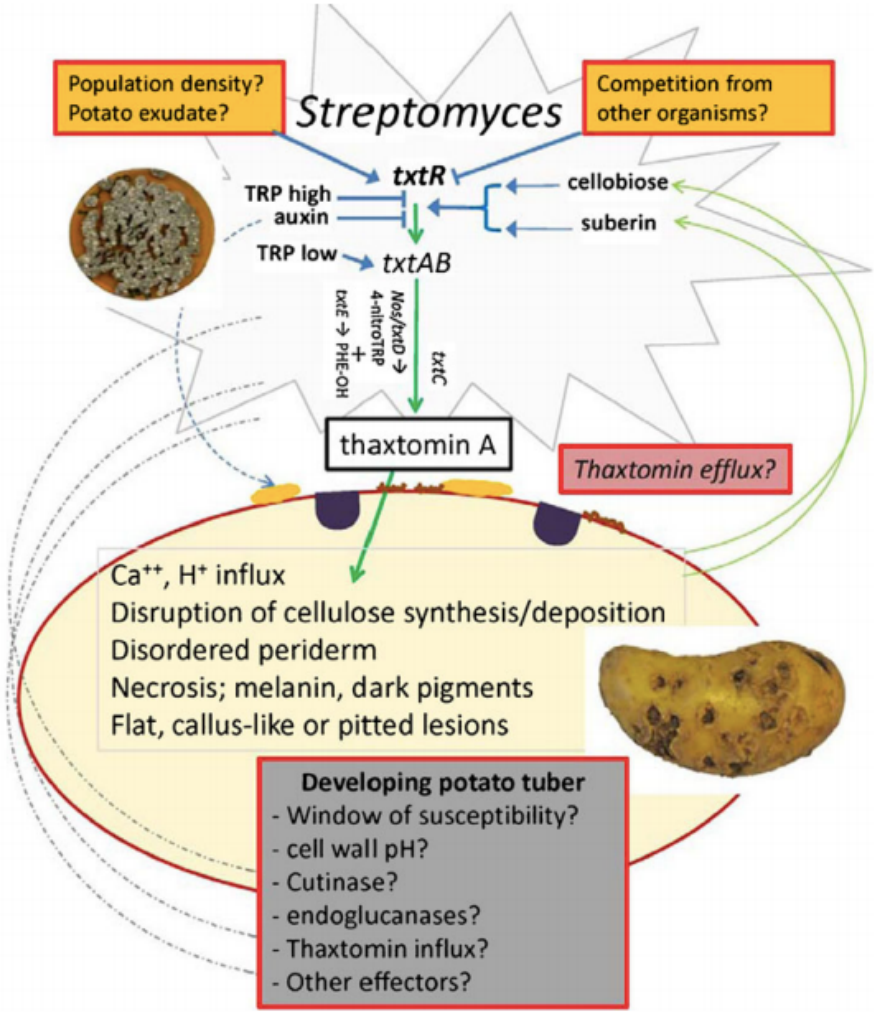


Figure 1: An overview of the current knowledge of common scab induced by thaxtomin. Boxes with red outline represent current unknown topics of debate of the cause of common scab by *Streptomyces spp* (Merete W., 2012).

4 Methodology

The work was performed in a microbiology laboratory facility in the Crop Research Institute (Prague) using selected 134 strains of potato cultivars, which were obtained from various fields in the Czech Republic and South America from years 2014 to 2019 (Table 1). The original stock had 164 strains, however the remaining strains were not chosen either due to contamination, no obtained DNA from the extraction, or old age of the glycerol preservation conserve.

Sample	Location	Year isolated	Sample	Location	Year isolated	Sample	Location	Year isolated
14HB22D	Havlickova Borova	2014	14HB8C	Havlickova Borova	2015	3CH6	Chile	2019
14HB4D	Havlickova Borova	2016	14HB5D	Havlickova Borova	2015	1CHIV	Chile	2019
14HB4B	Havlickova Borova	2015	14HB6D	Havlickova Borova	2015	1CH13	Chile	2019
14HB14D	Havlickova Borova	2015	14HB40D	Havlickova Borova	2015	14SL27	Slavkov	2014
14SL5	Slavkov	2015	14HB5C	Havlickova Borova	2015	1CH7	Chile	2019
14HB18D	Havlickova Borova	2014	14HB2B	Havlickova Borova	2016	3A6	Argentina	2019
14HB19D	Havlickova Borova	2014	14HB17B	Havlickova Borova	2015	8CH12R2	Chile	2019
14HB1B	Havlickova Borova	2015	14HB41D	Havlickova Borova	2015	14TU9	Trutnov	2015
14HB21C	Havlickova Borova	2015	14HB38D	Havlickova Borova	2015	14SL24	Slavkov	2015
14SL14	Slavkov	2015	14HB43D	Havlickova Borova	2015	8CH11R2	Chile	2019
14HB7C	Havlickova Borova	2014	14HB32D	Havlickova Borova	2015	25P1	Peru	2019
14HB27C	Havlickova Borova	2015	14HB37D	Havlickova Borova	2015	4C5	Chile	2019
14HB19C	Havlickova Borova	2014	14HB27D	Havlickova Borova	2015	14HB13B	Havlickova Borova	2015
14HB11C	Havlickova Borova	2015	14HB36D	Havlickova Borova	2015	14SL31	Slavkov	2015
14HB24B	Havlickova Borova	2014	14HB29D	Havlickova Borova	2014	14SL16	Slavkov	2015
14SL11	Slavkov	2015	14HB1D	Havlickova Borova	2014	14HB33D	Havlickova Borova	2015
14HB18B	Havlickova Borova	2015	14HB44D	Havlickova Borova	2015	14HB34D	Havlickova Borova	2015
14SL13	Slavkov	2015	14HB6C	Havlickova Borova	2015	14HB28B	Havlickova Borova	2015
14SL7	Slavkov	2014	14TU13	Trutnov	2015	14HB20C	Havlickova Borova	2015
14HB8D	Havlickova Borova	2016	14TU15	Trutnov	2015	14SL19	Slavkov	2015
14HB16C	Havlickova Borova	2015	14VE10	Velhartice	2015	8CH19R3	Chile	2019
14HB20D	Havlickova Borova	2016	14VE12	Velhartice	2015	14HB32C	Havlickova Borova	2015
14HB22C	Havlickova Borova	2015	14VE13	Velhartice	2015	14HB13D	Havlickova Borova	2016
14HB15C	Havlickova Borova	2015	14VE9	Velhartice	2015	14HB2D	Havlickova Borova	2015
14HB21B	Havlickova Borova	2015	14TU10	Trutnov	2015	14HB39C	Havlickova Borova	2015
14HB25C	Havlickova Borova	2015	14TU11	Trutnov	2015	14HB25B	Havlickova Borova	2015
14HB17C	Havlickova Borova	2015	14TU5	Trutnov	2015	14HB27B	Havlickova Borova	2015
14HB16D	Havlickova Borova	2015	14TU6	Trutnov	2015	14SL10	Slavkov	2015
14HB21D	Havlickova Borova	2015	1PIV	Peru	2019	14HB10B	Havlickova Borova	2015
14SL3	Slavkov	2015	1P4	Peru	2019	14HB44C	Havlickova Borova	2015
14HB9C	Havlickova Borova	2015	30P3	Peru	2019	14HB45D	Havlickova Borova	2015
14HB13C	Havlickova Borova	2015	14TU7	Trutnov	2015	14HB11B	Havlickova Borova	2015
14HB12B	Havlickova Borova	2015	14SL20	Slavkov	2015	14HB45C	Havlickova Borova	2015
14HB10C	Havlickova Borova	2015	14SL22	Slavkov	2015	14HB31C	Havlickova Borova	2015
14HB7B	Havlickova Borova	2015	11CH8	Chile	2019	14HB47C	Havlickova Borova	2015
14HB15B	Havlickova Borova	2015	7CH6	Chile	2019	14HB39D	Havlickova Borova	2015
14HB14C	Havlickova Borova	2015	30P4	Peru	2019	14HB34C	Havlickova Borova	2015
14HB25D	Havlickova Borova	2015	30VII	Peru	2019	14SL17	Slavkov	2015
14HB10D	Havlickova Borova	2016	30PX	Peru	2019	14HB42C	Havlickova Borova	2015
14HB26D	Havlickova Borova	2015	14SL29	Slavkov	2015	14HB33C	Havlickova Borova	2015
14HB28D	Havlickova Borova	2015	14TU4	Trutnov	2015	14VE2	Velhartice	2015
14HB3D	Havlickova Borova	2016	7CH8	Chile	2019	14VE4	Velhartice	2015
14HB11D	Havlickova Borova	2015	14SL28	Slavkov	2015	14HB20B	Havlickova Borova	2015
14HB15D	Havlickova Borova	2015	14TU8	Trutnov	2015	14SL8	Slavkov	2015
14HB16B	Havlickova Borova	2015	1P6	Peru	2019			

Table 1: Table with all 134 strains, which were used for final sequencing and their location of origin and year of isolation.

The experiment was aimed at determining the pathogenicity of 134 isolated strains through the presence/absence of the genes responsible for the production of the main pathogenicity determinant of the common scab thaxtomin (using primers *stx1 a/b* and *TxtBC1_F/R*) and other virulence genes such as tomatinase, fascinase, and necrotizing protein (*Tom 3/4*, *P19 f/r* and *Nec f/r*, respectively) (Table 2). The phylogenetic position of the strains was determined based on the 16S rRNA gene. All the strains were collected between years 2014 and 2019 in the various fields of South America (Peru, Chile, Argentina) and Czech Republic: Havlickova Borova (HB), Slavkov (SL), Trutnov (TU), Velhartice (VE). All the strains were stored in glycerol preservation conserved at -70°C. Collection strains *Streptomyces scabiei* DSM 41658, *Streptomyces acidiscabies* DSM 41668, *Streptomyces europaeiscabiei* DSM 41802, and *Streptomyces turgidiscabies* DSM 41838 were used as a control.

Primer	Gene	Function
16S	16S rRNA gene	universal primer, used for determination of phylogenetic positions of the strains
stx1 a/b	Thaxtomin gene	primers were based on the DNA sequence of the txtABgenes of S. acidiscabies and were designed toamplify a 402-bp fragment of the txtABintergenic regionamplifies the region of txtAB gene, which is important for determining pathogenicity in scab causing bacteria (R. Flores-González, I., et al., 2007)
TxtBC1_FR	Intergenetic region	Production of thaxtomin A involves the nonribosomal peptide synthetases (NRPSs) TxtA and TxtB, both of which contain an adenylation (A-) domain for selecting and activating the appropriate amino acid during thaxtomin biosynthesis. TxtC (Kopecky, unpublished)
Tom 3/4	Tomatinase gene	genes responsible the mechanisms of pathogenicity: for pothagenicity factors (L. A. Wanner, 2006)
P19 f/r	Fascinase gene	S. turgidiscabies contains a plant fasciation operon constituted by sixfasgenes and two methyltrans-ferase genes. The fas operon is not detected in S. scabies and S. acidiscabies (M. Aittamaa et al., 2010)
Nec f/r	Necrotizing protein	genes responsible for the mechanisms of pathogenicity: necrosis-inducing protein. It allows the nonpathogen *Streptomyces lividans* to necrotize and colonize potato tuber disks and produce scablike symptoms on immature potato tubers (R. A. Bukhalid, et al., 1998)

Table 2: Names of primers, respective genes and their description (by author, 2021).

4.1 Preparation and bacteria cultivation

4.1.1 Inventory sorting and Solid Medium Preparation

The cultures were taken from stocks stored in -70°C freezer. The strains were inventoried, and a table was produced (Table 1). The solid agar medium was prepared with 1 liter of distilled water to 18.2 grams of R2A Agar for microbiology (Ph.Eur; VWR Chemicals). Once prepared, the mixture was divided into three 500 mL bottles with screw caps and agitated. The sterilization was done for 15 minutes at 121°C in an autoclave PS20A (Chirana). The autoclave sticker was put on the jars to indicate if the desired temperature was reached by showing black stripes. Agar medium was then partly cooled down to room temperature to avoid water condensation on the Petri dishes.

Figure 2: Sterilized Petri dishes filled with solid agar medium, ready to be planted with samples in a Flowbox (photo by author, 2020).

The Petri dishes along with all the other needed materials (pipette, tips (autoclaved in advance), jars with agar medium (autoclaved in advance), permanent

marker, a stand for samples, single-use inoculation loops (10 µl Disposable Inoculating Loops, Biologix Group Limited), ethanol) were sterilized under the hood in a flow box equipped with an ultraviolet lamp for at least 30 minutes. The work was done with clean gloves (sterilized with 70% ethanol between the samples) and new tips and loops were used for each sample. Each Petri dish is labeled accordingly with a permanent marker. The liquid agar was distributed among the Petri dishes under the hood with airflow left there to solidify, and if not used immediately were stored in containers in the fridge upside down to prevent the lid from fogging up (Figure 2).

4.1.2 Bacterial strains cultivation

The cultures of individual *Actinobacteria* strains were transferred from the stocks on Petri dishes with the solid agar medium in a flow box. The cultures were taken out from -70°C freezer 30 minutes in advance, in order for them to take the liquid form. Approximately, 50ml of the culture were pipetted onto the Petri dish, then spread around with an inoculation loop (10 µl Disposable Inoculating Loops, Biologix Group Limited) and streaked with lines to separate individual colonies. Homogenous growth in R2A solid medium (Ph.Eur; VWR Chemicals) for all strains is essential for accurate results in further testing. The cultures were grown upside down in a Q-cell incubator (type, mark, country) at 27-28.5°C for five days, the Petri dishes were checked for contamination. The cultures grew differently, some already had developed spores. However, some were contaminated and contained other types of bacteria, which you could tell by the glistening shine they reflected upon the light (Figure 3). Those samples were regrown again either by selecting an individual uncontaminated colony or from the original stock, and if the result remained the same the culture was discarded (Table 1 only contains uncontaminated strains). The successfully grown Petri dishes were put upside down in bags and stored in a separate refrigerator to avoid drying out of the agar.

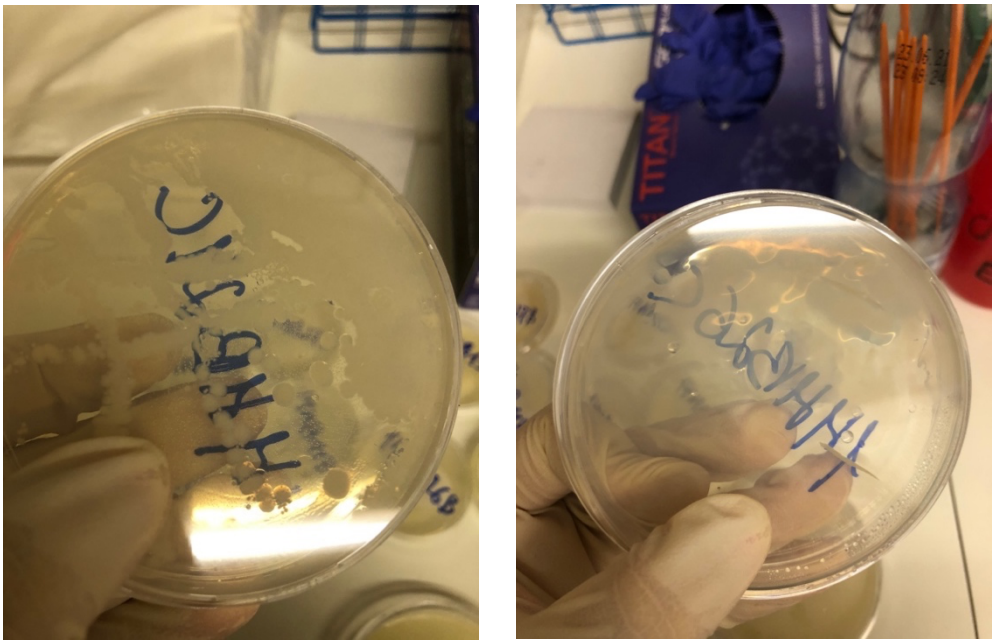


Figure 3: Petri dish with contaminated sample 14HB26C grown in solid agar medium, which clearly shows glistening shine and differentiates from typical actinobacteria appearance. (photo by author, 2020).

4.1.3 Liquid Medium

The following goal was to regrow the strains in a liquid medium.
R2A liquid culture medium was prepared according to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) R2A MEDIUM (Leibniz Institute DSMZ 2007), no agar was added to the mixture to maintain the liquid form of the medium. Liquid medium R2A was prepared by dissolving the individual components in 1 liter of distilled water: 0.50 grams of yeast extract (Bacto™, ref: 212750), 0.50 grams proteose peptone (Bacto™, ref: 211684), 0.50 grams of casamino acids (Difco™, ref: 228820), 0.50 grams of glucose (PENTA, CAS: 50-99-7), 0.50 grams of soluble starch (PENTA, CAS: 9005-25-8), 0.30 grams of Na-pyruvate (SIGMA, P2256-100G), 0.30 grams of K_2HPO_4 (PENTA, CAS: 16788-57-1), 0.05 grams of $MgSO_4$ x 7 H_2O (PENTA, CAS: 10034-99-8). After the solution was thoroughly mixed, the medium was evenly

distributed into 250ml Erlenmeyer flasks with ribbed sides (20 flasks, 50ml each). The ribbed flasks were used for better mixing afterwards. All flasks are covered with thick tissue and secured with an elastic band. Sterilization was performed with a PS20A autoclave (Chirana) at 121 ° C for 15 minutes.

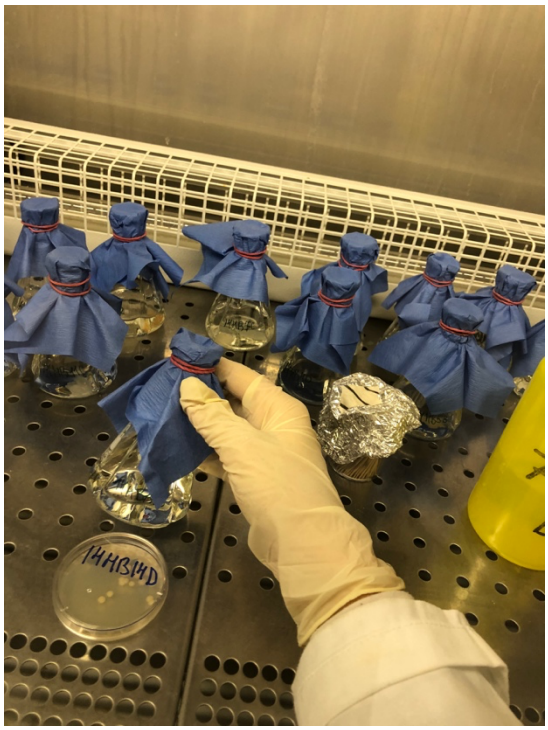
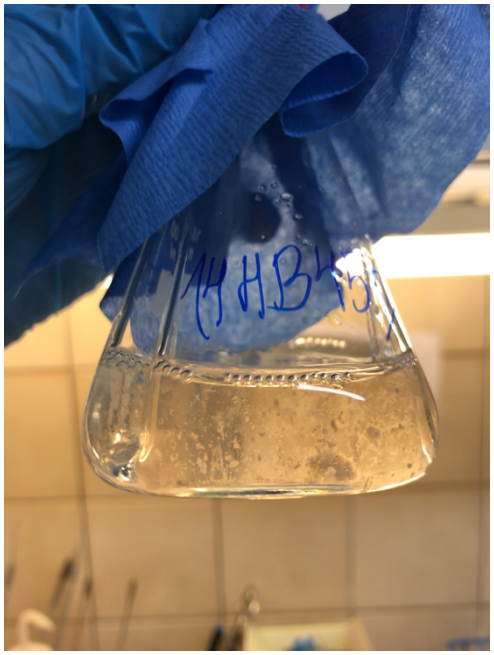


Figure 4a (left): Sterilized 250ml Erlenmeyer flasks filled with liquid medium, ready to be planted with bacterial cultivars grown in agar solid medium. (photo by author, 2020). Figure 4b (right): Planted 250ml Erlenmeyer flasks in the automatic shaker on 28°C and 200 rpm. (photo by author, 2020).

The Erlenmeyer flasks were taken out of the autoclave and chilled, then were brought together with all the other equipment (autoclaved toothpicks; inoculation loops (10 µl Disposable Inoculating Loops, Biologix Group Limited), ethanol, permanent marker, etc) in a flow box for UV light sterilization for at least 30 minutes. Following that, the flasks were planted with culture samples that were grown previously in the Petri dishes, one by one using a new clean toothpick every time to cut out a portion of agar with spores or high concentration of bacteria and carefully transfer it into the Erlenmeyer

flask with the liquid medium (Figure 4a). The flasks were labeled with a permanent marker according to the sample. Lids were open as little as possible and gloves were regularly sterilized to avoid contamination. After all the samples were transferred, the flasks were put in an automatic shaker at 28°C and 200 rpm for about 3-4 days (Figure 4b).

4.1.4 Cryotubes – new conserves



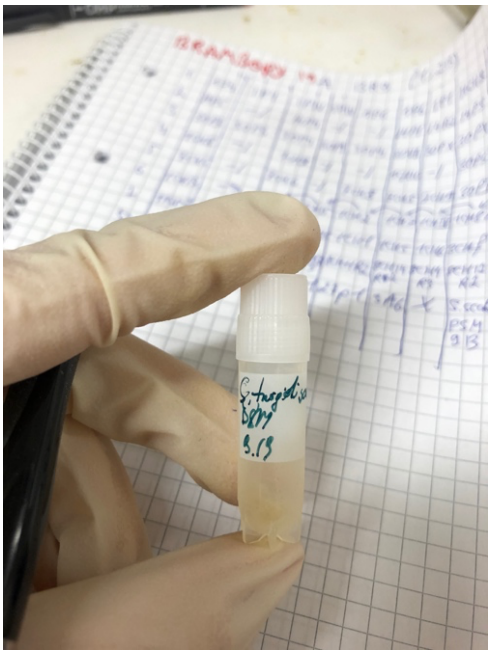


Figure 5 (left): Clear sample 14HB45D in a liquid medium, which visibly has the clear and see-through solution with no signs of contamination. (photo by author, 2021).
Figure 6 (right): Labeled cryotube sample from -70°C freezer (photo by author, 2020).

Following the procedure after 3 to 4 days, the planted Erlenmeyer flasks were checked for growth and contamination: all the bacteria have grown, and if the solution was clear with 1 visible kind of organism, then that sample was pure (Figure 5). Some grown samples developed cloudy and dark solution; therefore, they were replanted in a new Petri dish and to a new liquid medium again (the samples, which then ended up

being contaminated again were discarded from the experiment and the original stock). After samples passed the contamination check, 750µl glycerol preservation cryotubes were prepared and autoclaved PS20A (Chirana) for 15-20 minutes at 121°C. Those were used for making new conserves for long-term storage by adding 750µl of sample to a labeled cryotube. For each sample, two cryotubes were made and stored in -70°C freezer (Figure 6).

4.2 DNA isolation and Electrophoresis

4.2.1 DNA Isolation using SIGMA-ALDRICH Kit

Gen Elute Bacterial Genomic DNA kit (Wizard Genomic DNA Purification kit, QIAGEN, Germany) was used for the isolation of the DNA from the first couple groups of samples. The steps followed exactly the instruction provided by the kit. However, at the very last step instead of a provided collection tube, a 2ml Eppendorf tube was used for more convenient storage. After the isolation, the DNA samples were stored in -20°C freezer.

The successful extraction and quantity of DNA were measured by a spectrophotometer NanoPhotometer P300 (Implen) and gel electrophoresis.

4.2.2 DNA Isolation using DNeasy® PowerLyzer® Microbial Kit

For the remaining samples, commercial extraction kit DNeasy PowerLyzer Microbial Kit (QIAGEN, Germany) was used. The exact official protocol was followed. This kit was more preferable to the one previously stated for many reasons. First of all, this DNA isolation protocol was much more time efficient taking an hour and a half to complete versus approximately three hours with the Wizard Genomic DNA Purification kit. More crucially, this procedure did not require the use of the SpeedVac centrifugation step, which dried the samples and made DNA isolation less successful/prominent. After

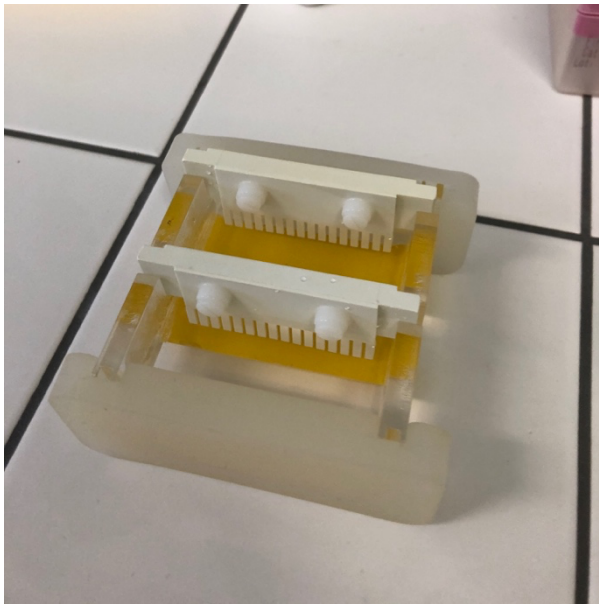
the isolation was complete the samples were put in -20°C freezer for storage or used immediately for electrophoresis.

4.2.3 Electrophoresis

Electrophoresis gel was used to check the results of DNA extraction. Work with electrophoresis materials was done in nitrile gloves for safety reasons and to avoid contamination of another workspace. To make 1 gel for 30 samples following solution was mixed: 0.4g of agarose (SIGMA, A9539-250G) and 40ml of TAE buffer. The solution was agitated and heated up without letting it boil till it became homogeneous, then 5µl of SYBR coloring was added and agitated again. Gel stand is then assembled, consisting of a plastic form, two thick rubber gums, and two comb separators (Figure 7). The agarose buffer solution was poured into the form and left at room temperature for 30 minutes to solidify. Subsequently, the combs and gums were taken out and the form with solidified gel was placed on a stand with TAE buffer for electrophoresis. Possible bubbles were removed by gently shaking the mold, TAE buffer was added if necessary, to completely submerge the gel (Figure 8).

The samples for electrophoresis were prepared on a sterile microscopic plate (labeled with a permanent marker). The following procedures were done rapidly to avoid drying out of the samples. In each designated hole (1 hole/1 sample) were added 2µl of ‘PCR loading buffer’, then added 2µl of DNA samples using a new tip every time.

In the first hole of each row of the gel 5µl of the ladder, solution (GeneRuler 1kb Plus DNA Ladder) was added. Ladder is a set of DNA molecules with known lengths, which is used to identify the length of the tested samples. The mixed DNA samples from the microscopic plate were pipetted into the corresponding gel holes. The list was made in advance to note down which sample corresponds to which hole. The electrophoresis box was closed with a cover, cords plugged in accordingly, and turned on for 15-18 min (67 volts, 94mAmps). The gels were scanned on a scanner using UV light, pictures were saved and labeled.



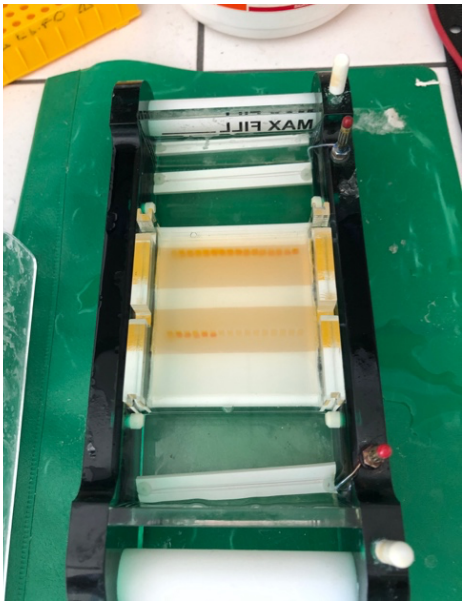


Figure 7 (left): On a photo is an assembled gel stand with rubber sides and combs, already filled with gel solution.
Figure 8 (right): Solidified gel in a stand covered completely in TEA buffer. The holes in the gel are already filled with 4µl of DNA + PCR loading buffer solution from a microscopic plate.

4.3 Polymerase Chain Reaction and sequencing

4.3.1 16S Polymerase Chain Reaction and conditions

With the DNA extracted, it was necessary to amplify/multiply specific segments of the DNA strand for further analysis. Universal primer 16S was used as a control primer since all bacteria have it. 25µl PCR was used with AccuPrime *Taq*DNA Polymerase (ThermoFischer Scientific) with the following mix proportions for one sample: 2.5µl of 10xAccuprime Buffer II; 1.25µl of DMSO; 0.5µl of 10mM 16S 27f (5'-AGAGTTTGATCMTGGCKAG-3');

0.5µl of 10mM pH' (5'-AAGGAGGTGATCCAGCCGCA-3')); 0.1µl of AccuPrime *Taq*DNA Polymerase; 1µl of diluted DNA (30x with distilled H_2O); and 19.15µl of distilled H_2O. The designated work surface was sterilized and all the components except for DNA were brought in for mixing. Depending on the number of samples, a proportionate solution was mixed in a sterilized 2ml Eppendorf tube and vortexed thoroughly until homogenous, then distributed to 0.2ml PCR test tubes (24µl per tube). DNA was distributed in a separate room (to not contaminate the polymerase) under the hood to each respective PCR test tube. The tubes were labeled according to the sample. All the tubes were put in a thermal cycler (Bio-Rad) with a specific protocol: 1) initial denaturation: 95°C for 2:00 min; 2) denaturation: 95°C for 30sec; 3) annealing: 56°C for 30sec; 4) extension: 68°C for 1:30min; 5) cycle number: go to 2 for 25 times; 6) extension: 68°C for 2:00 min; 7) soak: 4°C forever; 8) end.

After the end of the cycle, the samples were checked on electrophoresis gels, scanned and stored in -20°C freezer.

4.3.2 Temperature control for stx1 a/b, Tom 3/4, P19 f/r, TxtBC1_F/R, N f/r primers for PCR

The specific primers, which were ordered (stx1 a/b, Tom 3/4, P19 f/r, TxtBC1_F/R, N f/r) had a wide range of possible temperature that could be used in a thermal cycle: for stx1 a/b, Tom 3/4, P19 f/r, it was 54-61°C; for TxtBC1_F/R it was 49-58°C; for N f/r 60-67°C. To find out the temperature that works best for each primer, a gradient PCR was performed using the same sample for all the temperatures to determine the best conditions. The four strains that were used were *Streptomyces* isolates *Streptomyces turgidiscabies, Streptomyces europeascabies, Streptomyces acidiscabies, Streptomyces scabies*.; therefore, for each primer and isolate there are 8 samples, making it 160 samples.

The results showed that for *stx1 a/b*, *Tom 3/4, TxtBC1_F/R*, and *N f/r* primers the temperature did not make a difference, therefore the highest tested temperature was used for further analysis (61°C for stx1 and Tom, 58°C for txt, 67°C for N). For P19 f/r,

the highest temperature appeared more prominent on gels than all the others, therefore 61°C was used for all the other samples.

4.3.3 GoTaq ® G2 flexi DNA Polymerase

The GoTaq ® G2 Flexi DNA Polymerase (Promega, USA) was used for all further PCR procedures. Throughout the experiment, it was found to be the most efficient and prominent on the gels. One of the reasons for that is that the PCR mix contains Green GoTaq ® Flexi Buffer (Promega, USA), which contains dyes (a blue and a yellow dye), therefore during the electrophoresis it was not necessary to mix the sample with the 'PCR loading buffer' before putting it in the gel.

The PCR mix with the new polymerase for 25µl/sample contained: 5µl of Green GoTaq ® Flexi Buffer, 0.5µl of 10mM Nucleotide mix, 0.5µl of forward primer, 0.5µl of reverse primer, 0.125µl of GoTaq ® G2 Flexi DNA Polymerase, 17.374µl of distilled H_20, and added separately last 1µl of DNA sample.

4.3.4 stx1a/b; Tom 3/4; P19 f/r Polymerase Chain Reactions conditions

Primers stx1 a/b, Tom 3/4 and P19 f/r followed the same PCR protocol since time and temperatures were the same: 1) initial denaturation: 95°C for 2:00 min; 2) denaturation: 95°C for 30 sec; 3) annealing: 61°C for 40 sec; 4) extension: 72°C for 40 sec; 5) cycle number: go to 2 for 34 times; 6) extension: 72°C for 5:00 min; 7) soak: 12°C forever; 8) end. The samples were then either used immediately for electrophoresis gels or stored in -20°C freezer.

Sequences for: stx1a 5'-GTGGACCGTGGAGCATCT-3'; stx1b 5'-CAGTTCGGCGTAACTCAGC-3'; Tom3 5'-GAGGCGTTGGTGGAGTTCTA-3'; Tom4 5'-TTGGGGTTGTACTCCTCGTC-3'; P19f 5'-GTAGGTGGGACCAGGTGAGA-3'; P19r 5'-GGGGCTGACGGAACTACAC-3'.

4.3.5 TxtBC1_F/R Polymerase Chain Reaction conditions

For primer TxtBC1_F/R following protocol was used: 1) initial denaturation: 95°C for 2:00 min; 2) denaturation: 95°C for 30 sec; 3) annealing: 58°C for 40 sec; 4) extension: 72°C for 2:00 min; 5) cycle number: go to 2 for 34 times; 6) extension: 72°C for 5:00 min; 7) soak: 12°C forever; 8) end. The samples were then either used immediately for electrophoresis gels or stored in -20°C freezer. Sequences: TxtBC1_F 5'-CCTTCACACCCTGRACAT-3'; TxtBC1_R 5'-AGTTCGGTGAAGTTGGGY-3'.

4.3.6 Nf/r Polymerase Chain Reaction conditions

For primer Nf/r following protocol was used: 1) initial denaturation: 95°C for 2:00 min; 2) denaturation: 95°C for 30 sec; 3) annealing: 67°C for 40 sec; 4) extension: 72°C for 40 sec; 5) cycle number: go to 2 for 34 times; 6) extension: 72°C for 5:00 min; 7) soak: 12°C forever; 8) end. The samples were then either used immediately for electrophoresis gels or stored in -20°C freezer. Sequencing: Nf 5'-ATGAGCGCGAACGGAAGCCCCGGA-3'; Nr 5'-GCAGGTCGTCACGAAGGATCG-3'.

4.3.7 Sequencing analysis

After the PCR procedure for all the primers across all the samples has been finished and electrophoresis gels were made to check the success of it, the results were sent to another laboratory for sequencing analysis. Special autoclaved plates were prepared, and all the samples were transferred onto a designated slot on the plate (the list was made in advance to know which sample is going to be where). This is a very important step in the experiment as the results of sequencing will tell us exactly the species, genus, family, and classification of bacteria present in the samples. It is the most common that *Streptomyces* species from Actinobacteria group cause potato common scab decease.

During the first batch of sequencing on 12/01/21 all 16S, TxtBC1_F/R, P19 f/r and partly N f/r primers samples were sent. And during the second batch of sequencing all stx1 a/b, Tom 3/4, and remaining N f/r primers samples were sent.

5 Results

5.1 Results of the PCR gene primers testing

16S rRNA gene sequences were determined by amplification with primers 16Seu27f and pH. The specific PCR results demonstrated partial sequences of stx1 a/b and TxtBC1_F/R, which code for thaxtomin phytotoxin in common scab-causing bacteria. As a result, 96 strains tested positive for stx1 a/b, which is 72% from the total (being the highest percentage of all, and only 12 tested positive for TxtBC1_F/R, which is 9% from the total. Together only 8 strains tested positive for both stx1 a/b and TxtBC1_F/R intergenic region, out of which strain 4C5 is from Chile (South America) and the rest are from Havlickova Borova and Velhartice (Czech Republic). For detailed results in the respective locations, see Figure 9.

N f/r genes were positive in 63 samples (47%), out of which only 8 strains were from South America and the rest were from the Czech Republic. Each site contained these genes: 40/82 in Havlickova Borova, 10/18 in Slavkov, 3/6 Velhartice, 3/10 in Trutnov, 2/7 in Peru, 4/10 in Chile, and 1/1 in Argentina. N f/r genes were also the most frequent in stx1 a/b '+' strains. Genes P19 f/r and Tom 3/4 both tested very low with 15 and 20 positives, respectfully. For detailed results see Table 3. Gene Tom 3/4 was absent in Argentina and Velhartice. In Havlickova Borova 'stx1 a/b + txtB/C; Tom3/4; P19' tested similar 37-38% and in Chile the same types resulted in similar 45-50%, however there are no other clear patterns throughout the results (Table 4).

sample	16S	stx1 a/b	txt B/C	Tom 3/4	P19 f/r	N f/r
14HB22D	+	+	-	-	-	+
14HB4D	+	+	-	-	-	+
14HB4B	+	+	-	-	+-	-
14HB14D	+	+	-	-	-	+
14SL5	+	+	-	-	-	+

14HB18D	+	+	-	-	-	-
14HB19D	+	+	-	-	-	+-
14HB1B	+	+	-	-	-	+-
14HB21C	+	+	-	-	-	+
14SL14	+	+-	-	+-	-	-
14HB7C	+	+-	-	-	-	-
14HB27C	+	+-	-	-	-	-
14HB19C	+	+-	-	-	-	-
14HB11C	+	+-	-	-	-	+
14HB24B	+	-	-	-	-	-
14SL11	+	-	-	+-	+-	-
14HB18B	+	-	+-	-	+-	-
14SL13	+	-	-	-	-	-
14SL7	+	-	-	+	-	+
14HB8D	+	-	-	-	-	+-
14HB16C	+	-	-	-	-	+
14HB20D	+	+	+	+	-	+
14HB22C	+	-	+-	-	-	-
14HB15C	+	-	-	+-	+-	-
14HB21B	+	-	+-	-	-	-
14HB25C	+	-	-	-	-	-
14HB17C	+	-	-	-	-	-
14HB16D	+	-	-	-	-	-
14HB21D	+	+	+	+	-	+
14SL3	+	+	-	-	-	+
14HB9C	+	+	-	-	-	+-
14HB13C	+	+	-	-	-	+
14HB12B	+	+	+-	-	-	-
14HB10C	+	+	-	-	-	-
14HB7B	+	+	-	-	-	-
14HB15B	+	+	+-	-	-	+-
14HB14C	+	+	-	-	-	+-
14HB25D	+	+	-	-	-	-

14HB10D	+	+	-	-	+-	-
14HB26D	+	+	-	-	-	-
14HB28D	+	+	-	-	-	+
14HB3D	+	+	+-	-	+-	+
14HB11D	+	+	-	-	-	+
14HB15D	+	+	-	-	-	+
14HB16B	+	-	-	-	-	+
14HB8C	+	-	-	-	-	-
14HB5D	+	-	-	-	-	+
14HB6D	+	-	-	-	+	-
14HB40D	+	-	-	-	-	-
14HB5C	+	-	-	-	-	-
14HB2B	+	-	-	-	-	-
14HB17B	+	-	-	-	-	-
14HB41D	+	-	-	-	-	-
14HB38D	+	-	-	-	-	+
14HB43D	+	-	-	+	-	+
14HB32D	+	-	-	N/A	N/A	N/A
14HB37D	+	-	-	N/A	N/A	N/A
14HB27D	+	-	-	-	-	+
14HB36D	+	-	-	-	+-	+-
14HB29D	+	-	-	N/A	N/A	N/A
14HB1D	+	-	+-	N/A	N/A	N/A
14HB44D	+	+-	-	+	-	+-
14HB6C	+	+-	-	-	-	-
14TU13	+	+	-	-	-	+
14TU15	+	+-	-	-	-	-
14VE10	+	+-	-	-	-	+
14VE12	+	+-	-	-	+	-
14VE13	+	+-	+-	-	+	-
14VE9	+	+	-	-	N/A	-
14TU10	+	-	-	N/A	N/A	N/A
14TU11	+	-	-	N/A	N/A	N/A

14TU5	+	+-	-	-	-	-
14TU6	+	-	-	N/A	N/A	N/A
1PIV	+	-	-	N/A	N/A	N/A
1P4	+	-	-	N/A	N/A	N/A
30P3	+	+	-	+-	+-	-
14TU7	+	+	-	+-	+-	-
14SL20	+	+-	-	-	-	+
14SL22	+	+-	-	-	-	-
11CH8	+	+-	-	-	+-	-
7CH6	+	+-	-	+	-	-
30P4	+	+-	-	+	-	-
30VII	+	-	-	N/A	N/A	N/A
30PX	+	+	-	+	-	-
14SL29	+	+	-	-	-	-
14TU4	+	+	-	-	-	+
7CH8	+	+	-	-	-	+
14SL28	+	-	-	N/A	N/A	N/A
14TU8	+	+	-	-	-	+
1P6	+	+	-	-	-	+
3CH6	+	+-	-	-	-	+
1CHIV	+	+-	-	-	-	+
1CH13	+	+-	-	-	-	+
14SL27	+	+	-	+-	-	+
1CH7	+	-	-	N/A	N/A	N/A
3A6	+	+	-	-	-	+
8CH12R2	+	+	-	-	-	-
14TU9	+	+	-	-	-	-
14SL24	+	+	-	-	-	-
8CH11R2	+	+	-	-	-	+
25P1	+	+	-	-	-	+
4C5	+	+	+	+	-	-
14HB13B	+	+	-	-	-	-
14SL31	+	+	-	-	-	+

14SL16	+	+	-	-	-	+
14HB33D	+	+	-	-	-	+
14HB34D	+	+	-	-	-	-
14HB28B	+	+	-	+	-	+
14HB20C	+	+	-	+	-	+
14SL19	+	+	-	-	-	+-
8CH19R3	+	+	-	-	-	+
14HB32C	+	+	-	-	-	-
14HB13D	+	+	-	-	-	+
14HB2D	+	+	-	-	-	-
14HB39C	+	+	-	-	-	+-
14HB25B	+	+	+	-	+	-
14HB27B	+	+	-	-	-	+
14SL10	+	+	-	-	-	+
14HB10B	+	+	-	-	-	+-
14HB44C	+	+	-	-	-	+
14HB45D	+	+	-	-	-	-
14HB11B	+	+	-	-	-	-
14HB45C	+	+	-	-	-	-
14HB31C	+	+	-	-	-	-
14HB47C	+	+	-	-	-	-
14HB39D	+	+	-	+	-	+
14HB34C	+	+	-	+	-	+
14SL17	+	+	-	-	-	-
14HB42C	+	+	-	-	-	+
14HB33C	+	+	-	-	-	+
14VE2	+	+	-	-	-	+
14VE4	+	+	-	-	-	+
14HB20B	+	+	-	-	-	+
14SL8	+	+	-	-	-	+
S. turgidiscabies (+control)	+	+	+	+	+	+
distilled H_20 (-control)	-	-	-	-	-	-

summary	134	96	12	20	15	63

Table 3: Detailed PCR results for all the 134 tested strains (+/- controls are not included in the calculations). N/A strains with Tom 3/4, P19 f/r, and N f/r were not tested due to coming out negative for stx1 a/b and/or TxtBC_1 primers/genes.

5.2 Taxonomical determination of the strains

Sequences of 16S rRNA genes amplified from the individual strains were aligned using SILVA Incremental Aligner v.1.6.0. (Quast et al., 2013) with GenBank sequences of type strains of known-to-date pathogenic *Streptomyces* species causing common scab of potato. The best-fit model of nucleotide substitution was selected using jModelTest v.2.1.(Darriba et al., 2012). A phylogeny was inferred using maximum-likelihood analysis in FastTree 2.1.10. (Price et al., 2009). The phylograms were finalized in R software with annotation of the presence/absence of tested virulent genes and location (R Core Team, 2020). The resulted phylogenetic tree can be found in Figure 10. (This work has been done by Daria Rapoport to allow further analyses of the results by author).

Among *Actinobacteria* isolated from potato peel coming from different localities in the Czech Republic, various genera were determined which included: *Streptomyces, Glycomyces, Nonomuraea, Streptosporangium, Lentzea, Saccharothrix, Nocardioides, Kribbella, Micromonospora, Catellatospora, Krasilnikoviella, Promicromonospora, Microbacterium, Brevibacterium, Paenarthrobacter, Pseudarthrobacter, Allostreptomyces* (Figure 11). Some strains with detected *stx* gene belonged to another gram-positive phylum *Firmicutes (*genus *Bacillus)*; and gram-negative phylum *Proteobacteria (*genera *Phyllobacterium, Pseudomonas, Azamonas, Methylorubrum, Duganella, Agrobacterium, Acinetobacter),* (Figure 12).

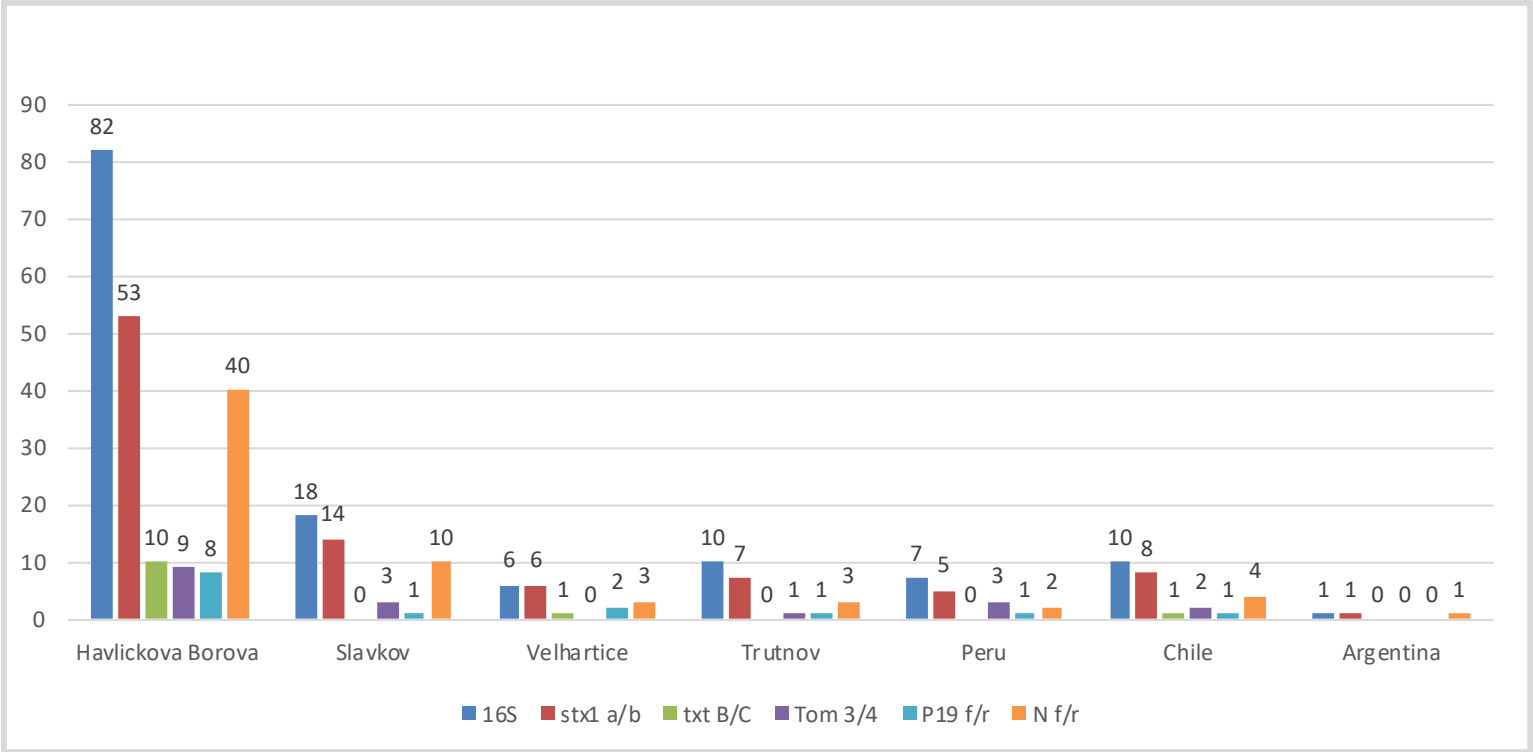


Figure 9: The number of stains with the selected genes determined by PCR with specific primers for individual strains in the studied locations.

All strains tested from South America belonged to the phylum *Actinobacteria* except for the strains 1CHIV and 11CH8 from Chile, which both belonged to *Firmicutes (*genus *Bacillus).* Moreover, the majority of them belonged to *Streptomyces* except for the strains 1CH9, 8CH12R2, and 8CH19R3 from Chile, which belonged to *Brevibacterium, Micromonospora, and Nonomuraea*, respectively. All the other gram-positive and gram-negative genera were determined from the Czech Republic. The genus *Brevibacterium* was only found in 1 sample in Chile, however the genus *Micromonospora* was also found in the fields of Havlickova Borova in the Czech Republic (14HB2D, 14HB25B, 14HB27D, 14HB28D, 14HB37D, and 14HB45D), as well as *Nonomuraea*, which was found in Havlickova Borova (14HB25D, 14HB32D, 14HB38D), Slavkov (14SL16, 14SL20, 14SL28) and Velhartice (14VE11).

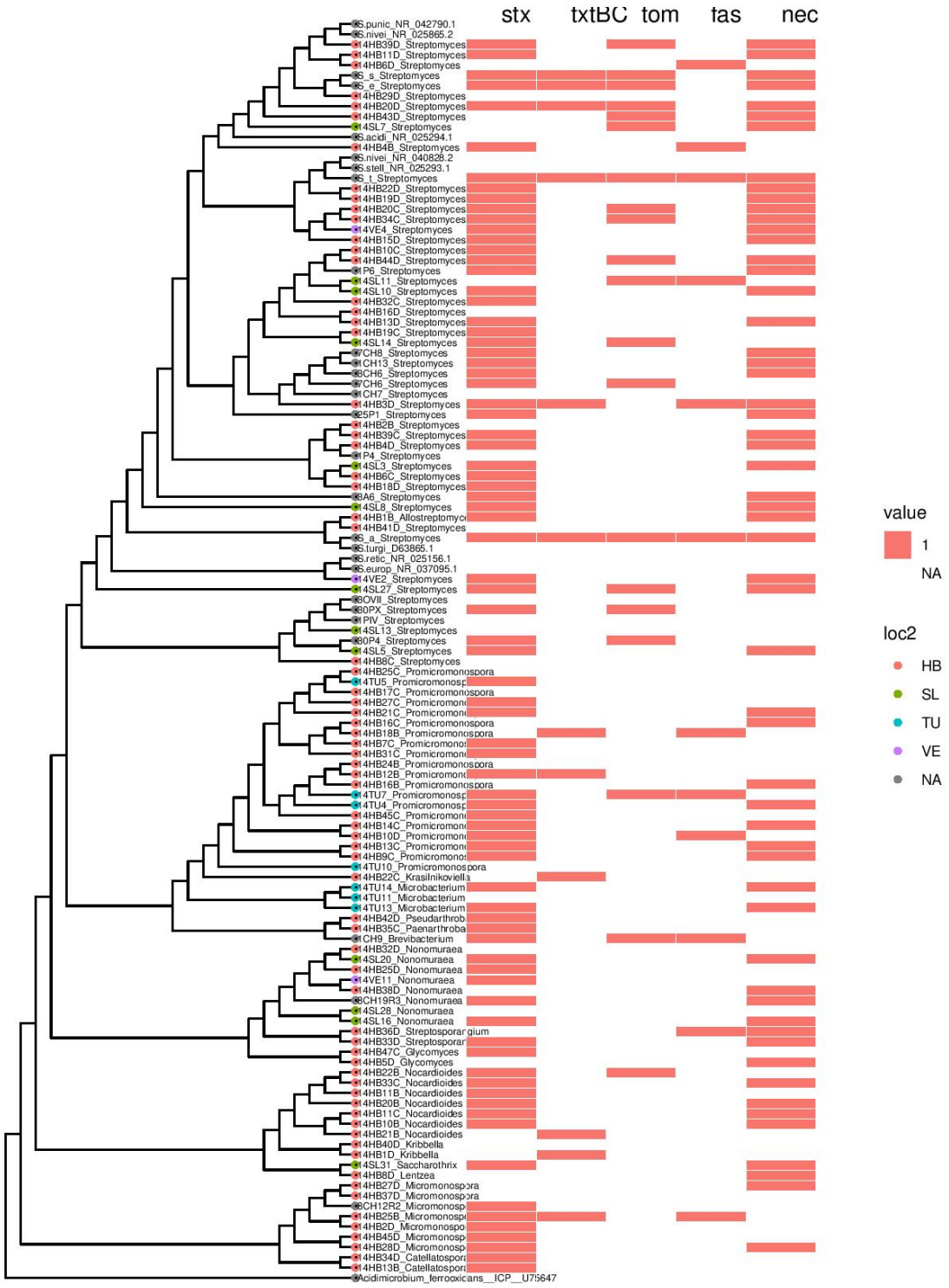


Figure 10: Phylogenetic tree of 16S rRNA genes of bacterial strains coming from different locations: HB-Havlickova Borova, SL-Slavkov, TU-Trutnov, VE-Velhartice, NA – controls. The tree includes also 16S rRNA gene sequences of *Streptomyces* spp. type strains, which are known to cause common scab coming from GenBank database.

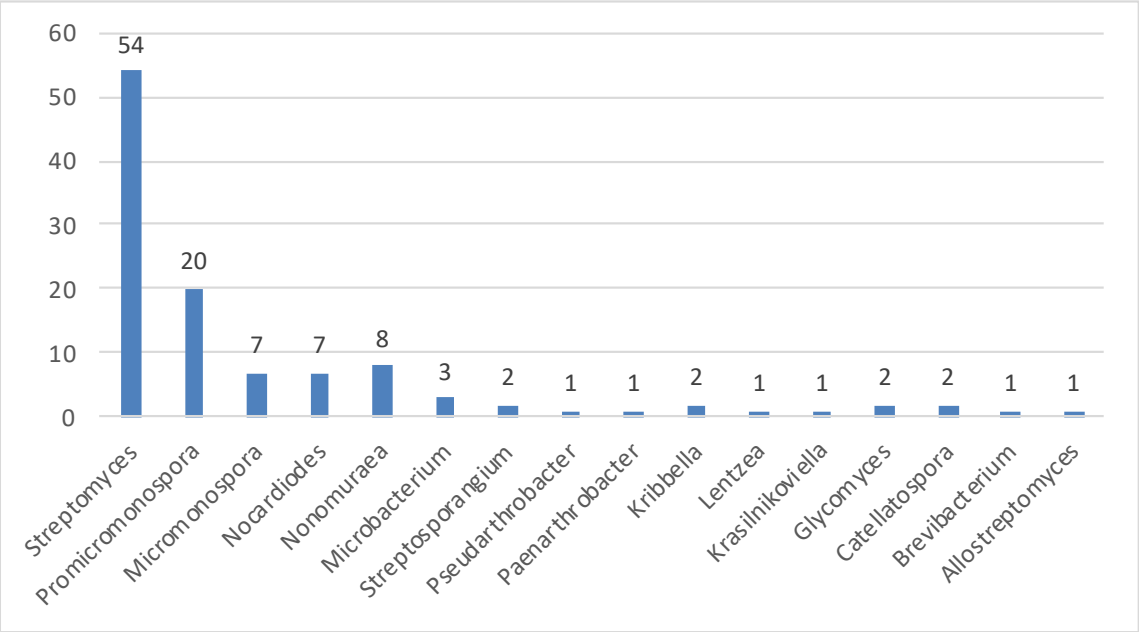


Figure 11: The number of genera from the phylum *Actinobacteria* among the studied strains.

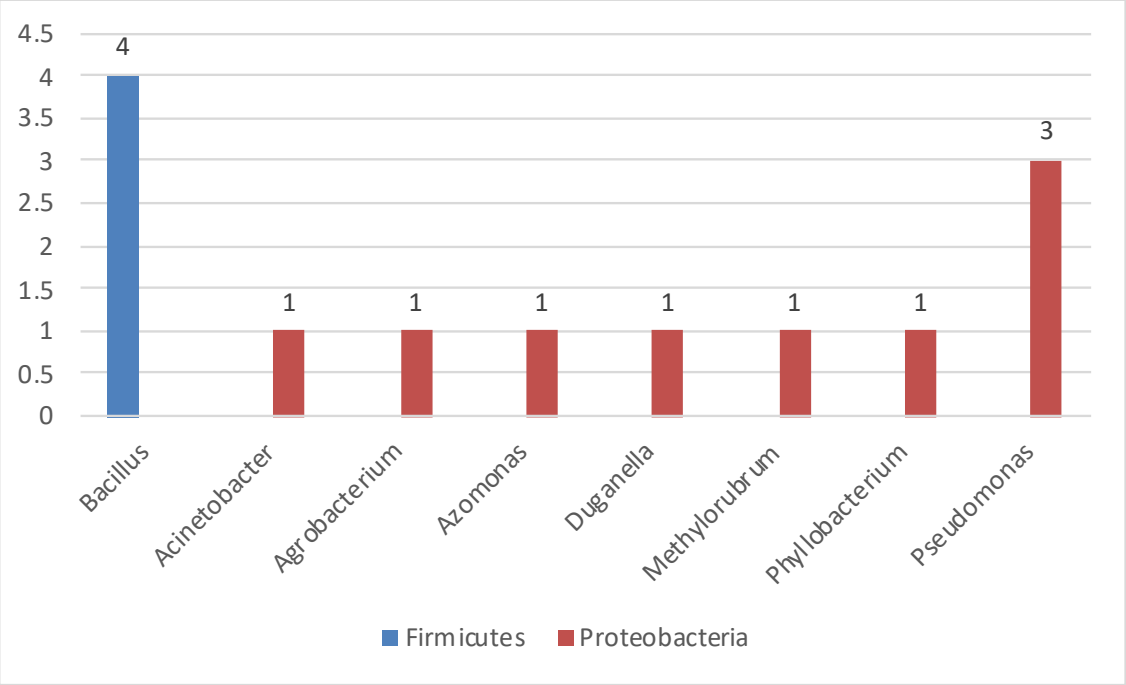


Figure 12: The number of genera from other phyla than *Actinobacteria* found among the studied strains.

Occurrence types:	Havlickova Borova	Slavkov	Velhartice	Trutnov	Peru	Chile	Argentina
stx1 a/b	65%	78%	100%	70%	71%	80%	100%
stx1 a/b + N f/r	57%	67%	75%	50%	50%	60%	100%
stx1 a/b + txt B/C	38%	0%	58%	0%	0%	45%	0%
stx1 a/b + Tom 3/4	37%	47%	0%	40%	57%	50%	0%
stx1 a/b + P19 f/r	37%	42%	67%	40%	43%	45%	0%
Tom 3/4 + P19 f/r	11%	12%	0%	10%	29%	15%	0%

Table 4: Percentage of types of gene occurrence in the studied locations (by author, 2021).

6 Discussion

16.1 Taxonomy comparison

The 16S gene sequences were obtained to compare taxonomic relatedness between all studied strains. Stx1 a/b and txt B/C primers show the presence of genes coding for thaxtomin, which have been known only for *Streptomyces spp.* (R. R. King, et al., 2009). In our study, only 72% of isolated *Streptomyces* strains were *stx*- positive, but we found as well as 65% of *Promicromonospora*. 5 out of 7 *Micromonospora* and 6 out of 7 *Nocardioides* were *stx*- positive too. Moreover, only 12 out of 123 isolates were *txtB/C*-positive. We do not know if the strains are responsible for the disease.

Interestingly, 3 isolates of *Promicromonospora* and 1 *Nocardioides* has *txtBC* intergenic region, but only 1 of them is *stx*- positive. Theoretically, all of the *stx*-positive strains should be also *txtB/C*-positive*,* probably with different length. Moreover, the pathogenic island types based on the presence/absence of different virulence factors and their organization differed between different streptomycetes, which is supported by another research (Li et al., 2019). The pattern of presence-absence of the different genes was expected to differ between strains. This supports the hypothesis that sites differ in the dominant type of the pathogenic island (Loria et al.2006; Lerat et al., 2009).

The surprising result is that genes coding for thaxtomin phytotoxin was detected not only in the genus *Streptomyces*, previously described as the only causing agent of potato common scab (Loria R., 1995), but also in *Allostreptomyces, Brevibacterium, Catellatospora, Glycomyces, Microbacterium, Micromonospora, Nocardioides, Nonomuraea, Paenarthrobacter, Promicromonospora, Pseudarthrobacter, Saccharothrix, Streptosporangium,* which include also gram-negative bacteria*.* This proposes that thaxtomin phytotoxin might be produced by other than *Streptomyces* genus (F.G. Healy et al., 2000). It is strongly suggested that horizontal gene transfer between various pathogens is the cause of the occurrence of thaxtomin genes in other bacteria than *Streptomyces*.

16.2 Location comparison

Pathogenic islands seem to differ from location to location without any obvious overall pattern according to our results. N f/r gene was interestingly, proportionally present in all the sites in both South America and the Czech Republic. In South America, gene P19 was present in Peru and Chile, but not in Argentina. 5/6 locations had at least 1 occurrence type missing, which increases the uniqueness of pathogenic island further. These random occurrences suggest that the presence of common scab and the pathogenic island type depend more on the geographical location and local conditions, rather than on the taxonomy.

According to M. Aittamaa (2010), *fascinase* gene (P19 f/r) is only found in *S. turgidiscabies*, and it's not present in *S. scabies and S. acidiscabies*, and according to L.A. Wanner (2006) tomatinase (Tom 3/4) is also found in *S. turgidiscabies*, which suggests that locations (Peru, Chile, Slavkov, Havlickova Borova) with higher percentage of that occurrence type (Table 4) will have *S. turgidiscabies* as one of the dominant bacteria in pathogenic islands. N f/r gene was the highest positives with stx1 a/b among all others, moreover in all the locations 'stx1 a/b + N f/r' was at least 50%, and in Velhartice and Argentina all the way up to 75% and 100%, respectfully. This supports the study by R. A. Bukhalid et al. (1998) suggesting that *Nec* genes are involved in pathogenicity and in the genomes are linked to the thaxtomin A biosynthetic genes.

There were more samples available from the Czech Republic (especially from Havlickova Borova) than from South America, therefore the results available are not even and it would be possible to draw more significant conclusions upon analyzing a higher number of strains from both locations to equalize the sample pool.

7 Conclusions

According to the conducted research, it has been concluded that:

- Thaxtomin production gene stx1 a/b can be found not only in genus *Streptomyces* but also in *Allostreptomyces, Brevibacterium, Catellatospora, Glycomyces, Microbacterium, Micromonospora, Nocardioides, Nonomuraea, Paenarthrobacter, Promicromonospora, Pseudarthrobacter, Saccharothrix, Streptosporangium,* as well as other gram-positive phyla *Firmicutes (*genus *Bacillus)*; and even gram-negative phyla *Proteobacteria (*genera *Phyllobacterium, Pseudomonas, Azamonas, Methylorubrum, Duganella, Agrobacterium, Acinetobacter).*

- Pathogenic island types based on the presence/absence of different virulence factors and their organization differed between different streptomycetes and between different locations. This supports our hypothesizes that the presence of potato common scab depends also on the environmental conditions such as origin site or a local pathogenic (bacterial) symbiosis; and that sites would differ in the dominant pathogenic island type.

- *Nec* genes are involved in pathogenicity and might be physically linked to the thaxtomin A biosynthetic genes.

8 References:

- Aittamaa, M., Somervuo, P., Laakso, I., Auvinen, P., Valkonen, J.P.T., 2010. Microarray-based comparison of genetic differences between strains of Streptomyces turgidiscabies with focus on the pathogenicity island. Molecular Plant Pathology no–no. doi:10.1111/j.1364-3703.2010.0064
- B. P. HUGHES. 1958 'The amino -acid composition of potato protein and of cooked potato'. Medical Research Council Department of Experimental Medicine, University of Cambridge
- Barka, E.A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., Clément, C., Ouhdouch, Y., Van Wezel, G.P., 2016. Taxonomy, Physiology, and Natural Products of Actinobacteria. Microbiology and Molecular Biology Reviews 80, 1–43. doi:10.1128/mmbr.00019-15
- Bártová, V., Bárta, J., Brabcová, A., Zdráhal, Z., Horáčková, V., 2015. Amino acid composition and nutritional value of four cultivated South American potato species. Journal of Food Composition and Analysis 40, 78–85. doi:10.1016/j.jfca. 2014.12.006
- Battistuzzi, FU., Feijao, A, and Hedges, SB. A genomic timescale of prokaryote evolution: insights into the origin of methanogenesis, phototrophy, and the colonization of land. BMC Evol Biol 2004; 4(1): 44
- Bignell DRD, Huguet-Tapia JC, Joshi MV, Pettis GS, Loria R (2010) What does it take to be a plant pathogen: genomic insights from Streptomyces species. Anton Leeuw Int J Gen Mol Microbiol 98 (2):179–194
- Boto, L., 2010. Horizontal gene transfer in evolution: facts and challenges. Proceedings of the Royal Society B: Biological Sciences 277, 819–827.doi:10.1098/rspb.2009.1679
- Bukhalid, R.A., Chung, S.Y., Loria, R., 1998. nec1, a Gene Conferring a Necrogenic Phenotype, Is Conserved in Plant-Pathogenic Streptomyces spp. and Linked to a Transposase Pseudogene. Molecular Plant-Microbe Interactions® 11, 960–967. doi:10.1094/mpmi.1998.11.10.960

- Flores-González, R., Velasco, I., Montes, F., 2007. Detection and characterization of Streptomyces causing potato common scab in Western Europe. Plant Pathology 0, 071202153352001. doi:10.1111/j.1365-3059.2007.01734.x
- Genomics of *Actinobacteria*: Tracing the Evolutionary History of an Ancient Phylum.
  Marco Ventura, Carlos Canchaya, Andreas Tauch, Govind Chandra, Gerald F. Fitzgerald, Keith F. Chater, Douwe van Sinderen. Microbiology and Molecular Biology Reviews Sep 2007, 71 (3) 495-548; DOI: 10.1128/MMBR.00005-07
- Healy, F.G., Wach, M., Krasnoff, S.B., Gibson, D.M., and Loria, R. (2000). The txtAB genes of the plant pathogen Streptomyces acidiscabies encode a peptide synthetase required for phytotoxin thaxtomin A production and pathogenicity. Mol. Microbiol. 38, 794–804.
- Hilario, E. & Gogarten, J. P. 1993 Horizontal gene transferof ATPase genes—the tree of life becomes a net of life. BioSystems31, 111–119. (doi:10.1016/0303-2647(93)90038-E)
- Hill J, Lazarovits G (2005) A mail survey of growers to estimate potato common scab prevalence and economic loss in Canada. Can J Plant Pathol 27:46–52
- King, R. R., and Lawrence, C. H. 1995. Characterization of 4-nitrotryptophans associated with the invitro production of thaxtomin A by Streptomyces scabies. Phytochemistry 40(1):41-43.
- King, R.R., Calhoun, L.A., 2009. The thaxtomin phytotoxins: Sources, synthesis, biosynthesis, biotransformation and biological activity. Phytochemistry 70, 833–841. doi:10.1016/j.phytochem.2009.04.013
- King, R.R., Lawrence, C.H., Calhoun, L.A., 1992. Chemistry of phytotoxins associated with Streptomyces scabies, the causal organism of potato common scab. J. Agric.Food Chem. 40, 834–837
- King, R.R., Lawrence, C.H., Clark, M.C., Calhoun, L.A., 1989. Isolation and characterization of phytotoxins associated with Streptomyces scabies. J. Chem.Soc., Chem. Commun. 13, 849–850.

- Kobayashi, A (Kobayashi, Akira); Kobayashi, YO (Kobayashi, Yuki Ohdaira); Someya, N (Someya, Nobutaka); Ikeda, S (Ikeda, Seishi). 2015. Community Analysis of Root and Tuber Associated Bacteria in Field-Grown Potato Plants Harboring Different Resistance Levels against Common Scab
- Koonin, E. V., Makarova, A. S. & Aravind, L. 2001 Horizontal gene transfer in Prokaryotes. Quantification and classification. Annu. Rev. Microbiol.55, 709–742. (doi:10.1146/annurev.micro.55.1.709)
- Labruyère, R. E. 1971. Common Scab and Its Control in Seed Potato Crops. Centre for Agricultural Publishing and Documentation, Wageningen, Netherlands.
- Law, J.W.-F., Letchumanan, V., Tan, L.T.-H., Ser, H.-L., Goh, B.-H., Lee, L.-H., 2020. The Rising of “Modern Actinobacteria” Era. Progress In Microbes & Molecular Biology 3. doi:10.36877/0000064
- Lawrence, C. H., Clark, M. C., and King, R.R. 1990. Introduction of common scabsymptoms in asceptically cultured potato tubers by the vivotoxin, thaxtomin. Phytopathology 80:606-608.
- Lerat, S., Simao-Beaunoir, A.-M., Beaulieu, C., 2009. Genetic and physiological determinants ofStreptomyces scabiespathogenicity. Molecular Plant Pathology 10, 579–585. doi:10.1111/j.1364-3703.2009.00561.
- Li, Y., Liu, J., Díaz-Cruz, G., Cheng, Z., Bignell, D.R.D., 2019. Virulence mechanisms of plant-pathogenic Streptomyces species: an updated review. Microbiology 165, 1025–1040. doi:10.1099/mic.0.000818
- Loria, R., Bukhalid, R.A., Creath, R.A., Leiner, R.H., Olivier, M., andSteffens, J.C. (1995). Differential production of thaxtomins by patho-genic Streptomyces species in vitro. Phytopathology 85, 537–541.
- Loria, R., Bukhalid, R.A., Fry, B.A., King, R.R., 1997. PLANT PATHOGENICITY IN THE GENUS STREPTOMYCES. Plant Disease 81, 836–846. doi:10.1094/pdis.1997.81.8.836
- Lynch, JM, and JM Whipps. 1991. Substrate flow in the rhizosphere. In: DL Keiser and PB Cregan (eds), The Rhizosphere and Plant Growth. Kluwer Academic Publishers, London. pp 15-24.

- Manzer, F. E., McIntyre, G. A., and Merriam, D. C. 1977. A new potato scab problem in Maine. Univ. Maine Tech. Bull. 85.
- Merete W. Dees & Leslie A. Wanner. (2012). In Search of Better Management of Potato Common Scab. 55:249–268 DOI 10.1007/s11540-012-9206-9
- Nouioui, I., Carro, L., García-López, M., Meier-Kolthoff, J.P., Woyke, T., Kyrpides, N.C., Pukall, R., Klenk, H.-P., Goodfellow, M., Göker, M., 2018. Genome-Based Taxonomic Classification of the Phylum Actinobacteria. Frontiers in Microbiology 9. DOI: 10.3389/fmicb.2018.02007
- Omura, S. Ivermectin: 25 years and still going strong. Int J Antimicrob Agents 2008; 31(2): 91–98.
- Powelson, M.L., K.B. Johnson, and R.C. Rowe. 1993. Management ofdiseases caused by soilborne pathogens. InPotato HealthManagement, ed. R.C. Rowe, 149–158. St. Paul, MN: AmericanPhytopathological Society.
- Rosemary Loria, Johan Kers, and Madhumita Joshi. 2006. Evolution of Plant Pathology in Streptomyces. Annual Review of Phytopathology. Vol. 44:469-487
- Ryan AD, and LL Kinkel. 1997. Inoculum density and population dynamics of suppressive and pathogenic Streptomyces strains and their relationship to biological control of potato scab. Biol Control 10:180-186.
- Slack SA (1991) A look at potato leafroll virus and potato virus Y: past, present, future. Badger Common Tater 43:16–21
- Wang, A, and G Lazarovits. 2004. Enumeration of plant pathogenic Streptomyces on post-harvested potato tubers under storage conditions. Can J Plant Patho126:563-572
- Wang, D.; Wang, C.; Gui, P.; Liu, H.; Khalaf, S.M.; Elsayed, E.A.; Wadaan, M.A.; Hozzein, W.N.; Zhu, W. Identification, bioactivity, and productivity of actinomycins from the marine-derived Streptomyces heliomycini. *Front. Microbiol.* 2017, *8*, 1147.
- Wang. A., Lazarovits G. (2005) Role of Seed Tubers in the Spread of Plant Pathogenic Streptomyces and Initiating Potato Common Scab Disease. Amer J. of Potato Res 82:221-230

- Wanner, L.A., 2006. A Survey of Genetic Variation in Streptomyces Isolates Causing Potato Common Scab in the United States. Phytopathology® 96, 1363–1371. doi:10.1094/phyto-96-1363
- Wanner, L.A., K.G. Haynes, C.A. Thill, J. Miller, R.G. Novy, D.L. Corsini, and J.L. Whitworth. 2006. Two years of the NationalCommon Scab Trials of Potato Varieties and Advanced Selec-tions. American Journal of Potato Research 83: 137
- Wilson CR (2004) A summary of common scab disease of potato research from Australia. Proceedings of the International Potato Scab Symposium 2004, Sapporo, Japan, Hokkaido University
- Yuting Li, Kapil Tahlan and Dawn R.D. Bignel (2020) Functional Cross-Talk of MbtH-Like Proteins During Thaxtomin Biosynthesis in the Potato Common Scab Pathogen Streptomyces scabiei. DOI: 10.3389/fmicb.2020.585456