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Genes for the biosynthesis of secondary metabolites in soil bacterial community interacting with plants

Bachelor´s Thesis

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BSc. Agriculture and food

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Declaration

I hereby declare that I have authored this master's thesis carrying the name „ Genes for the biosynthesis of secondary metabolites in soil bacterial community interacting with plants "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 master's thesis, I further state that I have not infringed the copyrights of third parties in connection with its creation.

In Prague on 21.04.2023 _____

Acknowledgments

This thesis could not have been completed without the assistance and input from numerous individuals. I would like to give my warmest thanks to my supervisor doc. RNDr. Markéta Marečková, Ph.D. who made this work possible her guidance and advice pulled me through all the stages of writing my thesis.

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I would like to express my deepest appreciation and thanks to all of my professors for doing a tremendous job in the past three years of our bachelor's program while facing a lot of difficulties such as the pandemic and switching to distance learning through those hard times.

Last but not least I would like to thank my family for all the support they have shown me throughout my whole life and for giving me the opportunity that wouldn’t possible without them.

Genes for the biosynthesis of secondary metabolites in soil bacterial community interacting with plants

Summary:

The thesis focuses on selected biosynthetic pathways and genes of secondary metabolites in bacteria, which participate in interactions with plants.

In the theoretical part, the definition of this group of metabolites, to be more precise about the siderophores and the importance of the group. Also will be described the chemical types of siderophores. About the genome that is presented in *Streptomyces scabie* and description of the usage potential to the crop plants.

The practical part describes the process of the cultivation of *Streptomyces scabie.* The practical part aimed at primers testing (forward & reverse), DNA dilution such as *Streptomyces scabiei DSM 41658* , the detailed description & characterization of the PCR application procedure and gels as a result of the laboratory work.

Keywords: Secondary metabolites, soil, DNA, primers, PCR test.

Table of content

1.Introduction

Secondary metabolites, also called specialized metabolites, toxins, by-products, or natural products, are organic compounds produced by bacteria, fungi, plants, or animals that are not directly involved in the growth, development, or reproduction of an organism. Instead, they usually mediate environmental interactions that may confer a selective advantage for an organism, and increase its survival or fecundity. Specific secondary metabolites are often limited to a narrow set of species within a phylogenetic group (Ludwig W., 2012) Bacterial production of secondary metabolites begins in the stationary phase due to the lack of nutrients or in response to environmental stress. The synthesis of secondary metabolites in bacteria is not essential for their growth, however, they allow them to better interact with their ecological niche (Ludwig W., 2012)

Secondary metabolites often play an important role in plant defense against herbivores and other interspecies defense mechanisms.

Most commerically used secondary metabolites are isolated from actinomycetes, microscopic fungi, and basidiomycetes, less often from other bacteria, plants, or animals.

This study will present a literature research characterizing the biological activity of selected secondary metabolites in bacteria and their potential impact on plants in the rhizosphere and on the soil surface, what role may have in their pathogenic interaction with other soil bacteria. Finally, some suggestions for the use of bacterial strains with specific genes involved in plant-microbe interactions will be given for practical use in the field applications.

2. Aims of the thesis

The present Bachelor's thesis aim was to evaluate the performance of a set of primers for PCR amplification of a specific target sequence. However, it was observed that amplification did not always occur in other samples, including the positive control Streptomyces scabiei.

The aim of the practical part is to test the newly developed primers, which will be seen in the first table, for the amplification of Streptomyces scabiei.

3. Literature research

This section is devoted to a review of the literature and a description of the basic terminology that is important for understanding and compiling the integrity of the bachelor's work and also for further description of the practical part.

3.1 Secondary metabolites in soil bacterial

Soil bacterial communities that interact with plants are known to produce a wide range of secondary metabolites with diverse functions, such as plant growth promotion, disease suppression, and stress tolerance (Raaijmakers and Mazzola, 2016). The biosynthesis of secondary metabolites is governed by complex gene clusters, which often contain numerous genes involved in the synthesis and transport of the compounds (Nett et al., 2009). These gene clusters are typically located on plasmids, which are mobile genetic elements that facilitate the rapid transfer of gene clusters between different bacterial strains or species (Nett et al., 2009). This allows for the rapid evolution and adaptation of the bacterial community to changing environmental conditions.

One example of a secondary metabolite produced by soil bacteria that interacts with plants is the compound 2,4-diacetylphloroglucinol (DAPG), which is produced by several species of Pseudomonas bacteria and has been shown to suppress plant pathogens and promote plant growth (Raaijmakers and Mazzola, 2016). The genes responsible for the biosynthesis of DAPG have been extensively studied and are known to be regulated by several transcriptional regulators and environmental cues (Gross and Loper, 2009).

Siderophores are another class of secondary metabolites produced by many soil bacteria to scavenge iron from the environment. Siderophores can also interact with plants, promoting plant growth and conferring resistance to plant pathogens (Dimkpa et al., 2009). The genes responsible for siderophore biosynthesis have been identified and characterized in several bacterial species, revealing a complex network of regulatory genes and environmental cues (Miethke and Marahiel, 2007).

Understanding the genes responsible for the biosynthesis of secondary metabolites in soil bacterial communities is essential for developing strategies to enhance plant-microbe interactions and promote sustainable agriculture. Ongoing research in this area is focused on elucidating the regulatory mechanisms involved in secondary metabolite production and identifying novel compounds with potential applications in agriculture and medicine (Raaijmakers and Mazzola, 2016).

3.2 The Growth and Cultivation of Bacteria

The study of the cultivation of microorganisms begins in 1830, when C. Cañard de Latour, K. Kützing and T. Schwann discovered the cause of wine fermentation (the presence of yeast cells).

There was no further progress in the field of cell culture until the 1850s, when L. Pasteur began his fundamental research: the study of the physiology of yeast and bacteria, the introduction of aseptic methods, minimal media, and the study of the nutritional needs of microorganisms and the role oxygen in their life processes. The works of L. Pasteur and his student M. Rollin established the need for the main and secondary components of the environment and energy sources. The first complete medium of a certain composition was obtained by M. Rollin in 1869 for the cultivation of fungi of the genus Aspergillus. The work is also of interest because Rollin determined the nutritional needs of mushrooms not only qualitatively, but also quantitatively.

Speaking about the remarkable works of L. Pasteur and M. Rollin, it should be noted that they did not have at their disposal the method of pure cultures, proposed by R. Koch somewhat later (in the 1870s) and giving significant advantages in growing microorganisms. Beginning with the exquisite work of Koch, culture methods have become a major concern of microbiologists.

The need of microorganisms for complex organic substances - growth factors - was first discovered by Vildieu in 1901, when he discovered vitamin B, or "bios factors", necessary for the growth of yeast.

In the 1930s With the introduction of the shaking flask method, the development of cultivation equipment began. This made it possible to apply the laboratory method for aeration of deep cultures, especially deep cultures of aerobic fungi, which were previously grown only on the surface of solid or liquid media. In surface cultures, the environment of the organism is heterogeneous, while in deep cultures it is homogeneous, which is easier to study and control. Subsequently, submerged cultures of aerobic fungi played a significant role in the development of industrial production of antibiotics. The growing technological importance of the culture of microorganisms significantly stimulated interest in this area and led in the 1940s and 1950s.

To the creation of fermenters with automatic regulation of environmental conditions. Since that time, thanks to the active development of the theory of continuous cultivation of the chemostat type, wide horizons have opened up not only for theoretical development, but also for the practical application of cell cultures. (Perth S. J, 1978.)

The choice of cultivation process depends not only on the needs of the organism, but also on what the culture will be used for, i.e., on the ultimate goal of the experiment. Many processes for cultivating microorganisms are known. They differ in:

1) the state of the nutrient medium (surface and deep);

2) the presence or absence of mixing (dynamic or static);

3) oxygen content (aerobic or anaerobic);

4) mode of action (closed, more often periodic, and open, more often continuous);

5) the number of fermenters (one-, two- and multi-stage);

6) control method (chemostat, turbidostat, oxystat, pH-stat and others).

Cultures of microorganisms can be divided into open and closed systems. An open system is a system in which all components can enter or leave it. A closed system is such a system in which at least one of the existing components can neither enter the system nor leave it. Consequently, all continuous cultures in which, on the one hand, the influx of the nutrient medium occurs, and on the other hand, the outflow of biomass and other products, are open systems. A simple batch culture containing a limited initial amount of nutrient substrate is an example of a closed system. In a closed system, the biomass growth rate should tend to zero, either due to a lack of a substrate, or due to intolerance of a toxic product with its further accumulation. Therefore, such systems are in an unstable state. In contrast, in open systems, there is always the possibility of establishing a stable state. (Adams R., 1983)

The isolation of Streptomyces scabies, as well as other streptomyces and related organisms, is carried out from soil samples enriched before sowing with substrates that create unfavorable growth conditions for other microorganisms.

The soil is enriched with $CaCO_3$. To do this, air-dry soil samples are mixed with $CaCO_3$ and incubated at +26°C for 7–9 days at 60% of full capacity. For 1 g of soil, 0.1 g of $CaCO_3$ is taken. In some cases, the soil is enriched with keratin and chitin.

The number of actinomycetes isolated from the soil can be increased by heating it before sowing for 8 hours to a temperature of +40°C–+45°C or treatment with a phenol solution. In the latter case, 10 ml of a 1.4% phenol solution is added to 0.1 ml of soil suspension. After 10 minutes, the suspension is brought to the desired consistency and sown on nutrient media.

To limit the growth of fungi and bacteria, antibiotics, sodium propionate, and rose bengal dye are added to nutrient media.

Starch, glycerin, casein, nitrates are used as sources of carbon and nitrogen.

Most often, for the isolation of Streptomyces scabies and other closely related species, a starch-casein nutrient medium, glycerol-argenin agar, and a medium with chitin are used.

To monitor the morphology of spore chains, 14-day-old cultures on starch-ammonia and oat agar are used. (Adams R., 1983)

3.3 PCR test

The polymerase chain reaction (PCR) is a versatile technique that has revolutionized molecular biology since its invention in 1983 by Kary Mullis, an American scientist who received a Nobel Prize for this invention. PCR is a powerful tool for amplifying low concentrations of specific DNA fragments in biological samples, making it one of the most accurate and sensitive methods for diagnosing infectious diseases today.

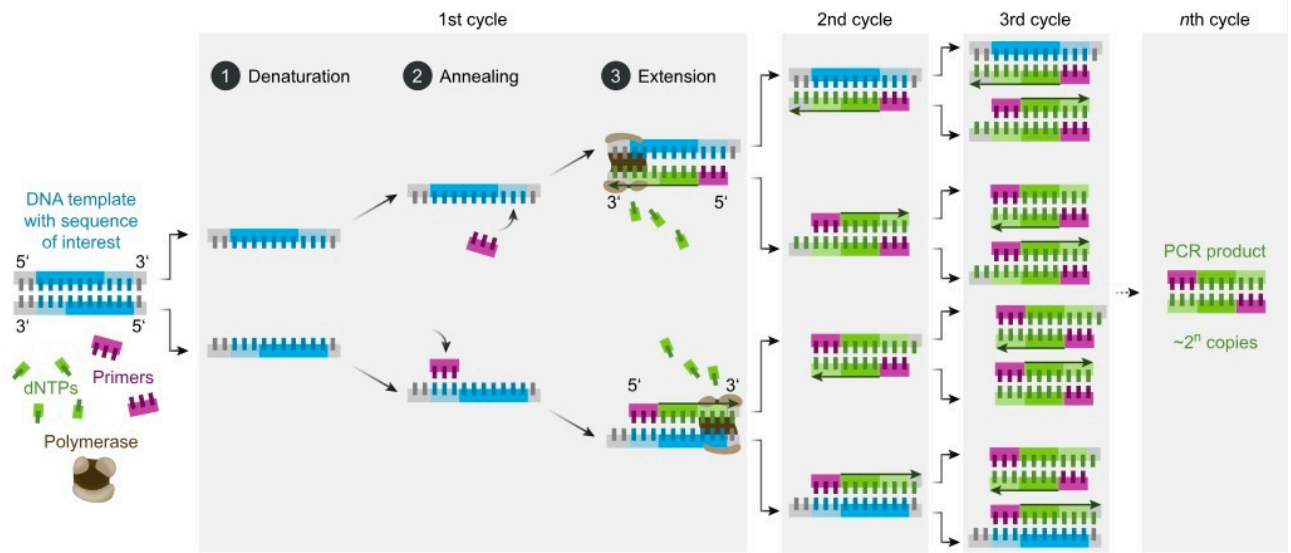


Figure 1: PCR test

The PCR method is based on the repeated doubling of a certain section of DNA in vitro with the help of enzymes. When optimal conditions are created, enough DNA is produced to visually detect the target DNA. The amplification process is highly specific and can selectively copy only that part of the target DNA that satisfies the given conditions.

In addition to simply increasing the number of copies of DNA, PCR can also facilitate several other manipulations of genetic material, such as introducing mutations, splicing DNA fragments, cloning genes, and isolating new genes. This universal method is widely used in biological and medical

practice, including for diagnosing hereditary and infectious diseases, establishing paternity, and studying gene expression in various tissues and organisms.

Thus, PCR is an important technique in molecular biology that has changed many areas of research. Its wide application makes it a powerful tool for researchers, clinicians and many other professionals in the scientific community.

3.3.1 Characteristic and steps of PCR test

The polymerase chain reaction (PCR) is a molecular biology technique that amplifies a DNA sequence, creating millions or billions of copies of a target DNA (Mullis, 1990). This method is widely used in various fields such as life sciences, paternity, and forensics. The main advantage of real-time PCR is its ability to provide rapid detection and quantification of target sequences with a wide dynamic range for quantification and multiplex amplification of multiple targets in a single reaction (Liu et al., 2021).

PCR can be used for various downstream applications such as gel electrophoresis, sequencing or cloning. However, one of the main problems of PCR is the possibility of obtaining false positive or false negative results. False positive results may be the result of contamination of the sample with foreign DNA molecules, and false negative results may be the result of inhibition of the PCR reaction by sample components (Ventura, 2007).

To prevent contamination, strict laboratory controls can be introduced and clean zones and sealed cartridges can be used. However, isolation of target DNA from complex samples remains a challenge, and optimal sample processing protocols are critical for efficient PCR analysis (Ventura, 2007).

Main stages:

- **Denaturation:** Vigorous heating of a reaction mixture to separate or denature a DNA strand. This process will result in the formation of a single stranded template for the next step. Denaturation step in the polymerase chain reaction (PCR) process in which heat is used to separate the two strands of DNA in the reaction mixture. This step is necessary to create a single-stranded template that can be used in the next step of the PCR process, which involves annealing the primers onto the template strand.

- **Annealing:** is the process of gradually cooling the reaction mixture in order to allow the primers to bind to their specific complementary sequences on the single-stranded DNA template, thereby facilitating the formation of stable primer-template duplexes.

- **Elongation:** is an instance of bringing the reaction mixture's temperature back up to a level that is ideal for Taq polymerase activity. By adding nucleotides to the 3' end of the primers and creating new DNA strands that are complementary to the template strands, the enzyme is able to lengthen the primers. (Ventura M., 2007)

3.4 Secondary metabolites and their application in agriculture

Secondary metabolites are biologically active substances produced by plants that serve a variety of functions. These compounds play important roles in plant storage and protection, with some having antimicrobial and antifungal properties. For example, essential oils, which are mixtures of secondary metabolites, can help protect plants against harmful microorganisms. Additionally, some secondary metabolites can form toxic compounds when decomposed during hydrolysis, such as hydrocyanic acid and coumarin.

Secondary metabolites also play a crucial role in protecting plants from biopathogens and are involved in hypersensitivity reactions in the form of phytoalexins (Fukushima et al., 2020). These compounds can also contribute to plant reproduction and seed dispersal by providing color to flowers and fruits through pigments such as anthocyanins, carotenoids, and betalains (Winkel-Shirley, 2001). Furthermore, secondary metabolites can inhibit the germination of seeds from competing plant species (Bais et al., 2003).

In agriculture, secondary metabolites are used to control insects and biopathogens such as fungi and bacteria. For instance, nicotine and anabasine are used as insecticides, while mustard oil glycosides can protect plants like radish, cabbage, and mustard against herbivores (Duke, 1983). Due to the complex molecular structure of many secondary metabolites, they cannot be easily synthesized chemically. As a result, plants will remain the primary source for the production of these valuable compounds for the foreseeable future.

3.4.1 Siderophores, compounds involved in iron acquisition

“Siderophores are secondary metabolites produced by various organisms to remove iron from the environment, making this important element available to the cell. Presenting a high affinity for ferric iron, siderophores are secreted to form soluble iron complexes that can be taken up by organisms. Siderophores have a complex chemistry that allows them to form the strongest chelating iron complexes.” (Albelda-Berenguer et al., 2016).

Siderophores are chemical compounds that chelate iron ions that are secreted by certain microorganisms. At a pH close to neutral (~ 7), Fe 3+ ions have a very low solubility in water, so microorganisms cannot absorb iron directly from the environment. Such ions are formed under aerobic conditions (for example, in soil) from Fe 2+ ions. The latter have a high solubility and can be absorbed by microorganisms. The siderophore isolated to the medium forms a complex with Fe 3+ ions, which subsequently enters the microorganism cell using active transport mechanisms. Many siderophores belong to the group of so-called nonribosomal peptides. Also the siderophores group are derivatives of hydroxamic acid, which is a very strong chelator.

Other strategies for increasing the solubility, and thus the bioavailability, of Fe 3+ ions are to increase the acidity of the medium, used, for example, by plant roots, or to reduce Fe 3+ ions extracellularly to more soluble Fe 2+ ions.

Examples of siderophores synthesized by microorganisms:

- Quinolobactin - Pseudomonas fluorescens,
- ferrichrome - Ustilago sphaerogena,
- enterobactin - Escherichia coli,
- bacillobactin - Bacillus subtilis,
- ferrioxamine B - Streptomyces pilosus,
- fusarin C - Fusarium roseum,
- vibriobactin - Vibrio cholerae,
- azotobactin - Azotobacter vinelandii,
- pseudobactin - Pseudomonas B 10
- erythrobactin - Saccharopolyspora erythraea,
- nocardamine - Pseudomonas stutzeri,
- pyoverdin - Pseudomonas aeruginosa,
- pyochelin - Pseudomonas aeruginosa and Burkholderia cepacia,
- pseudomonin - Pseudomonas fluorescens (Albelda-Berenguer et al., 2016).

The main function of siderophiles is the conversion of iron with proteins and a water-insoluble substance into the Fe3 form accessible to microorganisms. Most microorganisms of aerobic and facultative anaerobic origin synthesize at least one sidrophore. The relationship of siderophiles with the virulence of a microorganism has been proven, and approaches to the clinical use of their microorganisms have been developed. (Albelda-Berenguer et al., 2016).

In fact, the loss of the ability to synthesize siderophores is associated with the loss of virulence, which has been shown in bacteria of various species: Ervinia hirsantemi, Pseudomonas aeruginos, Vibrio anguillarum, etc. Active research on siderocytes began in the 90s, since then siderocytes of various groups have been identified and described microbes.(Albelda-Berenguer et al., 2016).

3.4.2 Chemical types of siderophores

The chemical nature of siderophores can be divided into 5 classes: catechites and phenolots "aryl caps", hydroxamates-hydroxycarbonate acids, carboxylates of dicarboxylic and tricarboxylic acids, siderophores of mixed types. The mixed-type siderophore corresponds in its design to two classes at the same time, so they were identified as a separate category. On fig. Below are the chemical structures of various classes of siderocytes. With one siderophore molecule providing six electron atoms for complex formation, an octahedral complex of FEL composition is obtained.

Complexation of the siderophore – Fe3 Fe3 ions have a coordination number of six, they are hard Lewis acids, therefore they salt six water molecules too strongly in water and form an octahedral (Fe(H2O)6)3+ aqua complex. Siderophore forms a strong complex bond with Fe3 through electron donor atoms, which are capable of displacing hydrogen molecules from the inner spheres of aquacomplexes. If one siderophore molecule has less than six electron atoms that participate in the complex formation, other compound complexes are formed, for example, for example, for rhodotorulic acid (Rhodotorula mucilaginosa) - Fe2L3, pyochelin (P. aeruginosa) - FeL and FeL2, cepabactin (Burkholderia cepacia) – FeL3

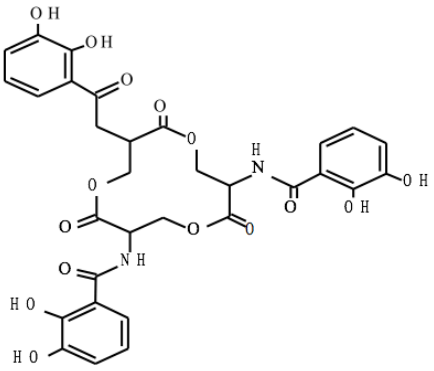
Fe3+ omplexes with siderocytes are characterized by a high thermodynamic potential, although there is no stabilization of the ligand fields, since oxygen atoms are hard bases for Lewis. In addition to Fe3+ ions, siderocytes can combine other metal cations, especially trivalent aluminum cations in less stable complexes. However, gallium is capable of forming stronger complexes with siderophiles than iron. The example of P. aeruginos shows that when gallium is introduced into the environment, siderophore activity is blocked and virulation and growth of bacterial populations are reduced. This fact opens up a new perspective for chemotherapy against microbes, as it confirms the evolutionary theory: if the goal of the action of an antibacterial drug is a commonly available

drug, the resistance of microorganisms in this drug does not develop.Divalent metal cations bind siderophores less preferentially. Within the framework of the theory of hard and soft Lewis acids and bases, this is explained by the fact that divalent cations are soft Lewis acids and prefer to interact with soft bases such as nitrogen and sulfur. Fe2+ ions also do not form sufficiently strong complexes with siderophores, which is explained not only by their belonging to soft Lewis bases, but also by the low ratio of the charge to the ionic radius, which makes an additional contribution to the destabilization of the complex structure (Raymond, K. N.,1979)

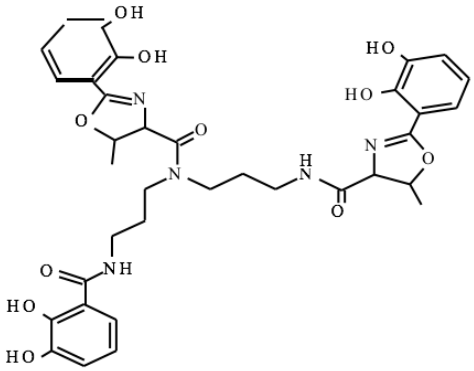
The quantitative assessment of the strength of complex compounds is carried out using the instability constants (Kn) and binding/stability:

Table 1. Chemical structures of siderophores

CATECHOLATE TYPE

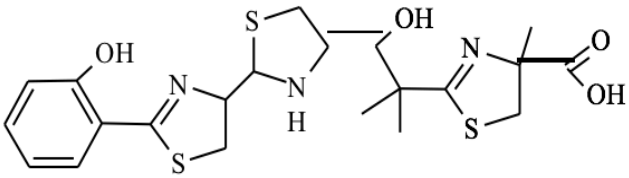


(*Streptomyces* spp.)

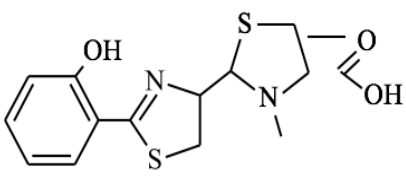


(*Vibrio cholerae*)

PHENOLATE TYPE

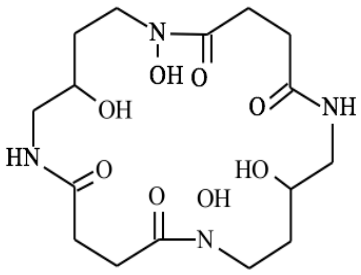


(*Yersinia pestis, Y. enterocolitica*)

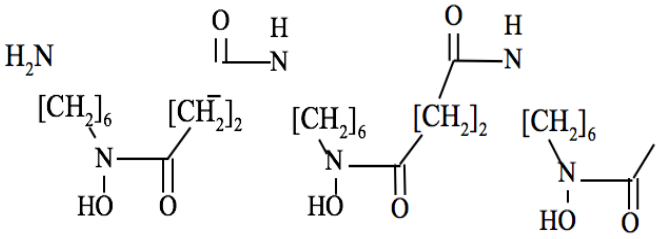


(*Pseudomonas aeruginosa*)

HYDROXAMATE TYPE

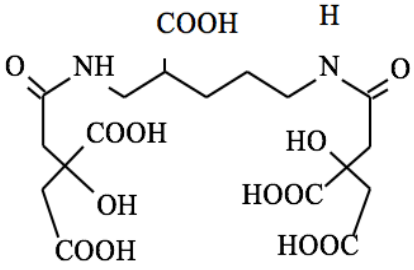


(*Alcaligenes denitrificans, Bordetella pertussis, B. bronchiseptica*)

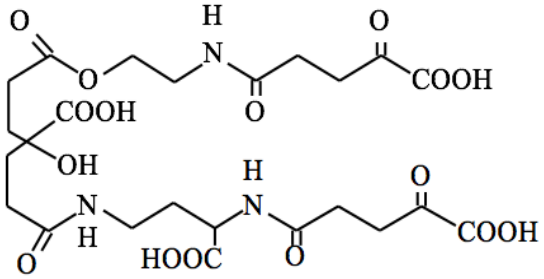


(*Streptomyces pilosus*)

CARBOXYLATE TYPE



(*Staphylococcus* spp.)



(*Erwinia chrysanthemi*)

Sources: Miethke M., Marahiel M. A. Siderophore-based iron acquisition and pathogen control. Microbiology and Molecular Biology Reviews, 2007, vol. 71, no. 3, pp. 413–451.

The coordination of Fe3+ ions is six and they are considered hard Lewis acids. This leads to strong solvation by six water molecules in aqueous solution, resulting in an octahedral Fe [(H2O)6]3+ aqua complex. Siderophores form strong complexes with Fe3+ due to their electron-donor atoms which replace water molecules in the inner sphere of the aqua complex. If a siderophore provides six electron-donor atoms, an octahedral FeL complex is formed, while less than six electron-donor atoms lead to complexes with different compositions, such as Fe2L3 for rhodotorulic acid, FeL and FeL2 for pyochelin, and FeL3 for cepabactin. These complexes exhibit high thermodynamic stability, despite the absence of ligand field stabilization, since oxygen atoms are hard Lewis bases. (Tseng, C. F.,2006; Carrano, C. J.,1978)

In addition to Fe3+ ions, siderophores can bind other metal cations, especially trivalent aluminum cations, into less stable complexes. However, gallium is able to form stronger complexes with siderophores than iron.

For example, P. aeruginosa, it was shown that the introduction of gallium into the medium blocks siderophores and the bacterial population decreases its virulence and growth activity.

This fact opens up new horizons for antimicrobial chemotherapy, as it confirms the theory of evolution: if the target for the action of an antimicrobial drug is a public product, then resistance in microorganisms to this drug does not develop.

Divalent metal cations bind siderophores less preferentially. Within the framework of the theory of hard and soft Lewis acids and bases, this is explained by the fact that divalent cations are soft Lewis acids and prefer to interact with soft bases such as nitrogen and sulfur. Fe2+ ions also do not form sufficiently strong complexes with siderophores, which is explained not only by their belonging to soft Lewis bases, but also by the low ratio of the charge to the ionic radius, which makes an additional contribution to the destabilization of the complex structure (Raymond, K.,1979)

Synthesis and transport of siderophores. Siderophore biosynthesis, the process by which siderophores are produced, can occur through two distinct pathways: nonribosomal peptide synthesis (NRPS) and the NRPS-independent pathway (NIS). The NRPS pathway involves the assembly of peptide siderophores, which are composed of nonproteinogenic amino acids and their derivatives. These siderophores are assembled without using RNA as a template (Miethke, M., 2007). The resulting peptides are typically short oligomers consisting of 2 to 48 amino acid residues.

Many siderophores are synthesized through the NRPS pathway, including yersiniobactin, enterobactin, and vibriobactin. Additionally, some antibiotics such as penicillin and vancomycin are also metabolites of the NRPS pathway. This pathway is important for the production of a wide range of biologically active compounds that play crucial roles in various biological processes.

The synthesis and modification of siderophores along the NRPS pathway is carried out step by step on the multimodal NRP synthetase. The functional role of NRP synthetase domains can be determined as follows:

1) activation of amino acids for peptide synthesis through the formation of thioesters;

2) formation of a peptide bond in the PCP domain (from the English peptidyl carrier protein - peptide carrier protein);

3) modification of amino acids, for example, epimerization, leading to isomerization L-amino acids to D-enantiomer;

4) interesterification of the peptide chain with subsequent release of final metabolites by hydrolysis or macrocyclization (Kohli, R.,2001)

The NIS pathway for the synthesis of siderophores occurs as a result of the condensation of various elements, usually dicarboxylic acids (succinate, citrate, α-ketoglutarate) with diamines, aminoalcohols, and alcohols (Challis, G. L.,2005) The NIS pathway synthesizes aerobactin, achromobactin, rhizobactin, vibrioferrin, etc. NIS synthetases are not homologous to

the NRPS pathway synthetases; they primarily catalyze the formation of amide and ester bonds between organic acids and amino groups/hydroxyl groups. groups of substrates.

For the use of siderophores, microorganisms have regulatory systems, including enzymes and transport systems that coordinate the processes of their biosynthesis, secretion, binding and release of iron. After the siderophore binds to Fe3+, the trapped iron ion is transported to the cytoplasm of the microorganism cell. Transport can be carried out in two ways, either the siderophore-Fe3+ complex dissociates on the cell surface and then the Fe3+ ion penetrates singly, or the complex does not dissociate and the Fe3+ ion is transported to the cytoplasm complexed.

In gram-negative bacteria, the siderophore-Fe3+ complex must overcome two membranes - the outer cell wall and the cytoplasmic one. Due to the fact that the siderophore-Fe3+ complex is quite large and is present in the medium at a low concentration, it cannot enter the cell through porin channels or by simple diffusion. To transport across membranes, Gram-negative bacteria have specialized receptor proteins that bind the Fe3+-siderophore complex and actively transport it into the periplasm against the concentration gradient. The most well studied proteins are FepA, FecA, FhuA, FpvA, FptA, which are receptors for enterobactin, citrate, E. coli ferrichrome, P. aeruginosa pyoverdin, and pyochelin (Buchanan, S.,1999) (Cobessi, D.,2005) The receptor proteins of the outer membrane have different affinities for the siderophore-Fe3+ complexes. If the receptor is specific to one ligand, like E. coli FepA to enterobactin-Fe3+, then such a receptor binds its siderophore-Fe3+ complex with high affinity. If the receptor is capable of recognizing siderophores of different nature, for example, the Cir receptor of uropathogenic strains of E. coli can recognize the Salmochelin-Fe3+Salmonella enterica complex, then such receptors bind siderophore-Fe3+ complexes with low affinity (Hantke, K.,2001)

In general, the structures of these receptor proteins are similar and have two functionally different domains: a cylindrical one, formed from 22 β-chains, and an N-terminal globular one, which includes up to 150 amino acid residues. The globular domain is located inside the cylindrical and forming a "plug" or "plug" for the internal channel of the b-cylinder. Removal of the globular domain opens the channel necessary for the interaction of the receptor with a protein complex consisting of three cytoplasmic membrane proteins, ExbB and ExbD, and the proline-rich TonB protein attached to the cytoplasmic membrane by its N-terminus. TonB interacts with ExbB and ExbD in the following stoichiometric ratios – 1 (TonB): 7 (ExbB) : 2 (ExbD) (Higgs, P.,2002) Through its C-terminus, TonB interacts with a conserved sequence

present at the N-terminus of all siderophore-specific receptors. The function of TonB is to transfer the energy created on the cytoplasmic membrane to the receptor of the outer membrane of the cell wall, as a result of which the latter undergoes conformational changes necessary for the process of active transport of siderophore-Fe3+ complexes through the outer membrane. The process of energy conjugation of two membranes occurs, and due to the electrochemical potential of the cytoplasmic membrane, active energy-dependent transport through the outer membrane of the cell wall occurs (Miethke, M.,2007) Delivery of Fe3+ ions to the cytoplasm is carried out using a system of periplasmic ABC transporter proteins. Until now, four subtypes of ABC transporters involved in the absorption of siderophore-Fe3+ complexes have been isolated in Gram-negative bacteria. Separately, the fifth subtype is proposed for the transport of salmochelin-Fe3+. The first subtype is the most common and consists of four proteins: a substrate-binding protein that has an extracytoplasmic localization, two integral membrane proteins that form a heterodimeric transmembrane channel, and a cytoplasmic ABC protein that interacts with cytoplasmic cytoplasmic sites with its subunits. membrane. An example of this subtype is the FepBDGC enterobactin-Fe3+ and FecBCDE Fe3+-dicitrate E. coli transporters. The E. coli FhuDCB transporter has a similar structure, but its transmembrane component consists of one FhuB polypeptide chain, which can be considered as a fusion of two “ordinary” transmembrane subunits. FhuDCB is the second subtype of ABC transporters of siderophore-Fe3+ complexes. The third subtype of ABC transporters consists of transmembrane lipoproteins and is found in Gram-positive bacteria and some Gram-negative bacteria (Vibrio spp., Campylobacter spp.) (. The fourth subtype was found in Yersinia and is intended for the transport of the Fe3+-yersiniobactin complex; it includes the YbtP and YbtQ proteins, respectively, the YbtPQ transporter. However, studies conducted on the nonpathogenic Y. enterocolitica NF-O strain showed that the transport of the Fe3+-yersiniobactin complex additionally requires a periplasmic protein. The fifth putative ABC transporter subtype is the four-domain IroC protein. It has been shown that E. coli fepB mutants can use salmochelin as a source of iron. Since IroC is the only cytoplasmic protein encoded at the iroA locus, it was concluded that it is responsible for the transport of the Salmochelin-Fe3+ complex (Zhu,M.,2005) Gram-positive bacteria lack the periplasmic space and the outer membrane of the cell wall; therefore, the process of iron transport begins with the interaction of the Fe3+-siderophore complex with the substrate-binding protein fixed in the cytoplasmic membrane . Transport across the cytoplasmic membrane is carried out by ATP-dependent transporters. Two subtypes of ATP-dependent transporters are known in Gram-positive bacteria. The first subtype is represented by lipid-

modifying proteins located on the outer side of the cytoplasmic membrane. The second subtype of ATP-dependent transporters was recently discovered in Mycobacterium tuberculosis and is designed to transport the carboxymycobactin-Fe3+ complex (Rodriguez, G.,2006) Eukaryotes with ABC-type transporters were not found; therefore, the transport of the siderophore-Fe3+ complex differs from prokaryotes. The main transporters of the Fe3+-siderophore complex are proteins of the MFS superfamily (from the English major facilitator superfamily, the superfamily of membrane transporters). MFS is a large group of secondary transporters that carry out transport by the mechanisms of uniport, symport, antiport. Four transporters are isolated in Saccharomyces cerevisiae: Arn1p for ferrichrome-Fe3+, Taf1p (Arn2p) for triacetylfusarinin-Fe3+ (TAFC, from English triacetyl fusarinine), Sit1p (Arn3p) for ferrioxamine B-Fe3+, Enb1p (Arn4p) for enterobactin-Fe3+ (Heymann, P., 2000)(Higgs, P., 2002)Micellial fungi and yeasts have a set of membrane-bound ferrisiderophore reductases; as a result, they can implement iron transport in a reductive way. In S. cerevisiae, membrane ferrireductases Fre1p and Fre4p, belonging to the flavocytochrome superfamily, were found, which are involved in the reductive uptake of the siderophore-Fe3+ complex, and the functions of the two homologues Fre5p and Fre6p have not yet been elucidated, but they are known to be activated at iron limit. The main iron transporters in S. cerevisiae are ferrireductases Fre1p and Fre2p, which can absorb Fe3+ ions, associated with citrate, ferrioxamine B, ferrichrome, TAFC, rhodotorulic acid. Ferrireductase Fre3p is 40 times less efficient in using these siderophores. Fre1p and Fre2p can absorb bacterial siderophores, for example, the Fe3+-enterobactin complex (Schroder, I., 2003) (Yun, C. W., 2001) The Fre activity increases in an acidic environment, which is associated with the generation of a proton gradient. Fre1p and Fre2p are also able to transport Cu2+ ions with reduction, which are then transported through the cytoplasmic membrane by the transporters CTR1 and CTR3 (Georgatsou, E.,1979) Like S. cerevisiae, Candida albicans has similar siderophore-bound iron transport pathways. Arn1p (CaArn1p) systems transport ferrichromes. All representatives of the genus Candida do not produce their own siderophores, but have transporters for the use of heterologous ones (Heymann, P.,2002) Absorption of heterologous siderophores is not the only mechanism of iron entry into the cell. It has been established that this transporter is necessary for invasion into the human epithelium, but is not necessary for the development of a generalized infection in mice (Heymann, P., 2002)

Evolutionarily, fungi, as well as many microorganisms, in addition to their own homologous siderophore, can use heterotrophors that synthesize other heterotrophic microorganisms. The possibility of using microorganisms in heteropathogenic siderophones allows micromicrobes to

better adapt to environmental and climate conditions, which is especially important for pathogenic microorganisms.

The capacity of bacteria to exploit heteropathogenic siderophores can boost their competitiveness within the body of a host. These siderophores can compete for available iron with the host's iron-binding proteins. Siderophores effectively bind and sequester this critical nutrient due to their high affinity for iron, depriving the host's iron-binding proteins and other competing bacteria of this crucial resource. Pathogens that create siderophores can take use of this to their advantage inside the body of the host.

3.4.3 Streptomyces scabiei and other pathogenic streptomycetes causing common scab of potatoes

A important prokaryotic pathogen of potatoes with a distinctive filamentous growth pattern is Streptomyces acidiscabie. It is one of several Streptomyces species, with S. scabies serving as the main causative agent, that can lead to common scab disease in potatoes. Under specific circumstances, other species, including Streptomyces acidiscabies and Streptomyces turgidiscabies, can also result in the illness (Lambert and Loria, 1989a,b; Miyajima et al., 1998). Streptomycetes are unusual bacteria that exhibit filamentous development, a high G + C concentration, and a positive gram stain. They can be recognized by their grey, smooth spores that are organized in spiral chains. They produce vegetative spores by severing aerial filaments. According to Lambert and Loria (1989), S. scabies is a diverse species that may be recognized from other streptomycetes that cause scabs by its morphological traits and patterns of sugar consumption.

The phytotoxin thaxtomin, a nitrated dipeptide toxin, is the main virulence component shared by all pathogenic streptomycetes (Lawrence et al., 1990). Numerous strains also possess the nec1 gene, which produces the necrogenic protein that is a second virulence factor (Bukhalid et al., 1998). These virulence factors can be found on a 325 kb chromosomal fragment known as a pathogenicity island (PAI), together with other virulence-related genes.

PAIs are known to carry clusters of pathogenicity-related genes in other plant and animal pathogenic bacteria (Kers et al., 2005). Pathogenicity can in some cases be passed on to related strains by horizontal gene transfer involving the entire PAI. Mobilization of the PAI gene cluster into *Streptomyces diastatochromogenes*transferred the scab-inducing phenotype to this non-pathogenic species, whereas transconjugants of Streptomyces coelicolordid not produce thaxtomin or necrotize potato tuber tissue (Kers et al., 2005). Horizontal transfer of the PAI from *S.*

scabies into specific receptive *Streptomyces*spp. perhaps accounts for the variability of apparent species associated with induction of the scab diseace. (Jan M. van der Wolf et al.,2007) Common potato scab is a plant disease. Actinomycetes are the responsible parties. These microorganisms have the capacity to develop branching mycelium. On potatoes, about ten species have been recognized. The most damage is caused by Streptomyces scabies. The condition is widespread. Stolons, roots, and, to a greater extent, potato tubers all exhibit disease symptoms. A white fluffy mycelium and sporulation layer is visible on recently excavated ones. The plaque rapidly vanishes when the tubers have dried. There are tuberculate folds surrounding the lenticels, which eventually develop into dry sores with a diameter of a few millimeters to one centimeter and a variety of shapes. The sores may split or combine to form a continuous crust of scaly skin. The pathogen is not active in relation to the plant's green portions.

According to the severity of ulcers, four forms of common scab are distinguished:

Flat scab - mainly found on young tubers. The peel or the uppermost layer of the periderm is affected. A brownish skin hardening or abrasion is formed on the tuber, initially light brown and then dark brown.

Mesh scab - the surface of the tuber is completely rough. The scab is formed in the form of small grooves intersecting in different directions, which resembles a grid in configuration.

Convex scab - initially has the appearance of cone-shaped depressions that are insignificant in depth. Later, the recesses rise, forming warty-like or scab-like growths on the surface of the tuber, up to 2.0 mm high.

Pitted or deep scab - most often found during the harvesting of potatoes. On the surface of the tubers, brown ulcers are formed up to 5 mm deep and up to 100 mm in size, surrounded by a torn peel. The form of ulcers is various.

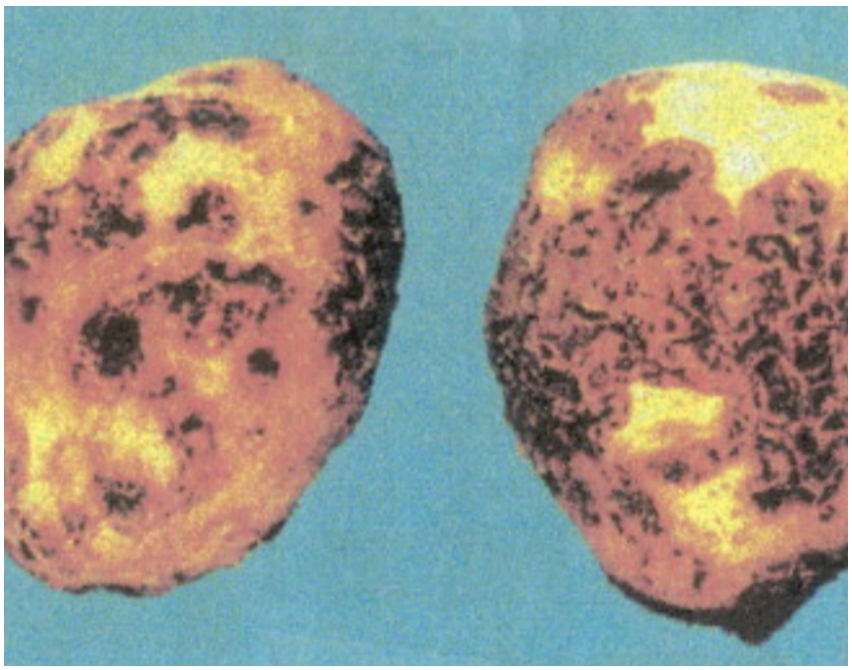


Figure 2. Potato scab caused by Streptomyces scabies.
Source: Photo-graph courtesy of R. Loria, Cornell University

The development of one or another type of common scab depends on the depth of exposure to the pathogen, varietal characteristics of potatoes, and environmental conditions.

Morphology

The causative agents of the disease are gram-positive bacteria of the Actinobacteria group ("actinomycete branch"), the order Actinomycetes (Actinomycetales), the family Streptomycetaceae, the genus Streptomycetes (Streptomyces). More than 10 species have been identified. The most harmful is Streptomyces scabies.

Streptomyces scabies is a species of bacteria, like all actinomycetes, tending to form branching hyphae developing into mycelium, as well as the formation of sporangia (sacs containing bacterial spores). The cell walls, like those of the entire heterogeneous group of Streptomycetes and related genera, contain L-diaminopicolic acid and glycine. Mycelium filaments do not break up into fragments and form aerial mycelium with long chains of bacterial spores.

- pathogens that cause
- common scab of potato

The bacteria Streptomyces scabies, like all streptomycetes, are obligate aerobes. They are undemanding to the nutrient substrate, they do not need growth factors. They are widely distributed in soils of various types and play an important role in mineralization processes. The specific smell of freshly plowed soil is determined precisely by their presence.

Streptomycetes actively develop in conditions of low soil moisture. In the soils of arid climatic zones, they numerically predominate over all microorganisms. Their ubiquitous distribution is closely related to the presence of enzyme systems that allow the destruction and use of various compounds.

The virulence of Streptomyces scabies is associated with the presence of cutinase, which hydrolyzes the polymer of the protective cuticular layer of the potato tuber. It has been established that all streptomycetes causing common potato scab suppress the formation of phytoalexins in tubers. Streptomyces scabies culture fluid filtrates inhibit the respiration of potato tubers. Phytopathogens live in the soil on organic residues and, under favorable conditions, switch to feeding on the underground organs of potatoes and other root crops. They penetrate into the tuber through lentils and mechanical damage. A white mycelium with helical conidiophores quickly forms on the affected organs.

Sources of primary infection are affected soil and planting material. Common potato scab does not develop in storage facilities. (Kiryushina et al., 2021).

Development conditions:

A common plant disease that might appear under specific circumstances is common potato scab. By lowering the air content and raising the amount of organic humus in the soil, the pathogen's viability can be inhibited. Actinomycetes can also be less active when trace elements like manganese and boron are present in adequate quantities.

However, a number of circumstances may have a role in the disease becoming active. These include the existence of plant residues that haven't decomposed, recent applications of organic fertilizer, excessive lime use, free calcium, and nitrites in the soil. A soil temperature between +25°C and +27°C, little soil moisture (soil drought), and a soil pH above 5.5 are ideal for the development of common potato scab.

3.4.4 Siderophores present in the genome of S. scabiei

Two new tris-hydroxamate siderophores, scapichelin and turgichelin, have been identified from the bacterial species Streptomyces antibioticus and Streptomyces turgidiscabies, respectively. On their gallium(iii) complexes, these metabolites' planar structures were identified utilizing mass spectrometry and NMR spectroscopy methods. Scabichelin and turgichelin's relative and absolute stereochemistry was determined using a variety of techniques, including a modified Marfey's approach, computer modeling, and NOESY NMR analysis.

The genomic sequence of the plant pathogen Streptomyces scabies 87.22 was further investigated, and it was discovered that a gene cluster comprising a gene encoding a nonribosomal peptide synthetase (NRPS) was present. A pentapeptide with structural resemblances to scabichelin and turgichelin is expected to be produced by this NRPS. Scabichelin is indeed produced by this cryptic gene cluster, as evidenced by comparison studies employing LC-MS/MS on culture supernatants from wild type S. scabies 87.22, a mutant strain with a damaged NRPS gene, and isolated scabichelin from S. antibioticus (Kodani, Shinya et al., 2013). This argues in favor of scabichelin's siderophoric properties.

4. Methodology

4.1 Cultivation of the bacteria and DNA extraction

The appropriate selection of dependable tools and the construction of ideal circumstances in the nutrient medium are just a couple of the variables that influence how well microorganisms reproduce and flourish in a regulated environment. Specific microbes can be grown and multiplied by researchers by carefully regulating these conditions in the lab.

Streptomyces scabiei, for instance, can be grown in a GYM medium at a temperature of 28 °C for a duration of 8 days. After the bacteria have developed and multiplied, their DNA can be taken out and used for additional research. Streptomyces scabiei's DNA can be taken out using a variety of techniques, including the DNeasy PowerLyzer Microbial Kit from Qiagen.

1.8 ml of bacterial culture are added to a 2 ml collection tube and centrifuged at 10,000 x g for 30 seconds at room temperature to extract DNA using this kit. The resulting cell pellet is then combined with 50 ml of Solution SL and resuspended in 300 l of PowerBead Solution. Using this method, Streptomyces scabiei's high-quality DNA may be extracted from the organism quickly and effectively.

Sample was homogenized by vortexing at maximum speed for 10 min and then the tube was centrifuged at a maximum of 10,000 x g for 30 s at room temperature. The supernatant was transferred to a clean 2 ml collection tube (provided). 100 ul of Solution IRS was added, and the mixture was vortexed for 5 s and incubated at 4°C for 5 min. Then, the sample was centrifuged at 10,000 x g for 1 min at room temperature. Avoiding the pellet, supernatant was transferred to a 2 ml collection tube, and 900 pl of Solution SB was added to the supernatant and vortexed for 5 s. 700 ul was loaded into an MB Spin Column and the tube was centrifuged at 10,000 x g for 30 s at room temperature.

Then 300 pl of Solution CB was added and the sample was centrifuged at 10,000 x g for 30 s at room temperature. The flow-through was discarded and the tube was centrifuged at 10,000 x g for 1 min at room temperature. MB Spin Column was placed in a new 2 ml Collection Tube. Then 50 µl of dH2O was added and the tube was centrifuged at 10,000 x g for 30 s at room temperature.

MB Spin Column was discarded and DNA was ready for downstream applications.

4.2. Preparation of the premix with primers

Water	13,125 µl	170,625 µl
5x Green GoTaq Reaction Buffer	5 µl	65 µl
DMSO	1,21 µl	16,25 µl
MgCl2 (25mM)	2 µl	26 µl
dNTP (10 mM)	0,5 µl	6,5 µl
GoTaq G2 Hot Start Polymerase	0.125 µl	1,625 µl
Primers (F) & (R)	2 x 1 µl	2 x 13 µl
Template	1 µl	13 µl

Table.2

Before working with primers, a forward and reverse DNA strand was given for attaching primers (Tab.1). In the table, we see the stage of primer attachment and data on the conditions that we used for this.

Scabichelin (1st adenylation domain)						
			Position (nt)	Length	Tm	GC
scab_F	5´-CATCGCGTACATGCTGG-3´	forward	933-949	23	59°C	58,80%
scab_R	5´-CGCGTTGAACATCATCCG-3´	reverse	1531-1548	24	59,9°C	55,60%

Table.3 Multiply x13

***Primers - forward (F) & reverse (R)**

Before diluting, the concentration of primers was 100 µM, diluted it 10 times (to 10 µM) so the concentration in the mixture (25 µl) was 400 nM.

The concentration of primers in premix was 400 nM. Before mixing all of the products, diluted both primers (400mM (primer) and 9µl (H2O) for the working solution of primer which is ready to use with the mixture.

After preparing the admixture in the laboratory, which will help us in the future to prepare the PCR test with the given DNA, I can start with the dilution of the DNA (Table.3).

4.3. Dilution of the DNA samples

3KA	Suppressive	From untreated soil
d3KA	Suppressive	From untreated soil
3UAF	Conductive	From treated soil
d3UAF	Conductive	From treated soil
2UA	Conductive	From untreated soil
d2UA	Conductive	From untreated soil
dStreptomyces scabiei DSM 41658	Pure culture	-

Table. 4

Environmental type strains were isolated from soil (fields) in Vyklantice.

1. 3KA & 3UAF dilution → 2 times diluted

2 µl H2O

2 µl DNA

2. 2UA dilution → 2 µl H2O & 1 µl DNA

3. *Streptomyces scabie dilution* → 29 µl H2O & 1 µl DNA

The DNA molecule is polar and readily soluble in polar water. Based on the principle of solubility, DNA is insoluble in relatively low-polarity ethanol, and even in ethanol-water mixtures, due to the reduction in the number of available water molecules (Tab. 3).

Extracted DNA from *Streptomyces scabiei* DSM 41658 and did PCR with newly designed primers for the first adenylation domain of NRPS (nonribosomal peptide synthetase) responsible for the biosynthesis of *scabichelin.*

4.4. Annealing Temperature for PCR

According to my research, the premix should be annealed at a temperature of 57°C, as illustrated in (Figure. 3). An essential PCR parameter that influences the specificity and effectiveness of the reaction is the annealing temperature. The target DNA can be amplified when the primers anneal to the complementary sequences on the DNA template at this temperature.

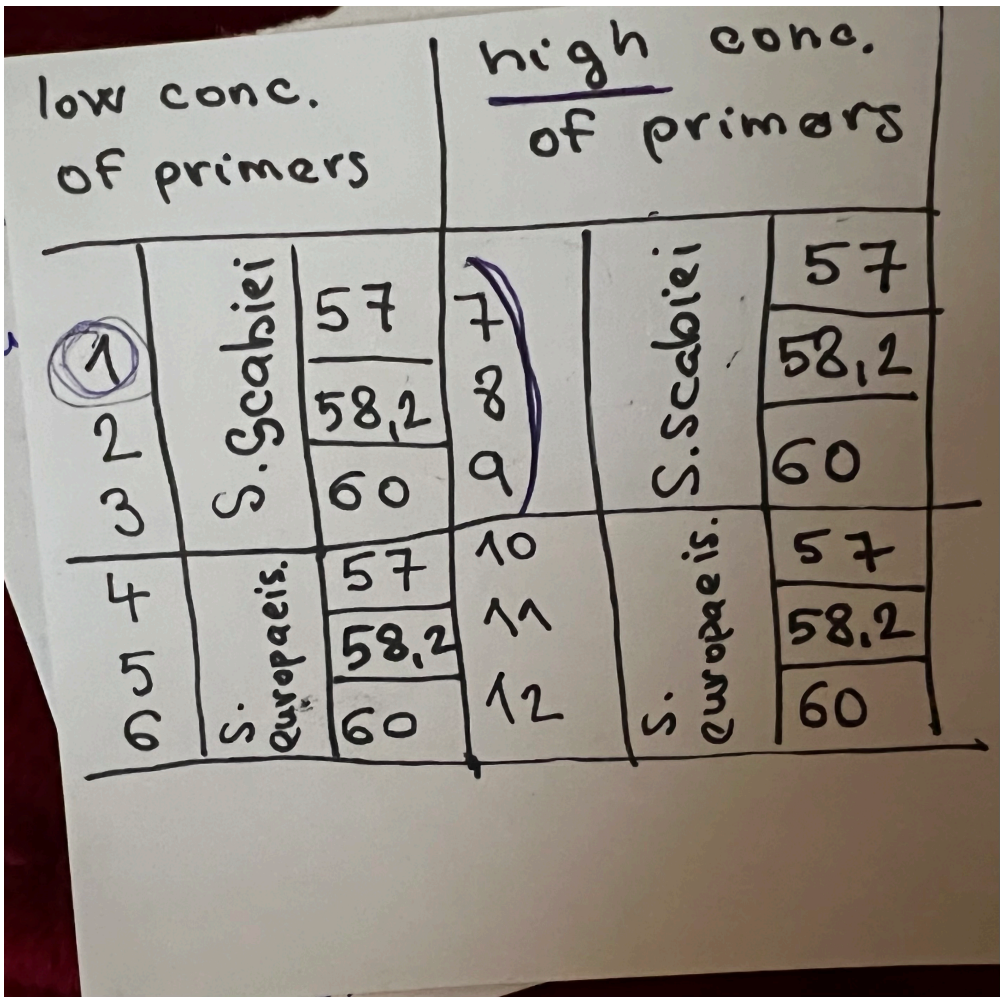


Figure.3

Was experimenting with high and low primer concentrations (2 microL), which is a crucial aspect of PCR optimization. The concentration of the primer can have an impact on the reaction's specificity and yield because too little primer can lead to insufficient amplification while too much primer can produce non-specific amplification.

To get the best results for your particular experiment, it's critical to optimize PCR settings such annealing temperature and primer concentration. You can increase the specificity and sensitivity of the reaction by figuring out the ideal circumstances, which will produce more trustworthy and accurate results.

4.5. PCR procedure

Since the beginning of my work in the laboratory, the goal has been to find the optimal temperature that will work and give a result and DNA that will show the desired result.
From the beginning of the work, a lot of PCR tests have been done to find the right temperature that would suit all DNA samples.
The results of the PCR reaction are usually clearly visible due to gel electrophoresis.
Polyacrylamide gel electrophoresis is a technique in which DNA fragments are passed through a gel under the influence of an electric current and separated by size. Additionally, a DNA marker is used to determine the size of the fragments in the sample.
Each strand of DNA contains many copies of the target fragment, rather than just one or a few. Because DNA is microscopic, it takes a lot of copies to see it with the naked eye. Therefore, PCR is very important: it leads to the formation of a sufficient number of copies of a DNA fragment so that we can see it and use it in further work.
After several tests were carried out and several results are seen, it was clear that the most suitable temperature for PCR tests was 57°C.
Once the optimal temperature for testing was found, the goal was to find the optimal DNA solution that would give the best results. Many trial tests were done before the best result of all was found (Figure.4).

4.6. Protocol of the PCR amplification procedure

Step	Temperature	Time	Number of cycles
Initial duration	95°C	2 min	1
Denaturation	95°C	30 s	35
Annealing	57-60°C	50 s	35
Extension	72°C	40 s	35
Final extension	72°C	5 min	1

Table. 5

5. Results

5.1 Electrophoresis

For the gel preparation, it was important to follow the laboratory rule of electrophoresis.
Until recently, the most common method was electrophoresis, based on the division of DNA molecules by size. In this case, the visualization of the results is carried out in an agarose gel plate, which is agarose frozen after melting in an electrophoresis buffer at a concentration of 1.5–2.5% with the addition of a special DNA dye, for example, ethidium bromide.

Rule:

For the preparation of 1% agarose gel, 50 ml of TAE buffer + 0,5 g of agarose were mixed in a glass bottle. The bottle was microwaved until the agarose was dissolved, cooled down to about 55°C and 5 µl of SYBR GREEN in DMSO (1:9) was added. The gel was poured into a gel tray, allowed to solidify, and placed into the gel box filled with TAE buffer until the gel was covered. 2 µl of the sample + 3 µl of loading buffer was added and prepared in a microtiter plate. Then, sample was mixed and loaded into the wells and then 5 µl of marker (1kb ladder) was loaded. The gel was running at 95 V and 95 mA until the samples reached 1/3 of the gel.. Then a result was visualized under UV light, and a picture of the gel was taken using GeneSnap software.
The frozen agarose forms a spatial lattice. When filling with combs in the gel form wells into which amplification products are added. The gel plate is placed in a horizontal gel electrophoresis apparatus and a constant voltage source is connected.
Negatively charged DNA begins to move in the gel from minus to plus. This makes shorter DNA molecules move faster than longer ones. The speed of DNA movement in the gel is affected by: agarose concentration, electric field strength, and temperature.
All molecules of the same size move at the same speed. After the end of electrophoresis, which lasts from 10 minutes to 1 hour, the gel is placed on a transilluminator filter that emits light in the ultraviolet range.
After the process of electrophoresis, it is possible to proceed to the preparation of the gel results.

5.2 Electrophoresis results

* The ladder, that was used for all of the gels - ZipRuler Express DNA Ladder 1 by Thermo Fisher Scientific. (Figure.3)

** Red numbers explaining the sizes of the brightest bands.

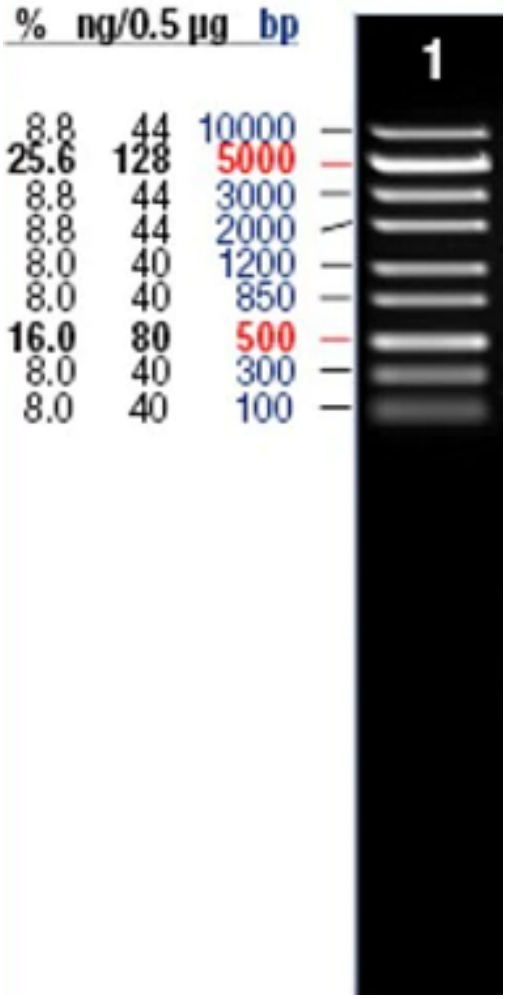


Figure. 4

6. Discussion

Gel N.1

The result of electrophoretic separation of PCR products. Agarose gel is stained with a ladder, which fluoresces under ultraviolet light. The expected size of the product (i.e. 650 bp).

This sample didn't work perfectly, because as a result could see only the leader and non-specific bands between DNA samples, it is not the right size (it's about 2000 bp). (Figure. 5)

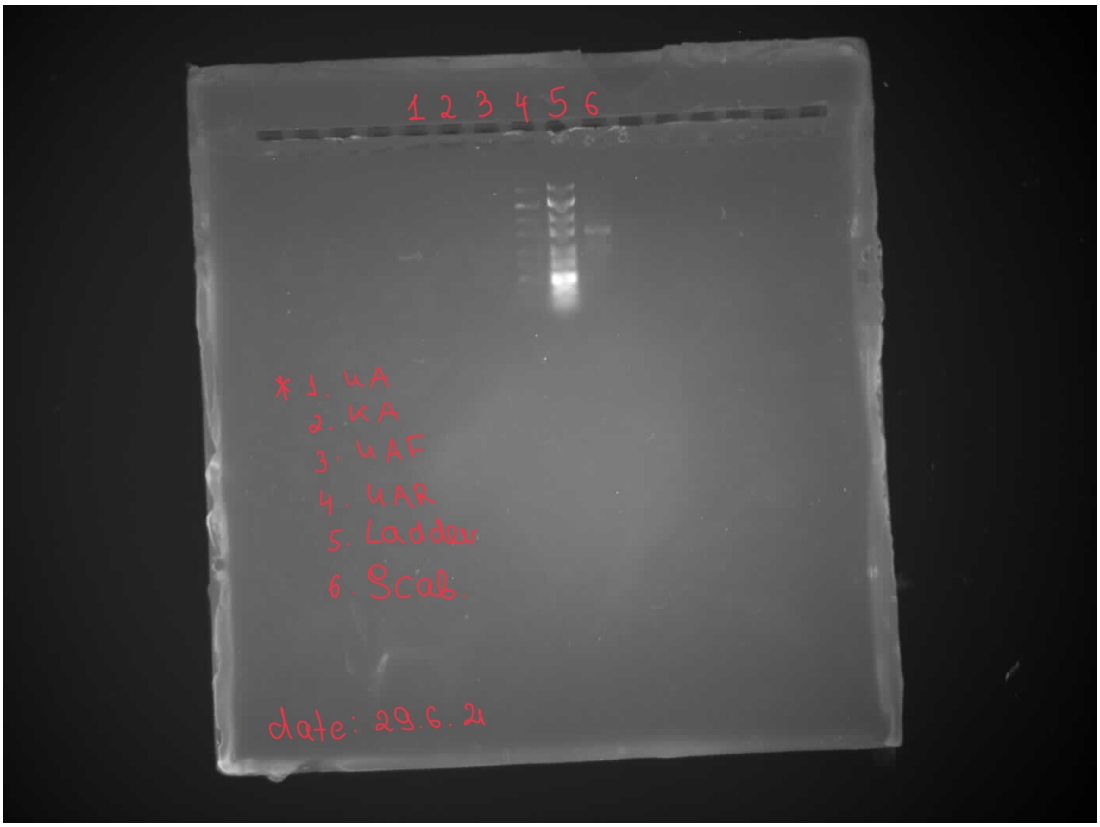


Figure. 5 **Length -500-300 bp**
Line/Row number 5 is ladder (Molecular Weight Marker)

Gel N.2

In the second trial, the resulting temperature gradient was changed from 58,2°C to the optimal (that was found after the last experiment) 57°C.

On the second sample of the gel, the sample with 3KA (i.e. 6) DNA sample worked, but for others it was hard to see any result clearly, because even the *Streptomyces scabiei* (Figure. 6) sample didn't work, so it was visible that I had no a new and good quality DNA sample.

For the next trial with PCR and electrophoresis, I have decided to cultivate a newly prepared culture of the previous trial (- *Streptomyces scabiei)* , but with higher concentration. (Figure. 6)

The PCR product in the line 5 - 3KA. It was visible that result is slightly improved compare to the first gel, but it wasn't that clear.

Figure. 6 **The length of the chain is 500 bp**

Gel N.3

Comparing the third gel with the previous one, new DNA dilution was performed toimprove the results.

After the DNA extraction and getting a new sample of DNA, it was visible that the result was slightly improved. From the first time, PCR products all of the DNA samples and the chain were visible, even though it wasn't the expected result because the PCR led to the production of nonspecific amplicons. The expected

length of the PCR product chain was around 650 bp (Figure.7).

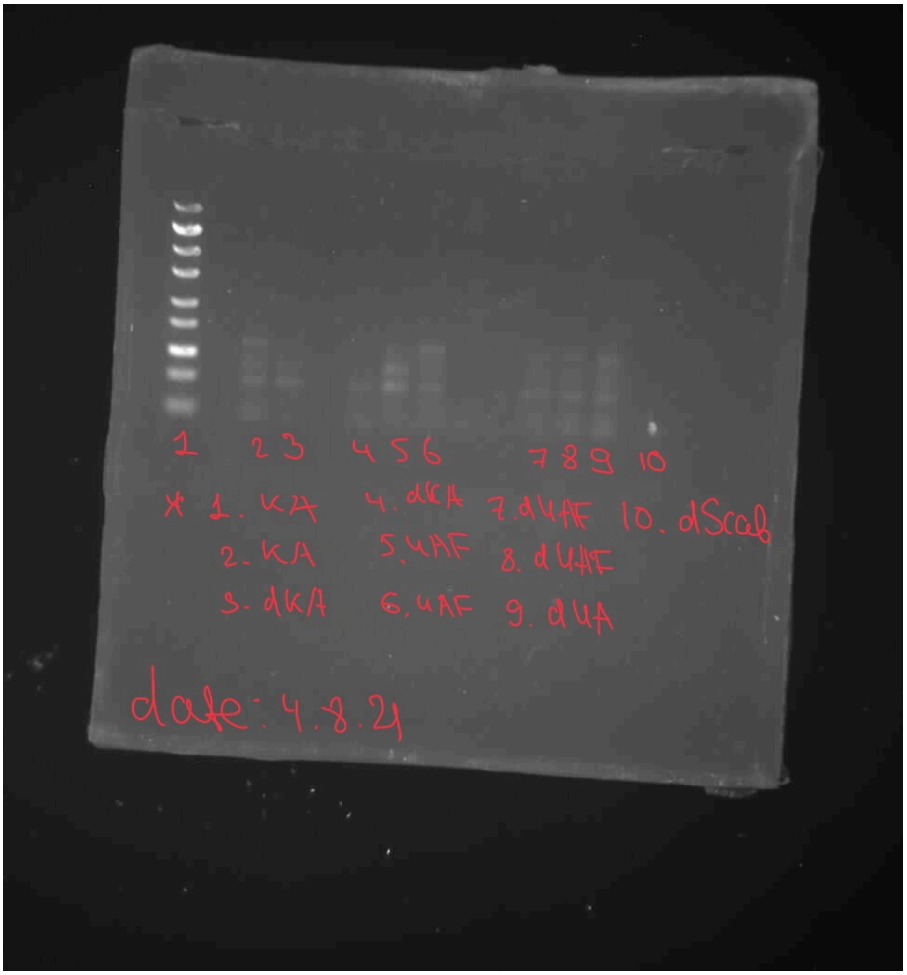


Figure.7 **Length - 650 bp**

Gel N.4

Main & final result:

The final result that was obtained (Figure. 4) after the new cultivation of the bacteria and new extraction of DNA so, it helped to get better results.

In the gel N.4, it was visible that the sample with diluted UA ((i.e. 10) DNA worked better than other samples, but other samples seemed to produce amplicons of the right size, although of the bad quality.

After the tests, it was found that the most appropriate result was with diluted DNA (dUA) that also was from conductive (severity of common scab of potatoes is high) and untreated soil.

That being said, the expected size of PCR products was about 650 bp.

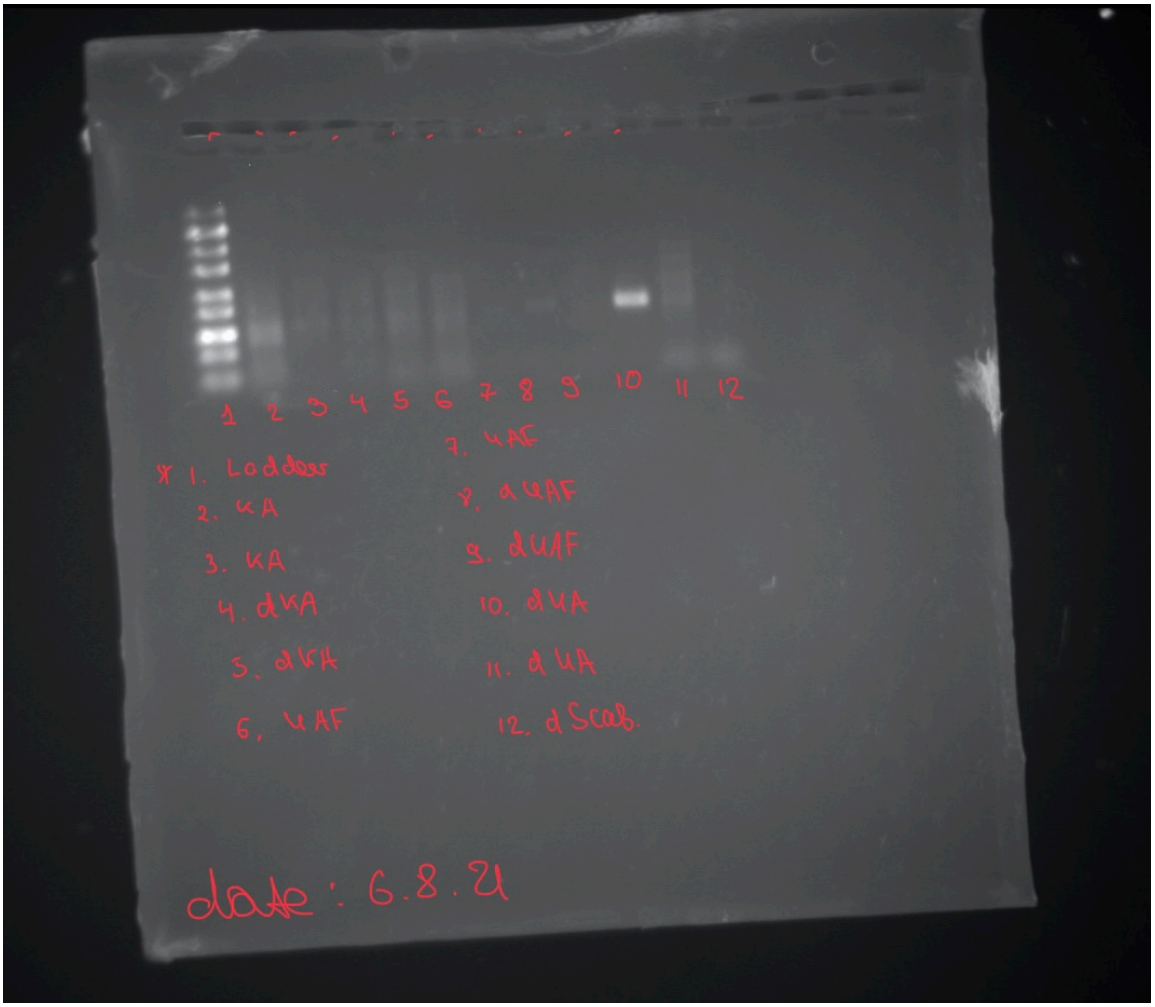


Figure. 8 **The length of the chain is 500 bp.**

7. Conclusion

As a result, I was able to amplify products of the right size in some samples (Figure. 4). As other samples (and sometimes even the positive control *Streptomyces scabiei*) did not work.
It would be beneficial to continue with attempts to optimize PCR (e.g. by modifying Mg concentration) in the future.
Moreover, the sequencing of the amplification would be appropriate to ensure that the expected product is amplifying. But in general, the results are promising and it seems that the primers could be used in various analyses.
Possibly, some modifications to the primers should be made to improve the specificity of primers in same samples.

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Enclosures