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Faculty of Science

Bachelor's Thesis

The interaction of *Streptomyces*-like bacteria and model microorganisms in secondary metabolite production, motility and hemolytic activities - Experimental

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Annotation

In this thesis, environmental and clinical *Streptomyces* strains were used to test the hypothesis if isolates from different isolation sources vary in frequency of beta-hemolytic activity. Moreover, their interaction with two human pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* was tested in parallel and T-type co-cultivations. For this, the responses (beta-hemolytic activity, growth, etc.) of both streptomycetes as well as model pathogens were evaluated. Furthermore, the frequency of observed interaction patterns with tested pathogens were compared between environmental and clinical *Streptomyces* strains as such study is lacking.

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1 Abstract

Streptomyces are Gram-positive bacteria which are widely distributed in soil, water and other natural environments. They show a complex developmental cycle and the production of bioactive secondary metabolites in general with antifungal, antibacterial or immunomodulatory activities. Recently, *Streptomyces* species have been isolated from various body parts of human (for example from skin and sputum) without direct prove of pathogenicity. Therefore, their role in mixed infections and hence the clinical importance of *Streptomyces* is unclear. The hypothesis is that they play a role in regulating inflammatory activity by producing secondary metabolites and controlling the spread of human pathogens. In this thesis, clinical and environmental strains of *Streptomyces* were tested on blood agar plates to evaluate their growth abilities and their hemolytic activities. Furthermore, their interactions (inhibition or activation of hemolytic activity and growth) with human pathogens (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) were analyzed using parallel and crossed tests. The results showed that more than a half (51.5 %) of the environmental streptomycetes and about three quarter (75.5 %) of the clinical isolates have the ability for beta-hemolysis, but the hemolytic zones of streptomycete strains from clinical origin were smaller. The co-cultivation with the pathogens showed that closer proximity of both pathogens increased the hemolytic zone size of *Streptomyces* likely as result of activation of metabolic activities. The pathogens were also influenced by the streptomycetes as both showed also an increase in beta-hemolytic activity in closer proximity to *Streptomyces*; however, this effect was more pronounced in *P. aeruginosa*. The influence on growth of the pathogens differed as *P. aeruginosa* showed more activations (increase in motility) and *S. aureus* was more often inhibited by *Streptomyces*. The interactions were very strain specific and there were always strains which showed other effects than the observed trend.

On the basis of this thesis, a future project can investigate the beta-hemolytic and inhibitory activities of selected streptomycete strains in more details.

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3 Introduction

3.1 Microbial interactions

3.1.1 General aspects and types

Microbial interactions are a key-strategy for colonialization and for successful establishment of a microbial population in a variety of different environments. During the process, the environment is first recognized followed by the transfer of molecules and genetic information. The exchange includes diverse mechanisms, such as secondary metabolites, siderophores, quorum sensing, biofilm formation, cellular transduction signaling and others, to establish in a community. In those communities the different species had many years of co-evolution which lead to adaptations and specializations. This resulted in a variety of relationships (Braga, et al., 2016).

The most prominent examples are: (I) the mutualistic relationship, where both organism benefit (Garcia & Gerardo, 2014), (II) the endosymbiotic relationship, in which one organism lives within the other (Keeling & McCutcheon, 2017), (III) the commensal relationship, where one organism benefits without affecting the other one (Dattagupta & Zielinski, 2011), (IV) the parasitic relationship, where one organism benefits and the other is harmed (Dattagupta & Zielinski, 2011), (V) the pathogenic relationship, in which one organism cause a disease by invading the host (Anon., 2004), and (VI) the competitive relationship, where both organisms are harmed (Maynard, et al., 2017).

3.1.2 Important types of interaction in soil

A very important cell to cell communication mechanism of microorganism is quorum sensing (QS). It involves the production, detection and response to so called autoinducers, which are the signaling molecules. Many different functions can be regulated using quorum sensing, for example biofilm formation, bioluminescence and DNA exchange. For instance, pathogenic bacteria can utilize QS to regulate the transcription and expression of their virulence genes (Rekha, et al., 2011). The following bacteria are some of various others which use QS signals for regulation of their virulence factors and host infection: *Bacillus cereus*, *Staphylococcus aureus*, *Vibrio* sp., and *Pseudomonas aeruginosa* (Rutherford & Bassler, 2012).

In general, quorum sensing depends on three basic principles. First, autoinducers are produced by the members of the community. At low cell density, they diffuse away and cannot be detected anymore, because their concentration is too low. At high cell density, local high concentrations of signaling molecules appear; hence they can be detected and the cells can response to the autoinducers. Secondly, receptors in the cytoplasm or in the membrane detect the signaling molecules. Third, the detection of the autoinducers leads to the activation of genes necessary for cooperative behaviors and autoinducers production (Rutherford & Bassler, 2012).

Gram-positive and Gram-negative bacteria use different types of autoinducer molecules. Autoinducing peptides (AIPs) are used by Gram-positive bacteria. After their production, the AIPs are processed and then secreted. They are recognized, over the threshold, by binding of the autoinducing peptides to a cognate membrane-bound histidine kinase receptor which consists of two compartments. This leads then to further activations. In contrast, Gram-negative bacteria use small molecules as autoinducers: either acyl-homoserine lactones or other molecules which are produced from S-adenosylmethionine. They can freely diffuse across the inner and outer membrane of the cell after production because they are so small. Mostly, these autoinducers bind to cytoplasmic receptors that are transcription factors and regulate the expression of genes in the QS regulon. However, in some cases they are detected using the two-component histidine kinase receptors as in Gram-positive bacteria (Rutherford & Bassler, 2012).

Secondary metabolites, which are also called natural products, play a very important role in communication between microorganisms (Figure 1). Secondary metabolites are organic compounds with a low-molecular-mass and in contrast to primary metabolites, they are not directly involved in growth, development or reproduction (Netzker, et al., 2015). Microorganisms are able to produce a large variety of secondary metabolites and about 170,000 of these natural products have already been characterized (Seyedsayamdost & Clardy, 2014). Many secondary metabolites can be used in medicine, for example as antibiotics. Due to this, an early explanation of the function of them was to provide the bacteria with the ability to compete against other microorganisms in a more effective way (Netzker, et al., 2015). However, many secondary metabolites modulate transcription patterns in a range of bacterial and eukaryotic cells below their inhibitory concentration (Yim, et al., 2006). They may function by binding to receptors in the cell followed by triggering different cellular responses. Therefore, they can be used as cell to cell signaling agents (Miao & Davies, 2010).

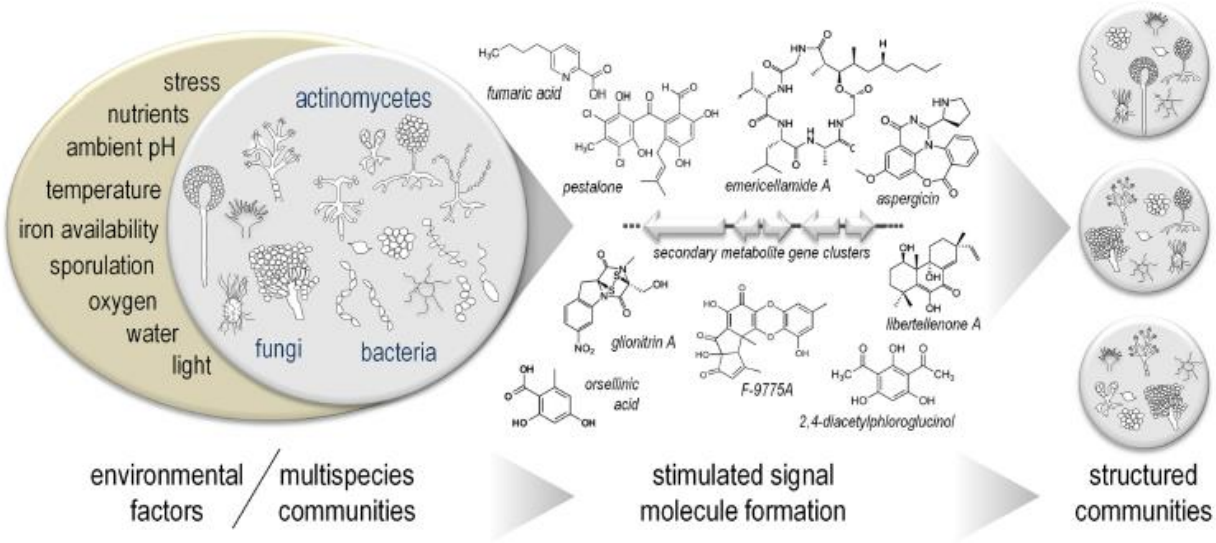

Figure 1. The scheme describes how secondary metabolites are used in microbial communities for communication and their structures **(Netzker, et al., 2015)**.

Siderophores are important for the bacteria, because they are used for the uptake of Fe^{3+}. All living cells need iron for their growth, but Fe^{3+} is an insoluble form and therefore it is unavailable. However, most bacteria have the ability to secrete siderophores which are typically small peptides (often non-ribosomally synthesized). These peptides have a high affinity for the Fe^{3+} and form complexes that can be taken up by the cell with a high efficiency. It is quite common for streptomycetes that they have the ability to produce more than just one kind of siderophore. A reason for this can be, that competing soil bacteria can possibly use siderophores produced by others exploiting uptake systems with corresponding specificity. Exactly this advantage is reduced by using more than one siderophore (Chater, et al., 2010).

3.2 Streptomycetes

3.2.1 Basic characteristics

The genus *Streptomyces* belongs to the *Streptomycetaceae* family, which is part of the *Actinomycetales* suborder *Streptomycinae* within the class *Actinobacteria* (Kämpfer, et al., 2014). *Streptomyces* is the largest genus of *Actinobacteria* (Hasani, et al., 2014) and includes more than 600 described species with a validated name. Main characteristics of *Streptomyces* are their complex developmental cycle and the production of bioactive secondary metabolites. They produce more than one third of clinical useful antibiotics of natural origin. Other produced

secondary metabolites may have in general antifungal or immunomodulatory activities. *Streptomyces* are aerobic, Gram-positive and catalase positive, but non-acid-fast bacteria. The G+C content of their DNA lies between 69 and 78 mol% and it is therefore relatively high (Kämpfer, et al., 2014).

The filamentous bacteria form extensively branched substrate and aerial mycelia with chains of three to many non-motile spores. Their vegetative hyphae, with a diameter of 0.5-2.0 μm, rarely fragments (Kämpfer, et al., 2014). The colonies of *Streptomyces* are slow-growing and often have a soil-like odor due to the production of the volatile metabolite geosmin (Hasani, et al., 2014). First, they have a smooth surface but develop later a weft of aerial mycelium that may appear floccose, granular, powdery, or velvety. The colonies are discrete and lichenoid, leathery, or butyrous. They produce a wide range of pigments responsible for the color of the vegetative and aerial mycelia; aside from that, colored diffusible pigments may be generated. Morphological characteristics are the basis to distinguish streptomycetes from other filamentous actinomycetes. In particular vegetative mycelium, aerial mycelium and arthrospores are used (Kämpfer, et al., 2014). However, a polyphasic approach and thus the combination of phenotypic and genotypic traits is necessary for elucidation of the genus and classification of *Actinobacteria* (Kämpfer & Glaeser, 2011).

Streptomycetes are chemoorganotrophic with an oxidative type of metabolism. Hence most species utilize a variety of organic compounds as sole carbon sources for energy and growth (Kämpfer, et al., 2014). In addition, they need inorganic nitrogen sources and mineral salts, and do not need vitamins and growth factors (Hasani, et al., 2014). Because of this, they generally belong to more easily and widely isolated bacteria from diverse environments. They usually degrade polymeric substrates such as casein, gelatin, hypoxanthine, and starch in addition to adenine and L-tyrosine. They also reduce nitrates to nitrites. Most members of this genus have an optimal growth temperature in the range of 25-35 °C. However, some species can grow at temperatures within the psychrophilic and thermophilic range. Their optimal pH range for growth is between 6.5 and 8.0 (Kämpfer, et al., 2014). Streptomycetes require less moisture than other bacteria and are very sensitive to water logged conditions. Furthermore, they can form arthrospores and are therefore more resistant to drought (Hasani, et al., 2014).

3.2.2 Life cycle

Streptomyces undergo a complex multicellular cycle of development and reproduction (Figure 2), which is unique for a bacterium. Its life cycle involves three phenotypic traits for detailed microscopic characterization. First, the vegetative (substrate) mycelium, which happens on the solid and in liquid medium. Secondly, the aerial mycelium with chains of arthrospores, and lastly the arthrospores themselves. The last two characteristics give the most diagnostic information for taxonomists. For example, the organization of the spore chain has played an important role in species descriptions for many years because of the enormous variety of spore chain morphologies (Kämpfer, et al., 2014).

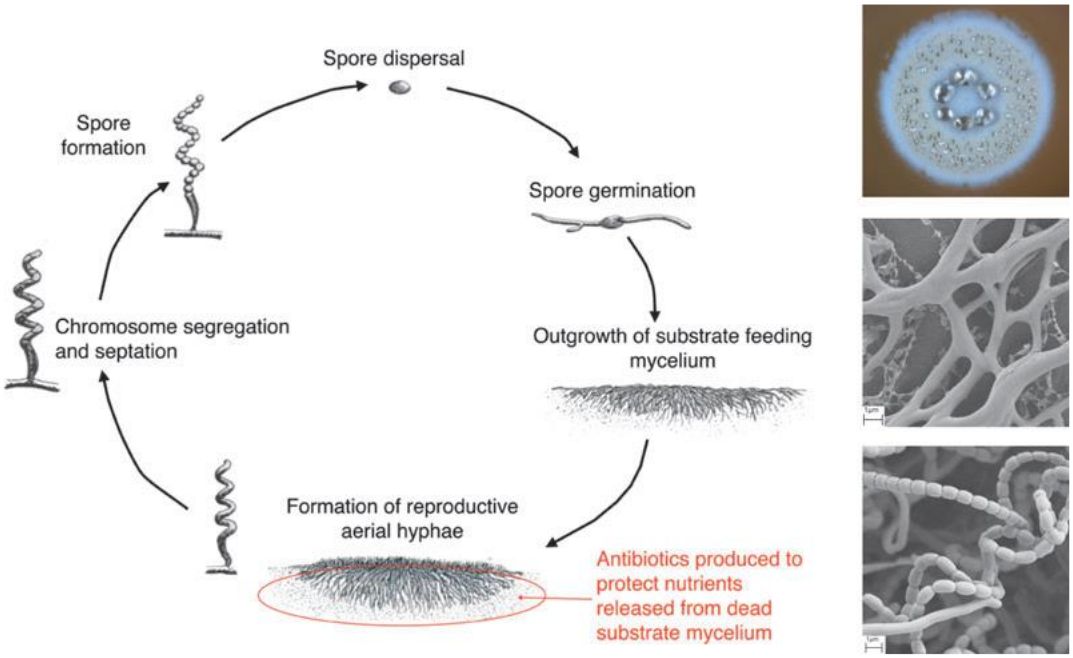

Figure 2. The *Streptomyces* life cycle. The pictures on the right show a colony of *Streptomyces* spp. isolated from *Allomerus* ants at 40x magnification (top), scanning electron micrographs of substrate mycelium (middle) and aerial hyphae and spore chains (bottom) of *Streptomyces coelicolor* at 400x magnification **(Seipke, et al., 2012)**.

The life cycle of *Streptomyces* starts with the spore germination, which may be induced by many factors including water, carbon dioxide, amino acids and vitamins. The germination begins, when the conditions are favorable, with swelling of the spore. Followed by the establishment of the spore polarity and by the development of one or more germ tubes (Kämpfer, et al., 2014). These germ tubes grow by tip extension and branching and form a network of hyphal filaments which grow into and across the surface of an agar plate. This network is called the substrate mycelium (Hasani, et al., 2014). The next step is the differentiation of the mycelia. This is initiated by a

complex regulatory cascade in response to physiological stress, primarily nutrient depletion. The result is the formation of aerial hyphae and additionally the production of extracellular proteins (Chater, et al., 2010) and secondary metabolites to protect the nutrient source (Kämpfer, et al., 2014). The extending aerial hyphae are supported by valuable nutrients, after lysis in many compartments of the substrate mycelium and the programmed cell death. Then the sporulation begins. It starts with the condensation and multiple replication of the chromosome in the apical compartments of the extended aerial hyphae, which begin to form multiple septa. Furthermore, synchronous cell division occurs and leads to prespore compartments with a single copy of the chromosome. The walls of these compartments thicken and change shape. The produced spores serve for long term survival (will stay in a quiescent state) and to expand across habitats until suitable conditions for germination arise (Kämpfer, et al., 2014). Tens of millions of spores are produced from only few milligrams (wet weight) of the colony's substrate (Chater, et al., 2010).

3.2.3 Presence and role in natural ecosystems

Streptomycetes are widely distributed in different natural environments (Hasani, et al., 2014), however, their major natural habitat is soil. Therefore streptomycetes are highly abundant in it (Kämpfer, et al., 2014) and they make 40% of soil bacteria when estimated by cultivation methods (Hasani, et al., 2014). In this habitat they can show the distinct mycelial growth. In soil there are multiple and often rapidly changing physical conditions, such as frost, drought, shifts in aeration, hydrostatic pressure, moisture tension, pH, and anaerobic conditions. However, streptomycetes are adapted to them for example by formation of spores. Due to these semidormant stages in the life cycle, they are able to survive for long periods in soil under frequently changing conditions (Kämpfer, et al., 2014).

Streptomycetes are very important for the initial decomposition of different organic materials in terrestrial habitats using a big variety of different enzymes such as cellulases, xylanases and lignocellulases (Chater, et al., 2010). For instance, most streptomycetes can degrade complex and fractious animal and plant materials, which are usually polymeric residues such as proteins (e.g. elastin and keratin), polysaccharides (e.g. cellulose, chitin, pectin and starch), lignocellulose and aromatic compounds. Moreover, they have the ability of biodegradation of other natural organic materials, such as wool, cotton and plant fibers, but they can degrade also synthetic substances

like plastics, hydrocarbons in emulsions and jet fuel (Kämpfer, et al., 2014). One important nutrient source of streptomycetes is chitin, which is the second most abundant polysaccharide in nature. Unlike other bacteria, they can use it as carbon as well as nitrogen source. However, chitin is insoluble, therefore they developed a complex extracellular enzymatic system for utilization including specialized chitin-binding proteins, chitinases and chitosanases, which cleave the molecule. In addition to chitin, streptomycetes can also degrade the plant cell wall components cellulose (which is the most abundant polysaccharide on earth) and diverse xylans (1,4-linked xylose polymers). Both are targeted using extracellular enzyme machineries for the hydrolysis of the polymers. Due to the ability of degrading cellulose and xylans, they are symbionts (along with other microorganisms) in the gut of termites (Chater, et al., 2010).

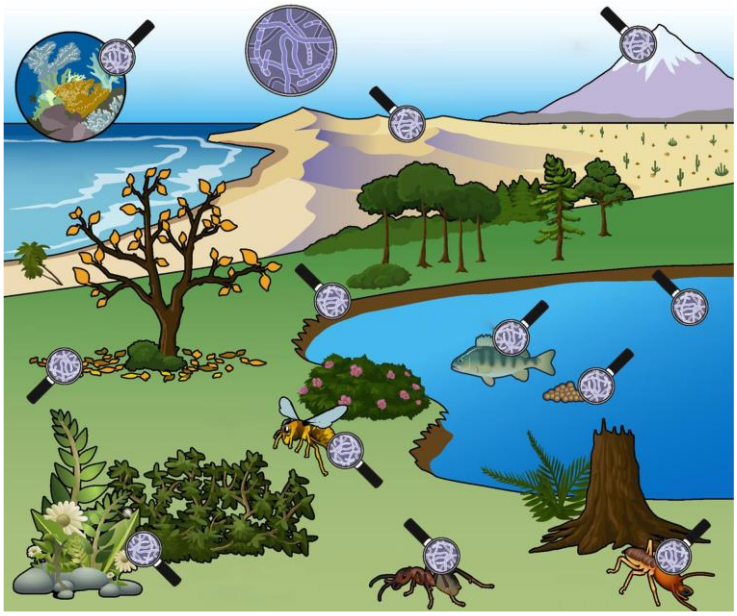

Figure 3. The most important habitat of free living actinomycetes is the soil where their role is the turnover of organic matter. However, they are also very common in marine and fresh water ecosystems. In addition, many actinomycetes live in symbiosis with other organisms such as plants, insects, fungi and animals. Theses symbionts benefit of secondary metabolites produced by the actinomycetes **(van der Meij, et al., 2017)**.

The production of the antibiotics and other secondary metabolites can also be an advantage for other organisms as it is the case in some symbiotic relationships of *Streptomyces* with other partner organisms (Figure 3). In these interactions, *Streptomyces* provides protection against infections of the partner caused by other microorganisms. For example, some streptomycetes produce antibiotics to protect plants against pathogens and the bacteria benefit from the plant exudates. Moreover, streptomycetes produce the antifungal compound candicidin for the leaf-

cutting ants so that the ants' fungus farms are not overgrown by fungi. Pine bark beetles obtain mycangimycin, also an antifungal agent, from their symbionts streptomycetes (Chater, et al., 2010). Furthermore, the microbiomes of marine organisms such as sponges, sea-cucumbers and seaweed contain also streptomycetes and some other actinomycetes (van der Meij, et al., 2017).

In contrast, *Streptomyces* is also known as a plant pathogen, for example *S. scabies* causes an economically significant disease which is known as the potato scab disease (Lambert & Loria, 1989). However, there are additional scab-causing species, for example *S. europaeiscabiei* and *S. stilliscabiei*, which are morphologically similar but differ genetically from *S. scabies*. Pathogenic isolates have in common the *txtAB* genes for biosynthesis of thaxtomins, which belong to phytotoxins. The symptoms of the common scab can range from brown spots on the surface up to dark pits that extend several millimeters into the tuber. These spots can be smaller or bigger so that they cover large areas. Due to this, the quality of the potatoes is much reduced and sometimes they can no longer be sold. Additionally to potatoes, common scab can also affect other tuber and root crops such as radish, beet, carrot, turnip and sweet potato (Wanner, 2006).

3.2.4 Presence and role in human microbiome

On the one hand, *Streptomyces* species have been isolated from various body parts of human without being the principle pathogen. Some examples are *S. violaceoruber*, *S. coelicolor*, and *S. albus* isolated from dental caries, blood, tonsils, skin, and sputum; *S. candidus* from the purulent exudate of a fractured patella and others from pus, liver abscess (Dunne, et al., 1998). Because they have been considered as commensals or random colonizers, the role of *Streptomyces* in mixed infections and hence the clinical importance of streptomycetes remained unclear (Dunne, et al., 1998). On the other hand, there are also few human pathogens known, *S. somaliensis* and *S. sudanensis*. Both are a significant cause of actinomycetoma in central Africa (Kirby, et al., 2012; Quintana, et al., 2008). Actinomycetoma is a severe and debilitating infection which can affect deep tissues and spread also to bones. The infection can result in tissue destruction and deformity and without surgical interventions it may be fatal (Kirby, et al., 2012) Very occasionally, other strains, for example *S. albus*, have been isolated from human actinomycetoma (Martín, et al., 2004). Furthermore, isolations of aerobic actinomycetes, mainly from sputum and wound, identified *S. griseus* as one of the four most frequent species occurring in 7.7 % of the samples.

The authors emphasized, that this species may represent potential pathogens, especially as primary pulmonary pathogens as *S. griseus* had not been reported to cause pulmonary infections before (McNeil, et al., 1990).

3.3 Interaction of streptomycetes with human pathogens

3.3.1 Production of secondary metabolites

As already stated above, streptomycetes produce a wide range of secondary metabolites, which are small molecules (between 100 and 3,000 Da) that are biologically active outside of its producer. There are at least 7,000 different secondary metabolites known which originate from *Streptomyces* isolates. Some well-known examples are antibiotics, fungicides and modulators of the immune response. Numerous gene clusters for the production of secondary metabolites are strain specific and some pathways are irregularly distributed over manifold of streptomycetes. Due to this, it is suggested that lateral gene transfer was involved to establish the secondary metabolic profile of streptomycetes. The genes for this bioactive compounds may be transferred by large linear plasmids which are common in streptomycetes. Additionally, there are examples known which show that these large linear plasmids are easily integrated into linear chromosomes (Chater, et al., 2010) or that big parts of the chromosome can be frequently rearranged (Birch, et al., 1990).

The production of antibiotics in streptomycetes is often species (or even strain) specific and helps them in the competition against other microorganisms, for example for accumulation of primary biomass. However, the synthesis of this secondary metabolites is usually activated after the outgrowth of the substrate mycelium and hence after accumulation of primary biomass as already stated above. Therefore, some antibiotics may increase the ability to defend against overgrowth by other microorganisms. One example of them is methylenomycin which inhibits the aerial growth more than the vegetative growth of sensitive streptomycetes (Chater, et al., 2010). To explain it in more details, there are two major types how secondary metabolites produced by actinomycetes act. The first type consists of metabolites acting synergistically against a target microorganism. Therefore, the antibiotic activity of different synergistic metabolites is bigger in combination than the sum of the individual activities. The second type of secondary metabolites

act contingently to overcome competition for nutrients with other microorganisms (Challis & Hopwood, 2003).

The discovery and production of microbial and fungal antibiotics changed medicine radically as these compounds are responsible for the successful treatment of a lot of different infectious diseases. Additionally, other applications for microbial compounds have been found which include not only human and animal therapy but also agriculture. Moreover, compounds of microbial origin are used for many non-infectious diseases (including for example cancer and heart disease). A very important role is the use in prophylaxis as well as in immunosuppression prior to invasive surgery (this lead to the development and success of organ transplantation). However, today the treatment of infectious diseases gets more and more difficult, because microbes acquire and spread the resistance against commonly used antibiotics by mutation or horizontal gene transfer (Miao & Davies, 2010).

The result of the increased resistance is that people are dying of infections which were previously treatable. Therefore, the search for novel therapeutic agents has to be intensified. Also today new compounds can be found, as only easily accessible compounds were found before. Moreover, only a small number of the bioactive molecules of *Actinobacteria* have been tested for therapeutic purposes. It is now known from genome sequencing that most *Streptomyces* spp. have the ability to produce many more structurally different bioactive compounds than it was suspected before (Miao & Davies, 2010). Many biosynthetic gene clusters are silent under standard laboratory growth conditions, hence there are many different approaches to activate them and to identify new secondary metabolites. A long known traditional approach is for example the co-cultivation of different microorganisms which should mimic the natural environment of the bacteria, their competition and communication. Another approach is the manipulation of the fermentation conditions (media composition, pH, temperature, rare elements, modulation of osmotic/salinity/ pressure, etc.) which is also known as “one strain many compounds” or short OSMAC approach. Alternative approaches are the usage of so-called “elicitors” which are external signals that challenge the bacterial cell to produce secondary metabolites or the genetic manipulation of the producing strain (Abdelmohsen, et al., 2015).

3.4 Hemolysis and hemolysins as virulence factors

3.4.1 General aspects and definitions

Hemoglobin is a protein in the blood which functions as oxygen (O_2) carrier from the lungs to the peripheral tissues and transports carbon dioxide (CO_2) back to the lungs. It consists of α-like and β- like globin chains which incorporate heme that also stabilizes the folding (Thom, et al., 2013). In general, heme proteins use the same cofactor Fe-protoporphyrin IX. However, they have variety of different functions in biological systems besides oxygen transport, such as electron transfer, catalysis and signaling. This is possible as the interactions between the cofactor and the polypeptide chains of the proteins can differ a lot (Lin, 2015).

Growing bacteria on blood agar (for example agar with defibrinated sheep blood) leads in case of many pathogenic bacteria to blood (hemoglobin) degradation and thus zones around the colonies. This is called hemolysis, where three main types of responses can be recognized. In beta-hemolysis, the blood is completely degraded and a clear zone arises. In comparison, alpha-hemolysis is only incomplete degradation of hemoglobin and results in a green zone surrounding the colony. The third type is gamma hemolysis which is also called non-hemolytic activity because these colonies do not show a zone around them (Ryan, et al., 2004).

Hemolysis can be induced by hemolysins as these proteins cause cellular lysis by targeting the membranes of cells and result in breakdown of blood cells. Therefore, hemolysins are one of the main virulence factors in pathogenic bacteria (Rajesh, et al., 2013). However, hemolysis can also be caused by other molecules. In particular, polyene secondary metabolites, which are mainly synthesized by streptomycetes and include important antifungal drugs along with some additional activities against parasites, enveloped viruses and pathogenic prions. They exert their antibiotic activity from the interactions with sterols within the membrane. They have an especially high affinity for ergosterol. At the same time, polyene antibiotics show serious side effects such as nephrotoxicity in human (Caffrey, et al., 2016). Examples are: (I) filipins, which lead to the formation of large aggregates within the erythrocyte membrane and thus to perforations that are permeable for hemoglobin; (II) amphotericin B and nystatin, which form smaller aggregates within the membranes, permeable for molecules of low weight (Knopik-Skrocka & Bielawski, 2002); (III) candicidin; (IV) pimaricin (Caffrey, et al., 2016).

3.4.2 Hemolysins in streptomycetes

Streptomyces coelicolor is a well-known secondary metabolite producing species isolated from the soil and it is not pathogenic. However, *S. coelicolor* is growing on blood agar and produces a hemolytic zone similar to beta-hemolysis shown by other pathogenic bacteria. The responsible molecule is *Streptomyces* hemolysin (S-hemolysin). The recombinant S-hemolysin showed in other catalytic tests also activities against human fibroblast cells (100 % cytolysis). One of the responsible genes for the hemolytic activity and production of the S-hemolysin is SCO1782 in *S. coelicolor*. This gene shows high sequence similarity (62 %) toward hemolysins from *Mycobacterium tuberculosis*. The deletion of the gene and therefore absence of S-hemolysin also affected the production of antibiotics and formation of spores. Other hemolysin-coding genes are SCO2534, SCO3882 (Rajesh, et al., 2013) and SCO4978. All genes are located on the genome of both *S. coelicolor* A3(2) as well as *Streptomyces* sp. TR42 (genome data not available yet) (Petříčková, et al., in prep.).

3.5 Aim of the thesis

Streptomycetes (common colonizers of human bodies) are probably able to control the microbiome due to their secondary metabolites. Because of this, the following hypotheses will be tested in this thesis: (I) do clinical and environmental streptomycetes vary in frequency of beta-hemolytic activity; (II) do they affect the behavior of model pathogens; (III) do beta-hemolytic strains possess the homologous genes responsible for the production of different types of hemolysins?

For this, a set of environmental and clinical streptomycete strains (maintained in the laboratory of supervisor) were used:

(a) To estimate and compare the frequency of beta-hemolysis in environmental and clinical streptomycete strains,

(b) To describe the response of streptomycetes phenotype in presence of potential human pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* (beta-hemolysis, growth, sporulation, etc.),

(c) To test the behavior of the pathogens (beta-hemolysis, growth, motility, etc.) in co-cultures of *P. aeruginosa* or *S. aureus* with streptomycete strains,

(d) To evaluate the presence of genes encoding different types of hemolysins (SCO1782, SCO2534, SCO3882, and SCO4978) in the genome of selected beta-hemolytic strains.

4 Materials and Methods

4.1 Cultivation

4.1.1 Cultivation media

MS agar:

The components from Table 1 were dissolved in 1 L tap water, and afterwards, the medium was sterilized using the autoclave (15 minutes at 120 °C).

Table 1. MS agar: Components and their amounts

Compound	Amount
Soya flour	20 g
D-Mannitol	20 g
Agar bacteriological no. 1 (Oxoid)	20 g

Blood agar:

KA Mueller-Hinton medium (KMH, Cat. No. 202, Dulab, spol. s. r. o.) with composition shown in Table 2 was used. The pH after dissolving the components in 1 L tap water is 7,3 (Mueller & Hinton, 1941).

Table 2. KMH agar (blood agar): Components and their amounts

Compound	Amount
Beef extract	4 g
Casein hydrolysate	17,5 g
Starch	1,5 g
Agar	12 g
Sheep erythrocytes (defibrinated blood)	5%

Solutions:

Glycerol 20% (v/v) in distilled water – sterilized by autoclaving (15 minutes at 120 °C)

4.1.2 Bacteria

4.1.2.1 Streptomyces

All streptomycete strains were provided from BCCO (Biology Centre Collection of Organisms) collection (available at: www.actinomycetes.cz). The environmental strains were isolated from different soils across the world, however, the majority of the clinical strains were isolated from decontaminated sputum (low respiratory tract). Table 3 shows all used *Streptomyces* strains and the corresponding performed tests. More strains were used to estimate the frequency of beta-hemolytic activity (data not shown). From those, most of beta-hemolytic strains were selected for the co-cultivation experiments with *Staphylococcus aureus* (STAU) and *Pseudomonas aeruginosa* (PSAE).

Table 3. Used *Streptomyces* strains with their origin, activity and corresponding performed tests (PCR corresponds to the test for hemolysin genes).

Strain No. BCCO 10	origin	activity	PSAE T-test	PSAE parallel	STAU T-test	STAU parallel	PCR
2	environmental	beta			x	x	x
6	environmental	beta	x	x		x	x
64	environmental	beta			x	x	x
68	environmental	beta	x		x	x	
70	environmental	beta		x		x	
72	environmental	beta	x	x	x	x	x
74	environmental	beta			x	x	x
75	environmental	alpha					x
121	environmental	beta			x	x	
122	environmental	beta		x	x	x	
125	environmental	beta			x	x	
126	environmental	beta		x	x	x	x
127	environmental	beta	x	x		x	
157	environmental	beta			x	x	x
162	environmental	beta			x	x	x
165	environmental	beta		x	x	x	x
166	environmental	beta		x	x	x	x
168	environmental	beta	x	x	x	x	x
169	environmental	beta			x	x	x
170	environmental	beta			x	x	x
171	environmental	beta			x	x	
470	environmental	beta			x	x	
474	environmental	beta	x	x	x	x	
485	environmental	beta			x	x	

489	environmental	beta			x	x	
490	environmental	beta			x	x	x
491	environmental	beta			x	x	x
492	environmental	beta	x	x	x	x	
493	environmental	beta			x	x	x
494	environmental	beta			x	x	
495	environmental	beta	x	x	x		
497	environmental	beta	x	x	x	x	x
517	environmental	beta	x	x	x	x	x
519	environmental	beta			x	x	x
524	environmental	beta			x	x	x
525	environmental	beta	x	x	x	x	x
526	environmental	beta	x	x	x	x	x
668	environmental	beta	x	x	x	x	x
669	environmental	beta	x	x	x	x	x
670	environmental	beta	x	x	x	x	x
672	environmental	beta	x	x	x	x	x
673	environmental	beta	x	x	x	x	x
675	environmental	beta	x	x	x	x	x
1499	environmental	beta	x	x	x	x	x
1500	environmental	beta	x		x	x	x
1503	environmental	beta	x	x	x	x	x
1508	environmental	beta	x	x	x	x	
1519	environmental	beta					x
1539	environmental	beta			x		x
1541	environmental	beta			x	x	x
1543	environmental	beta	x	x	x	x	x
1547	environmental	beta			x	x	x
1552	environmental	beta	x		x	x	x
1554	environmental	beta			x	x	
1555	environmental	beta			x	x	
1556	environmental	beta		x	x	x	
1557	environmental	beta			x	x	
1559	environmental	beta			x	x	
1560	environmental	beta			x	x	x
1561	environmental	beta		x	x	x	
1589	environmental	beta	x	x	x	x	x
1594	environmental	beta	x	x	x	x	x
1597	environmental	beta	x	x	x	x	
1598	environmental	beta	x	x	x	x	x
1603	environmental	beta			x	x	
1604	environmental	beta			x	x	
1605	environmental	beta			x	x	
1606	environmental	beta		x	x	x	

1607	environmental	beta	x	x	x	x	x
1608	environmental	beta			x	x	x
1615	environmental	beta			x	x	
1616	environmental	beta			x	x	x
1618	environmental	beta					x
1619	environmental	beta			x	x	
1622	environmental	beta		x	x	x	
1627	environmental	beta					x
1632	environmental	beta	x	x	x	x	x
1633	environmental	beta	x	x	x	x	x
1638	environmental	beta	x	x	x	x	x
1639	environmental	beta	x	x	x	x	x
1653	environmental	beta	x	x	x	x	x
1746	environmental	beta		x	x	x	
1747	environmental	beta			x	x	x
1748	environmental	beta			x	x	x
1749	environmental	beta			x	x	x
1750	environmental	beta			x		
1752	environmental	beta			x	x	x
2135	environmental	beta	x	x	x	x	
2152	environmental	beta	x	x	x	x	
2153	environmental	beta		x	x	x	
2154	environmental	beta	x	x	x	x	
2155	environmental	beta		x	x	x	
2156	environmental	beta	x	x	x	x	
2157	environmental	beta		x	x	x	
OS10141	clinical	beta	x	x	x	x	
OS17	clinical	beta	x	x	x	x	x
OS18	clinical	beta	x	x	x	x	x
OS19	clinical	beta	x	x	x	x	
OS20	clinical	beta	x	x	x	x	
OS21	clinical	beta	x	x	x	x	x
OS22	clinical	beta	x	x	x	x	
OS23	clinical	beta	x	x	x	x	
OS32	clinical	beta	x	x	x	x	x
OS33	clinical	beta	x	x	x	x	
OS3864	clinical	beta			x	x	x
OS4207	clinical	beta	x	x	x	x	
OS5666	clinical	beta			x	x	x
OS6180	clinical	beta			x	x	x
OS6215	clinical	beta			x	x	x
OS6643	clinical	beta			x	x	x
OS6672	clinical	beta			x	x	
OS6783	clinical	beta			x	x	x

OS6826	clinical	beta	x	x		x	x
OS6829	clinical	beta			x	x	
OS7188	clinical	beta			x	x	
OS8079	clinical	beta	x	x	x	x	
OS8305	clinical	beta	x	x	x	x	
OS8619	clinical	beta	x	x	x	x	
OS8917	clinical	beta	x	x	x	x	
TR01	clinical	beta	x	x	x	x	
TR02	clinical	beta	x	x	x	x	
TR03	clinical	beta	x	x	x	x	
TR07	clinical	beta	x	x	x	x	
TR08	clinical	beta	x	x	x	x	
TR09	clinical	beta	x	x	x	x	
TR11	clinical	beta	x	x	x	x	
TR12	clinical	beta	x	x	x	x	
TR15	clinical	beta	x	x	x	x	
TR16	clinical	beta	x	x	x	x	
TR17	clinical	beta	x	x	x	x	x
TR20	clinical	beta	x	x	x	x	
TR21	clinical	beta	x	x	x	x	x
TR24	clinical	beta	x	x	x	x	
TR25	clinical	beta	x	x	x	x	
TR28	clinical	beta	x	x	x	x	
TR29	clinical	beta	x	x	x	x	
TR31	clinical	beta			x	x	
TR33	clinical	beta	x	x	x	x	
TR34	clinical	beta	x	x	x	x	
TR35	clinical	beta	x	x	x	x	
TR37	clinical	beta	x	x	x	x	
TR38	clinical	beta	x	x	x	x	x
TR39	clinical	beta	x	x	x	x	
TR41	clinical	beta	x	x	x	x	
TR42	clinical	beta	x	x	x	x	x
TR43	clinical	beta		x	x	x	
TR44	clinical	beta	x	x	x	x	x
TR45	clinical	beta	x		x	x	
TR46	clinical	beta	x	x	x	x	x

4.1.2.2 Pathogens

Clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were used as opportunistic pathogens for the co-cultivation tests on the blood agar plates. Both strains were provided by Mgr. Kateřina Petříčková, PhD., from 1st Medical Faculty, Charles University in Prague.

S. aureus is a Gram-positive, round-shaped bacterium which does not sporulate. The yellow-pigmented bacterium is a facultative anaerobe and can therefore grow without the need of oxygen (Fuchs, 2014). They are part of the human microbiome. Especially it easily colonizes skin, which may result in life threatening infections such as serious wound, bloodstream, lung and catheter related infections (O'Gara, 2017). *S. aureus* can also be a pathogen causing food poisoning. The infections are often promoted by the bacteria using a lot of different virulence factors such as the production of hemolysins and heat-stable enterotoxins as well as biofilm formation. A worldwide problem are the antibiotic resistant strains such as the methicillin-resistant *S. aureus* (MRSA) (Fuchs, 2014).

P. aeruginosa is a Gram-negative, rod-shaped bacterium which is very abundant in soil and water. However, they are also very important opportunistic pathogens causing infections mainly in immunodeficient patients. *P. aeruginosa* can also show resistance against a range of different antibiotics (Fuchs, 2014).

4.1.3 Co-cultivation

All cultivation processes were performed in a laminar box. A sterile inoculation loop or disposal laboratory aids were used for re-inoculating the individual bacteria. Mueller-Hinton agar with 5% defibrinated sheep blood (Dulab, s. r. o., České Budějovice) was used for all cultivation experiments.

4.1.3.1 Determination of hemolytic properties

The goal was to get individual colonies to determine the hemolytic properties of the streptomycete strains. Therefore, the respective streptomycete was inoculated on the blood agar plate using the streak-plate technique where the sample is gradually diluted. Then the bacteria were cultivated at

28 °C. After 3-4 days, the colonies were observed and the growth and hemolytic activity were recoded.

4.1.3.2 Co-cultivation experiments

All co-cultivation experiments were performed with both, *S. aureus* and *P. aeruginosa*. Fresh overnight cultures of both strains grown on blood agar plates at 37 °C have been used for all co-cultivation experiments.

Both parallel tests (separated cultivation and parallel co-cultivation with one of the pathogens) were always performed on the same day and cultivated the same time to get results which can be well compared.

<u>Parallel test: on separated plates ("separated cultivation")</u>

The separated cultivation was performed on blood agar plates which were separated into two halves by a physical barrier. These plates were marked with two lines, one in each half, parallel to the boundary (Figure 4A). The lines were 2 cm apart from each other and each of them were 1 cm apart from the boundary. Then, the selected *Streptomyces* strain was inoculated as straight line on one side (on the line) of the Petri-dish with an inoculation loop and the pathogen was inoculated on the other side. After cultivation for 2-3 days at 28 °C, the growth, hemolytic activity and possible interaction were evaluated for all performed experiments.

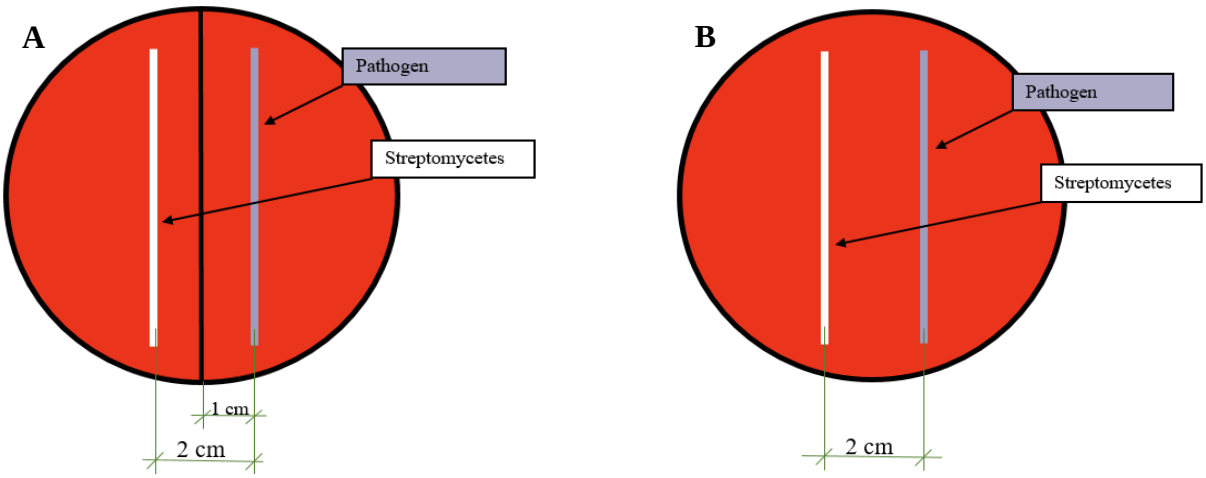

Figure 4. A: Schematic drawing of separated cultivation (on separated plates). **B:** Schematic drawing of parallel co-cultivation (on not separated plates).

Parallel test: on not separated plates (“parallel co-cultivation”)

The parallel co-cultivation was performed on normal blood agar plates. As before, the plates were marked with two parallel lines which were 2 cm apart from each other (Figure 4B). Then, the selected *Streptomyces* strain was inoculated as straight line (on one line) with an inoculation loop and the pathogen was inoculated on the other one. After cultivation for 2-3 days at 28 °C, the growth, hemolytic activity and possible interaction were evaluated for all performed experiments.

Crossed test (“T-type co-cultivation”)

In the T-type co-cultivation first, the selected *Streptomyces* strain was inoculated as straight line in the middle of the blood agar plate with an inoculation loop. Then they were cultivated at 28 °C. After 2-3 days, the respective pathogenic bacteria was added by inoculating it also as straight line but perpendicular to the *Streptomyces* (Figure 5). After cultivation for 2-3 days at 28 °C, the growth, hemolytic activity and possible interaction were evaluated for all performed experiments.

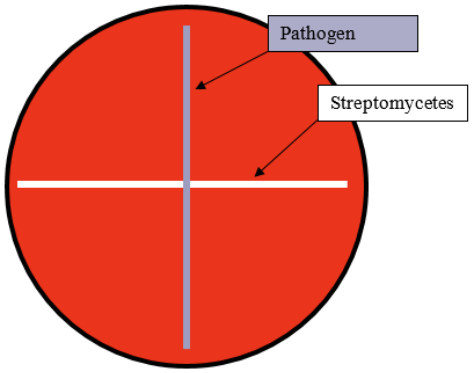

Figure 5. Schematic drawing of T-type co-cultivation

4.2 DNA extraction and PCR of hemolysin genes

About 100 *Streptomyces* strains (Table 3) were chosen to be tested for 4 different hemolysin genes using the following procedure.

4.2.1 DNA extraction

Most of the DNA have already been extracted. However, the Wizard Genomic DNA purification kit with modified protocol was used for the rest as following.

On the first day, the liquid cell culture was transferred to an Eppendorf tube and centrifuged for 3 minutes 13200 rpm at room temperature. The supernatant was discarded using a pipette and then 480 µL 50 mM EDTA were added to re-suspend the pellet. Depending on the amount of biomass, about 20 µL lysozyme were added to get a concentration of about 100 mg/mL. After incubation for 1-3 h at 37 °C (the lysed cells have gel/slime like consistence), the sample was centrifuged for 5 minutes at 12000 rpm at room temperature. Then the supernatant was discarded (the sample can be stored at -20 °C).

On the second day, 600 µL Nuclei lysis solution were added to the sample and mixed using a pipette. Then it was incubated for 5 min at 80 °C and for 5 min on ice. Afterwards, 3 µL RNase solution were added and the sample was briefly vortexed. After incubation for 3 h at 37 °C, 250 µL Protein Precipitation solution were added and vortexed for 20 seconds. Then, the sample was incubated for 5 min on ice and centrifuged for 14 min at 16000 rpm at 4 °C. The supernatant (about 500-700 µL) were transferred to a new 1.5 mL Eppendorf tube. Isopropanol was added to get a 1:1 (supernatant:isopropanol) ratio and the mixture was shaken gently. After incubation for 10 min at room temperature the sample was centrifuged for 14 min at 16000 rpm at 4 °C. Then, the supernatant was discarded and the pellet washed using 600 µL 70 % ethanol and gently mixed. Afterwards a centrifugation step was performed for 5 min at 16000 rpm at 4 °C and the supernatant discarded. Then again a centrifugation step was performed for 1 min at 16000 rpm at 4 °C and the supernatant was discarded. After evaporation of the ethanol, the pellet was re-suspended in 60 µL DNA rehydration solution and the genomic DNA was stored at 4 °C.

4.2.2 Determination of DNA concentration and dilution

The DNA concentration in the suspension was determined using NanoDrop 1000 instrument and then it was diluted using DNA rehydration solution to a concentration of about 150 – 250 ng/µL.

4.2.3 Quality check of the DNA using 16S rDNA PCR

The 16S rDNA PCR was performed to check the quality of the used streptomycete DNA. Only positive strains were used for further analysis of the presence of hemolysin genes. The primers for the 16S rDNA PCR (Table 5) were designed according to (Edwards, et al., 1989). The Master Mix for one reaction was prepared according to Table 4 (final volume of 25 µL per reaction).

Table 4. Master Mix for one reaction of 16S rDNA PCR

Compound	**Volume**
MilliQ water	10 µL
pAf 10 µM (working solution)	0.75 µL
pHr 10 µM (working solution)	0.75 µL
Fast Start Master (Roche) 2x with a final concentration of 30 U/ml	12.5 µL
DNA solution	1 µL

Table 5. Primers sequences (5´-3´) for 16S rDNA PCR and their amplicon size

	Primer forward (pA)	**Primer reverse (pH)**	**amplicon size**
16S rDNA	5´-AGAGTTTGATCCTGG CTCAG-3´	5´-AAGGAGGTGATCCAGC CGCA-3´	cca 1,800 bp

Then, the PCR was performed using the program shown in Table 6. Additionally, to the samples, each PCR was performed using one non-template control (NC) and one positive control (PC).

Table 6. Thermal cycling used for amplification of 16S rDNA PCR

16S rDNA PCR	
95 °C for 3 min	
94 °C for 1 min	34 cycles
61 °C for 0.5 min	
72 °C for 1.5 min	
72 °C for 5 min	

A 1 % gel was prepared, and the electrophoresis was performed as following: First 1 g of agarose were weighed into a 250 mL Erlenmeyer flask and 100 mL 1x TEA buffer were added. Then it was dissolved by mixing and boiling, then cooled down in a bath and poured into the form and solidified. The PCR products were loaded using a 6x Loading Dye (5 µL sample were mixed with 1 µL dye) and additionally 5 µL Gene Ruler Mix (both ThermoFisher Scientific) were loaded in separate pockets on the gel. The electrophoresis was run at 110 V and 265 mV for about 40 minutes. Then it was stopped and the agarose gel was stained by using and ethidium bromide bath, where the gel stained for 30 to 40 minutes. The bands were visualized using UV light.

4.2.4 PCR screening for presence of streptomycete hemolysin genes

The primers for the 4 hemolysin genes were designed using the genomic data of *Streptomyces* sp. strain TR42 in the laboratory of the cooperating group (Mgr. K. Petříčková, PhD., 1st Medical Faculty, Charles University in Prague). It was not known, which annealing temperature works best for these primers in case of other strains. Hence, first the PCR for each gene of the strain TR42 was performed using different annealing temperatures. The presented PCR protocol (Table 9) represents the conditions and thermal cycling for the final screening of the samples.
First, the Master Mixes were prepared (Table 7) using the primers shown in Table 8 and for each reaction 1 µL DNA template was added to get a final volume of 25 µL.

Table 7. Master Mixes for PCR of hemolysin genes. Coding for the reactions (4-numbered codes), corresponds to respective gene locus of both *S. coelicolor* A3(2) as well as *Streptomyces* sp. TR42

	Starting concentration	**PCR1 /1782**	**PCR2 /2534**	**PCR3 /3882**	**PCR4 /4978**
Milli Q water		10 µL	10 µL	10 µL	10 µL
Primer forward	10 µM	0.75 µL	0.75 µL	0.75 µL	0.75 µL
Primer reverse	10 µM	0.75 µL	0.75 µL	0.75 µL	0.75 µL
Fast Start PCR Master	5000 U/mL	12.5 µL	12.5 µL	12.5 µL	12.5 µL

Table 8. Primer sequence (5´-3´) for the different hemolysin genes and their amplicon size

	Primer forward	**Primer reverse**	**amplicon size**
PCR1/ 1782	5´-TCTAGAAGGAGCTGAA CCGAACGTG-3´	5´-ATGCATGGGCCAGCAGG AAAACAGTA-3´	870 bp
PCR2/ 2534	5´-TCTAGACCGACCGTCT CGTAAGCC-3´	5´-ATGCATTGAACGGGAAG TCCTCCAC-3´	1374 bp

PCR3/ 3882	5´-TCTAGATGGATGCCGC CCTACAGC-3´	5´-ATGCATAAGAGGCTGGC AATCGTGTC-3´	441 bp
PCR4/ 4978	5´-TCTAGACCCCTCACAT CCGATTTAC-3´	5´-ATGCATCAATTGGTGG GAGCTTGC-3´	834 bp

The PCR were performed using the programs shown in Table 9. Each PCR was performed using additionally a positive control (PC; using gDNA of strain TR42) and a non-template control (NC).

Table 9. Thermal cycling used for amplification of hemolysin genes

PCR1/1782		**PCR2/2534**		**PCR3/3882**		**PCR4/4978**	
95 °C for 3 min		95 °C for 3 min		95 °C for 3 min		95 °C for 3 min	
95 °C for 1 min	34 cycles	95 °C for 1 min	34 cycles	95 °C for 1 min	34 cycles	95 °C for 1 min	34 cycles
54 °C for 1 min		54 °C for 1 min		55 °C for 1 min		52 °C for 1 min	
72 °C for 1 min		72 °C for 1 min		72 °C for 1 min		72 °C for 1 min	
72 °C for 5 min		72 °C for 5 min		72 °C for 5 min		72 °C for 5 min	

The electrophoresis was prepared and performed as described above (4.2.3 Quality check of the DNA using 16S rDNA PCR).

4.3 Statistics

The results are shown as means ± SEM (for the indicated number of experiments). For statistical comparison, the two-tailed Student's t-test was used. In case of comparison of two parallel tests, the paired Student's t-test was applied; otherwise unpaired t-test was used. However, $p<0.05$ was considered to be significant in all performed tests.

5 Results

5.1 Co-cultivation

5.1.1 Frequency of beta-hemolytic activity in *Streptomyces*

Altogether, 249 *Streptomyces* strains were tested for their hemolytic activities, with about one third of clinical origin and the rest isolated from the environment. The screening resulted in 59.0 % of all strains showing beta-hemolytic activity. Interestingly, the frequency of beta-hemolytic activity was higher in clinical strains as there three quarters (75.6 %) of all clinical isolates showed this property then in environmental strains, where about half (51.5 %) of all isolates showed beta-hemolysis.

5.1.2 Co-cultivation with *Pseudomonas aeruginosa*

The co-cultivation experiments were performed as described above in part 4 Methods (4.1.3.2). After cultivation with both, *Streptomyces* (STR) and *P. aeruginosa* (PSAE), their growth width and zone of beta-hemolytic activity were measured with a ruler. These measurements were transformed into categories for better comparison and evaluation (Table 10). The categories of *Streptomyces* and *P. aeruginosa* differ because PSAE shows generally much smaller zones. Additionally, the sporulation of *Streptomyces* was examined.

Table 10. Transformation of *Streptomyces* (STR) and *P. aeruginosa* (PSAE) beta-hemolytic activity and growth width into different categories

category	STR beta-hemolytic activity / cm		PSAE beta-hemolytic activity / cm		PSAE growth width / cm
1	0.00 – 0.10	Note: in case of double zones only higher value was used for category	0.00 – 0.05	Note: in case of double zones only smaller value was used for category	0.00 – 0.40
2	0.11 – 0.30		0.06 – 0.10		0.41 – 0.50
3	0.31 – 0.50		0.11 – 0.20		0.51 – 0.60
4	0.51 – 0.70		0.21 – 0.30		0.61 – 0.70
5	0.71 – 1.00		0.31 – 0.40		0.71 – 0.80
6	1.01 – 1.30		> 0.40		> 0.80
7	> 1.30				

5.1.2.1 Parallel tests (separated cultivation and parallel co-cultivation)

The parallel tests were performed to compare the beta-hemolytic activities of both *Streptomyces* and *P. aeruginosa* in separated cultivation (on separated plates) and parallel co-cultivation (on not separated plates). Moreover, the growth of *P. aeruginosa* as well as the sporulation of *Streptomyces* were compared in these tests.

The beta-hemolytic activity of *Streptomyces* (parallel tests with *P. aeruginosa*) gave different results in the separated cultivation and parallel co-cultivation in case of frequency of the categories (Figure 6A). In the presence of the pathogen a higher abundance of categories 2 and 3 was observed in comparison to the strong dominance of category 1 (with the smallest beta-hemolytic zone) when both strains were growing separately. This trend can be also seen with the mean value of beta-hemolytic activity categories, which was 1.57 and 2.20, in separate cultivation and parallel co-cultivation respectively (Figure 7A and Supplementary Figure 1: separated cultivation and parallel co-cultivation). Hence the presence of *P. aeruginosa* increases the beta-hemolytic activity of *Streptomyces* (t-test, $p<0.001$). Furthermore, the mean value of beta-hemolytic activity of environmental *Streptomyces* strains is higher than the clinical one and hence the zones of environmental isolates are bigger in both, separated cultivation and parallel co-cultivation (t-test, $p<0.001$ in both cases). Figure 6B (green column) shows that the category of beta-hemolytic activity in parallel tests increased by 1 in about half of the strains and in some even by 2.

In general, the sporulation of the *Streptomyces* strains did not change regardless of the presence of *P. aeruginosa* (Figure 6B: violet column). However, there are few strains where the sporulation activity increased when growing in parallel co-cultivation with the pathogen.

The beta-hemolytic activity of *P. aeruginosa* shows trends similar to those of *Streptomyces* (Figure 6C) as the frequency of categories with high beta-hemolytic activity of *P. aeruginosa* is much greater in parallel co-cultivation than in separated cultivation. This trend can also be seen in the mean values of categories of beta-hemolytic activity of *P. aeruginosa* which were 1.93 and 2.73, in separated cultivation and parallel co-cultivation respectively (Figure 8A). Because of this the presence of *Streptomyces* increased *P. aeruginosa* beta-hemolytic activity (t-test, $p<0.001$). More than half of the tests showed an increase of category in beta-hemolytic activity of the

pathogen by 1 or more, however, there were also some strains where the category decreased (Figure 6B: orange column). There was also a difference in beta-hemolytic activity of *P. aeruginosa* between parallel co-cultivation with environmental or clinical *Streptomyces* strains (Figure 8A: parallel co-cultivation; t-test, $p<0.001$). The co-cultivation of *P. aeruginosa* with clinical isolates lead to about 1.5 higher mean category sizes compared to those with environmental strains (2.04 and 3.48, respectively).

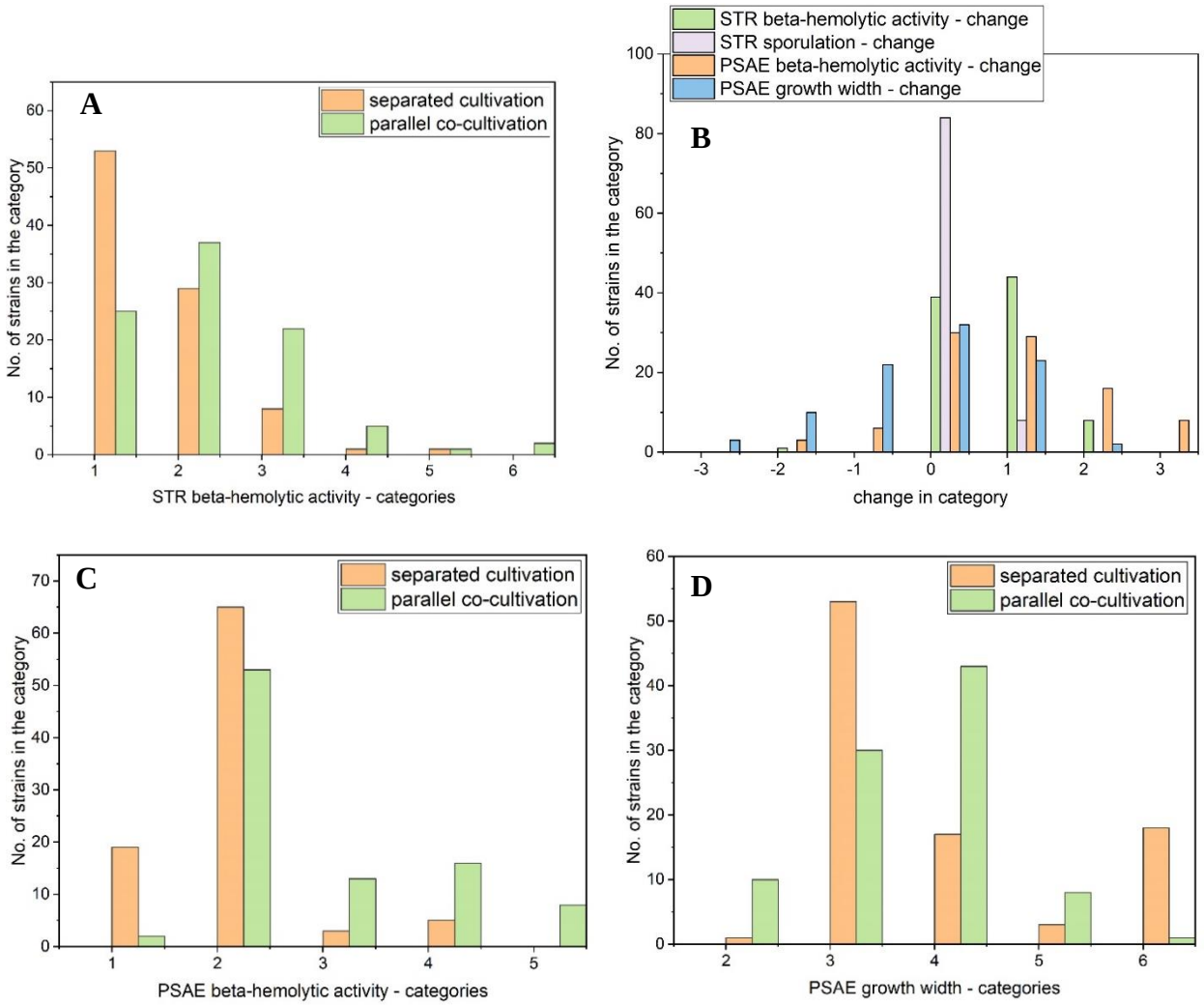

Figure 6. A: *Streptomyces* (STR) beta-hemolytic activity in separated cultivation and parallel co-cultivation with *P. aeruginosa* (PSAE). **B:** Category difference between separated cultivation and parallel co-cultivation experiment. **C-D:** *P. aeruginosa* beta-hemolytic activity (C) and growth width (D) in separated cultivation and parallel co-cultivation with *Streptomyces*.

The growth width of *P. aeruginosa* in co-cultivation with *Streptomyces* showed an opposite trend as the mean category value was higher in separated cultivation than in parallel co-cultivation, reaching 3.83 and 3.57, respectively (Figure 8B: blue column; t-test, $p<0.05$). The co-cultivation

with environmental *Streptomyces* strains lead to a higher growth width than with clinical isolates in both parallel tests (t-test, $p<0.001$ in separated cultivation, $p<0.05$ in parallel co-cultivation). The abundance of the growth width categories of *P. aeruginosa* is not normally distributed (Figure 6D). In separated cultivation the most abundant category was 3 followed by 6, and in parallel co-cultivation the most common category is 4 followed by 3 and 2. Additionally, there are about as many cases where the growth width category increased as decreased (Figure 6B). Hence the response of growth abilities of *P. aeruginosa* to the co-cultivation with streptomycetes was strain specific.

5.1.2.2 Crossed tests (T-type co-cultivation)

The T-type co-cultivations were performed to evaluate the growth pattern and beta-hemolytic activity of *Streptomyces* and *P. aeruginosa* in direct contact and to compare this results with parallel tests (separated cultivation and parallel co-cultivation) in which the bacteria were apart from each other.

Note for the graphs (Figure 7A and Figure 8A-B): in case of gradual activation/inhibitions of beta-hemolytic activities/growth width, the mean value of the respective property was used for each strain; activation and inhibition zones were not taken into account for the graphs as this data was collected and analyzed separately for each strain. Figure 7A and Figure 8A-B do not include standard deviations, therefore the Supplementary Figure 1, Supplementary Figure 2 and Supplementary Figure 3 were added.

The beta-hemolytic activity of *Streptomyces* in T-type co-cultivation is higher than in both parallel tests (separated cultivation and parallel co-cultivation; t-test, $p<0.001$ in both cases) (Figure 7A). Interestingly, there is a big difference between environmental and clinical strains in the T-type co-cultivation (t-test, $p<0.001$). The environmental strains have in average a zone of about 0.71-1.00 cm (the mean beta-hemolytic category was 5.32) and the clinical strains have in average a 0.6 cm smaller beta-hemolytic zone with the mean category equal to 2.23.

Additionally, some *Streptomyces* strains showed a gradual activation of beta-hemolytic activity near to *P. aeruginosa* as some zones were increasing from the edge to the contact with *P. aeruginosa* (Figure 7B). 27 (32.9 %) of the tested strains showed this behavior and the majority of them (26) were clinical isolates, which are over 50 % of all tested clinical *Streptomyces* strains.

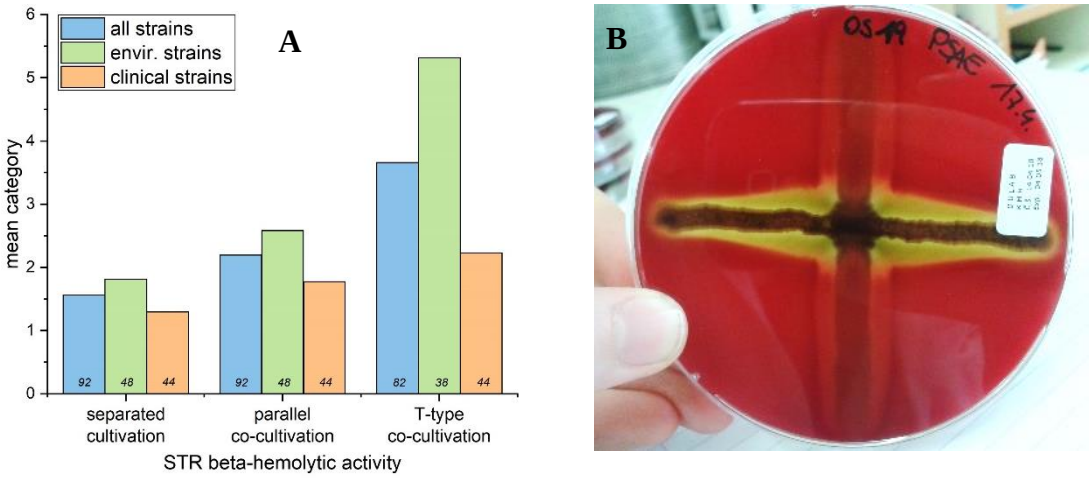

Figure 7. A: Beta-hemolytic activity of *Streptomyces* (STR) in T-type co-cultivation with *P. aeruginosa* (PSAE) - mean value of categories in parallel tests (separated cultivation and parallel co-cultivation) and T-type co-cultivation. The numbers inside the columns indicate the total number of evaluated tests. **B:** T-type co-cultivation of *Streptomyces* (horizontal) with *P. aeruginosa* (vertical) showing slightly activation of beta-hemolytic zone of *Streptomyces* (OS19).

Similarly, the beta-hemolytic activity of *P. aeruginosa* was greater in T-type co-cultivation than in parallel cultivation (separated cultivation and parallel co-cultivation) regardless of streptomycete origin (Figure 8A; t-test, $p<0.001$ in both cases). Clinical strains increased the beta-hemolytic activity of *P. aeruginosa* in T-type co-cultivation compared to separated cultivation, having mean values of 2.00 and 4.84 respectively. However, this trend was less pronounced in environmental strains (1.88 and 2.45, respectively) and hence the beta-hemolytic activity of *P. aeruginosa* in T-type co-cultivation with environmental or clinical strains differs a lot (t-test, $p<0.001$).

A slightly activation of *P. aeruginosa* beta-hemolytic activity (gradual increase) occurred next to *Streptomyces* in 46.7 % of T-type co-cultivation (Figure 8C). This happened mostly with isolates from the environment (31 of the total 38 cases). Furthermore, 2 strains (BCCO 10_number 1499, TR42 and hence one clinical and one environmental isolate) showed an activation zone of the beta-hemolytic activity of *P. aeruginosa* similarly to Figure 8D in T-type co-cultivation.

The growth of *P. aeruginosa* did not show such nice trend as its beta-hemolytic activity when comparing parallel and crossed cultivations (Figure 8B). Environmental strains decreased the growth width of *P. aeruginosa* in T-type co-cultivation compared to parallel co-cultivation (3.11 and 3.75, respectively; t-test, $p<0.05$). However, the clinical strains significantly activated the growth width of *P. aeruginosa* in the T-type co-cultivation when compared to the parallel co-cultivation (3.73 and 3.36, respectively; t-test, $p<0.05$). A very common property of *P. aeruginosa*

was its growth along the *Streptomyces* by activating the motility (Figure 8E). This behavior occurred in 85.4 % (70 cases) of the T-type co-cultivation experiments. However, the increase in motility differed: 22 of streptomycetes stimulated the motility of *P. aeruginosa* moderately (growth length along *Streptomyces* < 1 cm); whereas 3 environmental isolates stimulated *P. aeruginosa* motility severely (growth length > 3 cm). This property occurred equally in both, environmental and clinical isolates. A slightly inhibitory activity of streptomycetes (Figure 8F) was also observed in some T-type co-cultivations (9 cases and hence 11.0 %) as gradual decrease in growth width of *P. aeruginosa*.

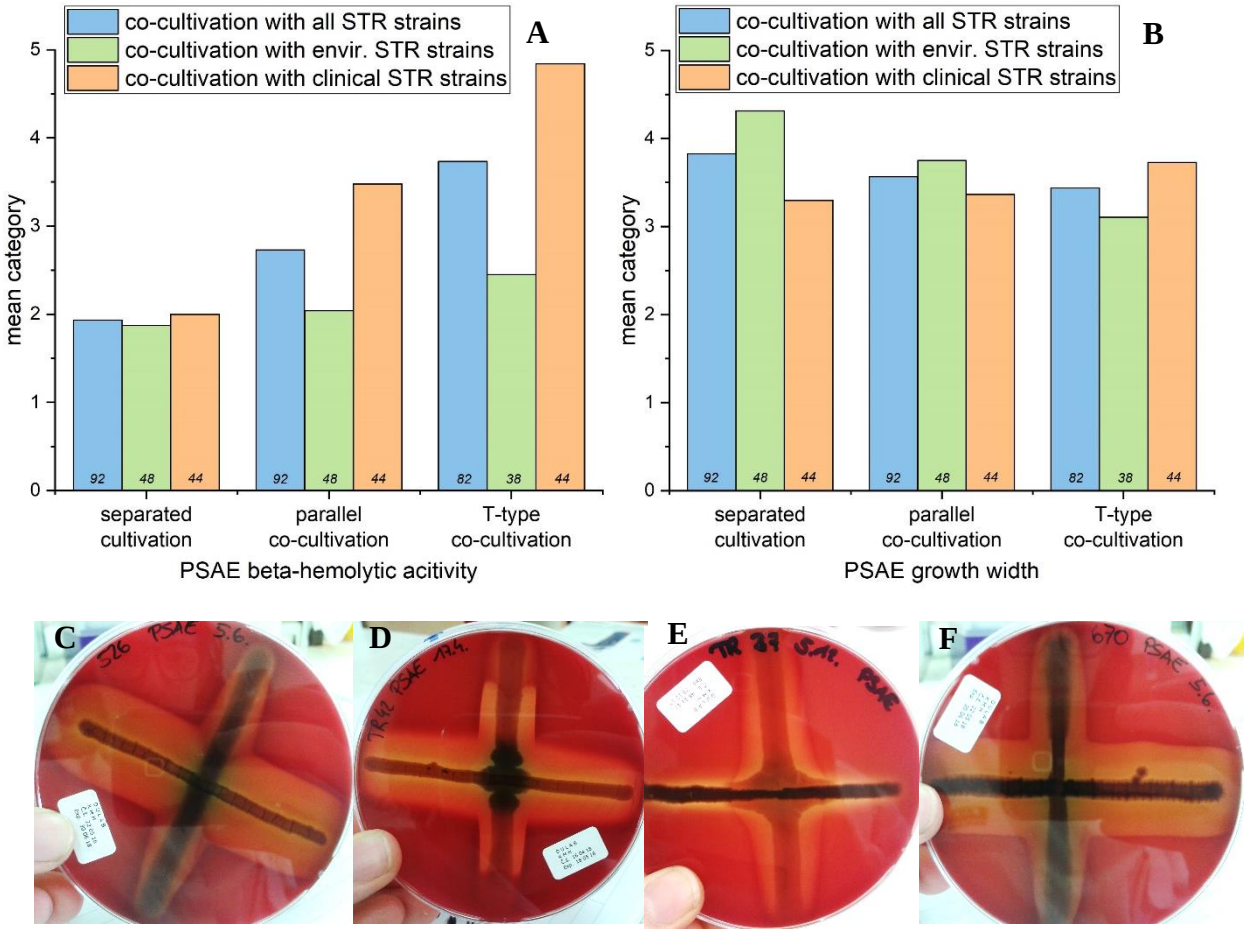

Figure 8. A-B: Beta-hemolytic activity (A) and growth width (B) of *P. aeruginosa* (PSAE) in co-cultivation with *Streptomyces* (STR) - mean value of categories in parallel tests (separated cultivation and parallel co-cultivation) and T-type co-cultivation. The numbers inside the columns indicate the total number of evaluated tests **C-F:** T-type co-cultivation of *Streptomyces* (horizontal) with *P. aeruginosa* (vertical). *P. aeruginosa* shows slightly activation of beta-hemolytic zone (C), activation zones (D), increase in motility (growth along *Streptomyces;* E) and slightly inhibition of growth (gradual decrease; F). C: 526. D:TR42. E: TR37. F: 670.

5.1.3 Co-cultivation with *Staphylococcus aureus*

The procedure of the co-cultivation experiments is shown in part 4 Methods (4.1.3.2). The evaluation included the measurement of growth width and zone of beta-hemolytic activity of *Streptomyces* (STR) and *S. aureus* (STAU) with a ruler. These measurements were transformed into categories for better comparison and evaluation (Table 11). The categories of *Streptomyces* and *S. aureus* differ because STAU shows smaller beta-hemolytic zones. Additionally, the sporulation of *Streptomyces* was examined.

Table 11. Transformation of *Streptomyces* (STR) and *S. aureus* (STAU) beta-hemolytic activity and growth width into different categories

category	STR beta-hemolytic activity / cm		STAU beta-hemolytic activity / cm		STAU growth width / cm
1	0.00 – 0.10	Note: in case of double zones only higher value was used for category	0.00 – 0.05	Note: in case of double zones only smaller value was used for category	0.00 – 0.20
2	0.11 – 0.30		0.06 – 0.10		0.21 – 0.30
3	0.31 – 0.50		0.11 – 0.20		0.31 – 0.40
4	0.51 – 0.70		0.21 – 0.30		0.41 – 0.50
5	0.71 – 1.00		0.31 – 0.40		0.51 – 0.60
6	1.01 – 1.30		> 0.40		> 0.60
7	>1.30				

5.1.3.1 Parallel tests (separated cultivation and parallel co-cultivation)

The parallel tests were performed to compare the beta-hemolytic activities of both *Streptomyces* and *S. aureus* in separated cultivation (on separated plates) and parallel co-cultivation (on not separated plates). Furthermore, these tests were used to compare the growth of *S. aureus* as well as the sporulation of *Streptomyces*.

The frequency of beta-hemolytic activity categories of *Streptomyces* shifted from the strong dominance of category 1 with the smallest zone in separated cultivation to higher abundance of categories 2 – 4 in parallel co-cultivation and hence larger zones (Figure 9A). Accordingly, the mean category value for separated cultivation was 1.73 and in parallel co-cultivation 2.09 (Figure 11). Because of this, the presence of *S. aureus* increases the beta-hemolytic activity of *Streptomyces* (t-test, $p<0.001$). Interestingly, the environmental streptomycetes show higher mean category value of their beta-hemolytic activity in both separated cultivation and parallel co-cultivation than clinical isolates (t-test, $p<0.001$ in both cases). One third of the streptomycete

strains increased the beta-hemolytic activity in presence of *S. aureus* (category change by 1) (Figure 9B: green column).

Similarly to the tests with *P. aeruginosa*, the sporulation of *Streptomyces* mostly did not change in presence of *S. aureus* (Figure 9B: violet column).

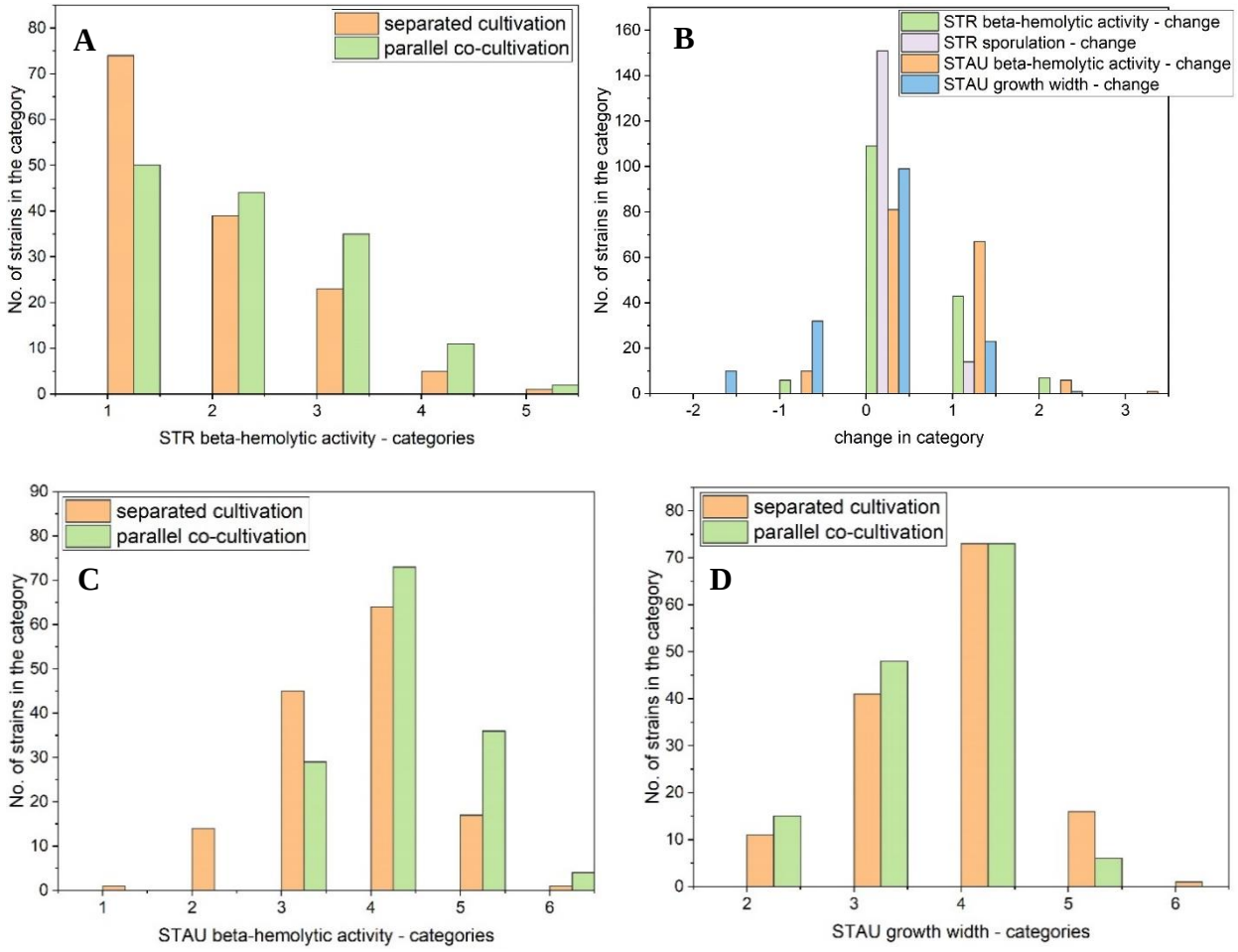

Figure 9. A: *Streptomyces* (STR) beta-hemolytic activity in separated cultivation and parallel co-cultivation with *S. aureus* (STAU). **B:** Category difference between separated cultivation and parallel co-cultivation experiments of *S. aureus* and *Streptomyces*. **C-D:** *S. aureus* beta-hemolytic activity (C) and growth width (D) in separated cultivation and parallel co-cultivation with *Streptomyces*.

The parallel co-cultivation of *Streptomyces* with *S. aureus* resulted in a shift of beta-hemolytic activity frequency of the pathogen towards higher zone sizes and hence categories 4 – 6 (Figure 9C). This effect can also be seen in the mean category value of beta-hemolytic activity of *S. aureus*, which is 3.60 in separated cultivation and 4.11 in parallel co-cultivation. Hence the presence of *Streptomyces* increases the beta-hemolytic activity of *S. aureus* (Figure 12A; t-test,

p<0.001). Nearly half of the strains increased their beta-hemolytic activity by 1 category (Figure 9B: orange column).

Interestingly, the parallel co-cultivation of streptomycete strains with *S. aureus* resulted in appearance of a double beta-hemolytic zone of the pathogen, which was observed in between primary zones of both strains. This zone seems to be related to the streptomycete metabolic activity (Figure 10). Altogether, 28 of the tested strains showed similar pattern of activation of beta-hemolytic zones as presented in Figure 10, most of them (20) were of environmental origin.

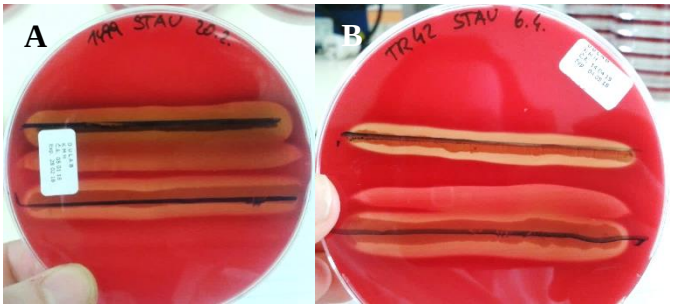

Figure 10. Activation of *S. aureus* (lower line) beta-hemolytic activity due to parallel co-cultivation with *Streptomyces* (upper line)

The growth width of *S. aureus* in co-cultivation with *Streptomyces* was in parallel-co-cultivation lower than in separated cultivation (Figure 9D) as the smaller categories are more common in parallel co-cultivation and the highest two categories are less abundant. The mean category follows this trend, with 3.68 in separated cultivation and 3.49 in parallel co-cultivation (Figure 12B). There is no difference in mean value of growth width with environmental and clinical streptomycetes in separated cultivation (t-test, p=0.73), but parallel co-cultivation with environmental strains resulted in a lower mean value than with clinical isolates (t-test, p<0.001). In about half of the tests, *S. aureus* changed the growth width in presence of *Streptomyces* (Figure 9B: blue columns) in both directions as there were increasing and decreasing categories.

5.1.3.2 Crossed tests (T-type co-cultivation)

The T-type co-cultivations were performed to evaluate the growth pattern and beta-hemolytic activity of *Streptomyces* and *S. aureus* in direct contact and to compare these results with parallel tests (separated cultivation and parallel co-cultivation) in which the bacteria were apart from each other.

Note for the graphs (Figure 11A and Figure 12A-B): in case of gradual activation/inhibitions of beta-hemolytic activities/growth width, the mean value of the respective property was used for each strain; activation and inhibition zones were not taken into account for the graphs as this data was collected and analyzed separately for each strain. Figure 11A and Figure 12A-B do not include standard deviations, therefore the Supplementary Figure 4, Supplementary Figure 5 and Supplementary Figure 6 were added.

The co-cultivation of streptomycetes with *S. aureus* resulted in a similar pattern as with *P. aeruginosa*. The T-type co-cultivation resulted in much higher beta-hemolytic activity of *Streptomyces* compared to parallel co-cultivation (Figure 11A; t-test, $p<0.001$), which suggested the activation of beta-hemolysis in the presence and especially in direct contact with the tested bacteria. The environmental *Streptomyces* showed larger beta-hemolytic zones than clinical isolates (with a mean values of 4.23 and 2.63, respectively; t-test, $p<0.001$). This trend was similar to the co-cultivation of streptomycetes with *P. aeruginosa*.

Additionally, some *Streptomyces* strains showed the activation of beta-hemolysis near *S. aureus* as some *Streptomyces* zones were gradually increasing from the edge to the close contact with *S. aureus* (Figure 11B). 15 (10.6 %) of the tested strains showed this behavior and nearly all of them (14) were clinical isolates.

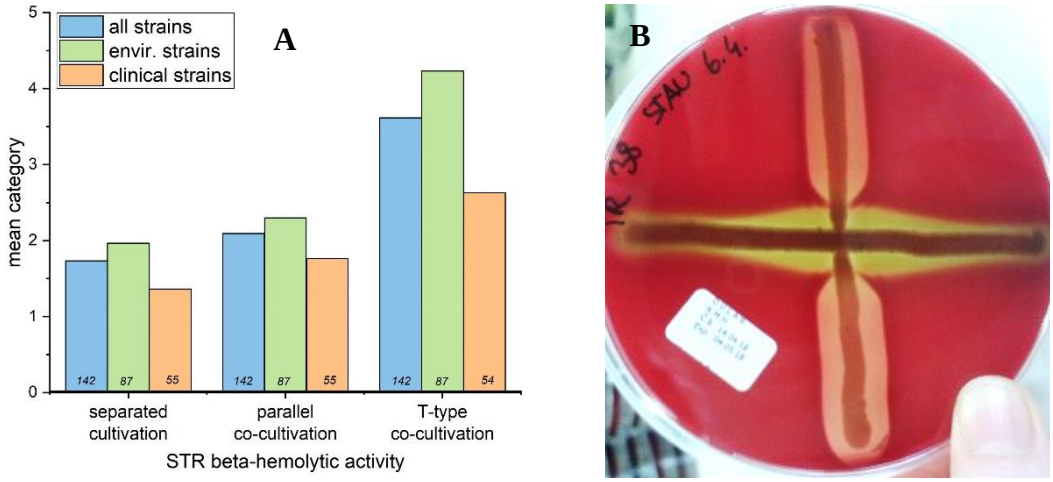

Figure 11. A: Beta-hemolytic activity of *Streptomyces* in co-cultivation with *S. aureus* - mean value of categories in parallel tests (separated cultivation and parallel co-cultivation) and T-type co-cultivation. The numbers inside the columns indicate the total number of tests. **B:** T-type co-cultivation of *Streptomyces* (horizontal) with *S. aureus* (vertical) showing slightly activation of beta-hemolytic zone of *Streptomyces* (TR38).

The beta-hemolytic activity of *S. aureus* in T-type co-cultivation with *Streptomyces* showed also similar results as the co-cultivation with *P. aeruginosa*. The close contact of the two bacteria resulted in larger zones compared to parallel co-cultivation (Figure 12A; t-test, $p<0.001$). Moreover, the co-cultivation with clinical streptomycete strains resulted in higher beta-hemolytic activity of *S. aureus* than with environmental strains (t-test, $p<0.001$) in T-type co-cultivation (similar to the parallel cultivations).

Additionally, various interactions were observed, for example a gradual decrease of the beta-hemolytic activity of *S. aureus* in close contact to *Streptomyces* similarly as in Figure 12C. This was mainly observed in co-cultivation with clinical isolates as 16 of the 17 strains (12.1 % of all) were of clinical origin. A gradual increase of the beta-hemolytic activity of *S. aureus* occurred also in 14.9 % of the cases (Figure 12D), but mainly with environmental strains (20 out of 21 strains were of environmental origin). Moreover, the activation of *S. aureus* beta-hemolysis by *Streptomyces* or maybe the synergy of beta-hemolytic molecules from both bacteria was observed quite rarely (5 environmental strains, Figure 12E-F). BCCO 10_numbers 1499, 1552 and 2155 were similar to Figure 12E and the numbers 1598 and 1615 looked similar to situation shown in Figure 12F.

The growth width of *S. aureus* decreased in comparison of T-type co-cultivation to parallel co-cultivation (Figure 12B, t-test, $p<0.001$) and the trend was similar when co-cultivated with environmental or clinical streptomycete strains. The growth width of *S. aureus* did not differ in T-type co-cultivation with environmental and clinical strains (t-test, $p=0.092$).

The additional growth behavior is more interesting as it varies a lot between the strains. The activation of motility of *S. aureus* and hence growth along environmental *Streptomyces* strains was observed in 12 cases (8.5 % of all and 13.8 % of environmental strains; Figure 12G). However, the motility was not as robust as in case of *P. aeruginosa*. Moreover, a partial inhibition (gradual decrease; Figure 12H) of the growth width of *S. aureus* was observed in case of 7 environmental *Streptomyces* strains (5.0 % of all and 8.0 % of environmental strains). Furthermore, weak (< 3 mm) or apparent (> 3 mm) growth inhibition of *S. aureus* occurred in about one quarter of all T-type co-cultivations (24.1 %, 34 cases; Figure 12I-J). Most of them (23 strains) inhibited the growth of *S. aureus* only weakly, by less than 3 mm apart from *Streptomyces*. However, 5 streptomycete strains inhibited the growth *S. aureus* much apparently, showing an

inhibition zone larger than 1 cm. Four of these strains (BCCO 10_numbers 491, 1543, 1606, 1632) were environmental isolates and one (TR42) was of clinical origin.

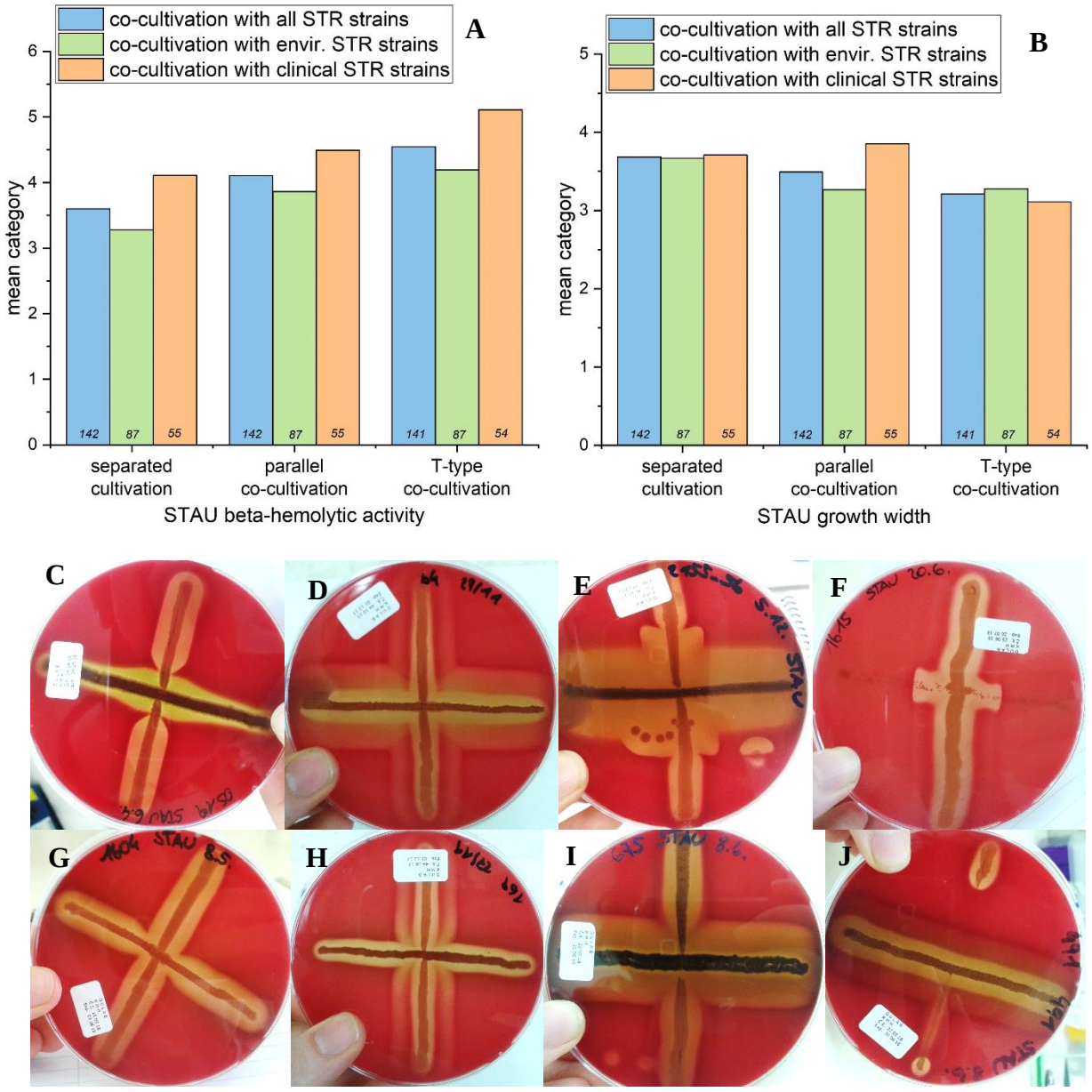

Figure 12. A-B: Beta-hemolytic activity (A) and growth width (B) of *S. aureus* (STAU) in co-cultivation with *Streptomyces* (STR) - mean value of categories in parallel tests (separated cultivation and parallel co-cultivation) and T-type co-cultivation. The numbers inside the columns indicate the total number of evaluated tests **C-J:** T-type co-cultivation of *Streptomyces* (horizontal) with *S. aureus* (vertical). *S. aureus* shows slightly inhibition of beta-hemolytic zone (A), slightly activation of zones (D), activation zones (E-F), increased motility (growth along *Streptomyces;* G), slightly inhibition of growth (gradually decrease; H) and inhibition of growth (I-J). C: OS19. D: 64. E: 2155. F: 1615. G: 1604. H: 169. I: 675. J: 491.

5.2 PCR screening for presence of streptomycete hemolysin genes

First, the quality of the streptomycete genomic DNA was evaluated by PCR amplification of 16S rDNA gene (amplicon size 1.8 kb). 73 different strains (Table 3: column "PCR") were tested, and just 7 of them gave negative results. This was considered as false negative as this gene is a house keeping gene for bacteria. The following strains had a negative result and therefore could not be used for further analysis: BCCO 10_numbers 1627, OS18, OS3864, OS5666, OS6783, TR17, TR21.

Secondly, the PCR for the 4 different hemolysin genes was performed with strain TR42 to test different annealing temperatures (Figure 13A). The amplification of the third gene (PCR3/3882) was unsuccessful and therefore not used for further screening. All others worked as expected and the annealing temperature was chosen as it can be seen in Table 9.

Then the streptomycete strains with positive 16S rDNA result were tested for the remaining three hemolysin genes. For the first hemolysin gene (PCR1/1782) 9 strains gave a positive result, which had the following BCCO 10_numbers: 75, 169, 170, 670, 673, 1747, 1748, 1749, 1752 (examples in Figure 13B). The PCR for the second hemolysin gene (PCR2/2534) gave just one positive result which was BCCO 10_1598. However, no strain gave a positive result for the fourth hemolysin gene (PCR4/4978).

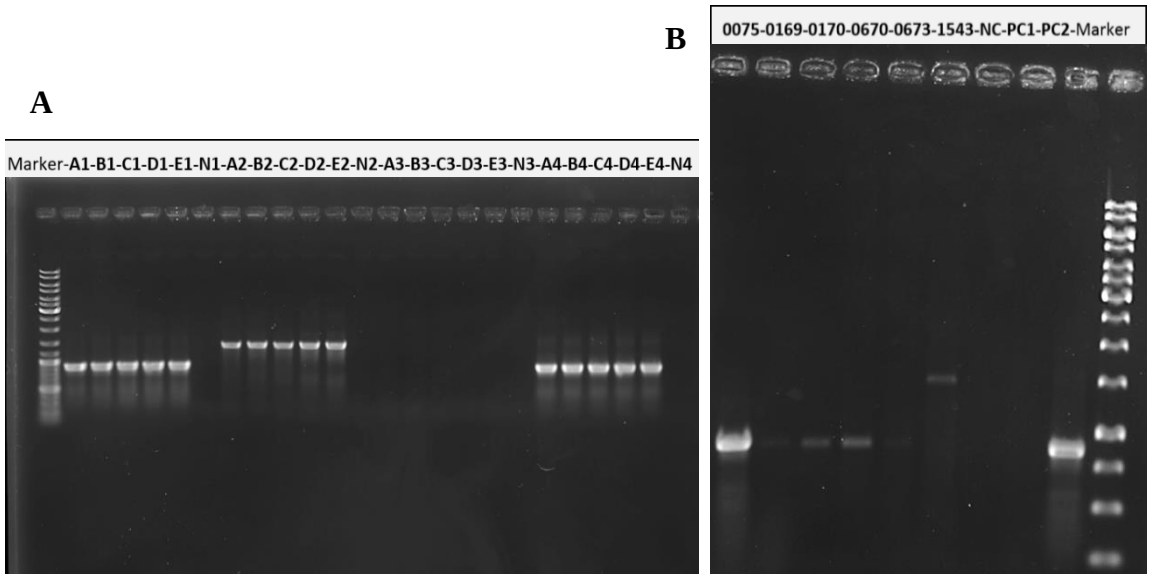

Figure 13. A: PCR with strain TR42 for 4 different hemolysin genes (noted as 1-4) at different annealing temperatures. **B:** PCR for the hemolysin gene number 1

6 Discussion

In this thesis, I focused on the evaluation of the frequency of beta-hemolysis in streptomycete strains (Gram-positive bacterium) from different isolations sources (environmental and clinical) as well as their interaction with two potential human pathogens *Pseudomonas aeruginosa* (Gram-negative bacterium) and *Staphylococcus aureus* (Gram-positive bacterium). The responses (and hence their beta-hemolytic activity, growth, etc.) of both *Streptomyces* and model pathogens were evaluated and compared in parallel and T-type co-cultivations (with close contact). Furthermore, the observed patterns of environmental and clinical streptomycete strains were compared as such study is lacking. Even though we assume that streptomycete clinical strains are not pathogenic, we were interested in the possibility that they are adapted to co-existence with such pathogens.

The frequency of beta-hemolysis was higher in clinical streptomycete strains than in strains of environmental origin, reaching 75.6 % and 51.5 %, respectively. This may indicate an adaptation of clinical streptomycetes to the human body as their new habitat, however, further research is needed to prove this hypothesis. Surprisingly, the environmental strains showed a relatively high frequency of beta-hemolysis as well. To the best of my knowledge, there is no comprehensive study which includes the beta-hemolytic potential of streptomycetes from any kind of habitat, hence these findings are considerable.

In contrast to the frequency, the beta-hemolytic activity of environmental streptomycete strains was higher as they had bigger zones than clinical strains. They differed already in separated cultivation with both *P. aeruginosa* and *S. aureus*. However, the difference was the highest in T-type co-cultivation with close contact to the pathogens. A reason can be that environmental and clinical streptomycete strains produce different molecules, which are responsible for beta-hemolysis, due to adaptation to their habitat. The differences might be either in the production of S-hemolysins (proteins which cause cellular lysis (Rajesh, et al., 2013)), siderophores which are small peptides that form complexes with iron (Chater, et al., 2010) or the palette of secondary metabolites (e.g. filipins, amphotericins, candicidins (Knopik-Skrocka & Bielawski, 2002; Caffrey, et al., 2016)). Very recently, the polyene antibiotic filipin was identified as the only molecule responsible for beta-hemolytic activity of one clinical isolate (TR42) (Petříčková, et al.,

in prep.). The results indicated that apparently large beta-hemolytic zones (> 1cm in diameter) in streptomycetes cultures might be created rather by cytotoxic secondary metabolites, f. e. polyenes, than by hemolysins (proteins). However, the beta-hemolytic compounds of other streptomycete strains have to be identified and characterized with further research.

In general, the response (beta-hemolytic activity) of *Streptomyces* was similar in co-cultivation with *P. aeruginosa* and *S. aureus*. However, the environmental streptomycetes showed a lower beta-hemolytic activity in co-cultivation with *S. aureus* than with *P. aeruginosa*. Strains of clinical origin showed the opposite trend as their beta-hemolytic activity was higher in co-cultivation with *P. aeruginosa* than with *S. aureus*. However, this cannot be completely confirmed as not the same strains were used for the co-cultivation with the two model pathogens, because some tests were not complete.

The presence of both model human pathogens *P. aeruginosa* and *S. aureus* increased the beta-hemolytic activity of *Streptomyces* (of environmental as well as clinical origin) in parallel and T-type co-cultivation. Furthermore, it led to a weak activation of the beta-hemolytic activity of *Streptomyces* in close contact with pathogens (T-type co-cultivation); thus the response was distance dependent. This is likely a result of the interaction of the two bacteria and might be due to competition for the nutrients.

The presence of model pathogens did not affect the growing properties (growth width, sporulation) of the streptomycete strains, maybe because they were inoculated 2-3 days prior to the pathogens and thus their cultures have already been developed.

The beta-hemolytic activity of *P. aeruginosa* and *S. aureus* showed a similar trend as it generally increased due to co-cultivation with *Streptomyces*, maybe as a result of competition for the nutrients. This showed that microorganisms in co-culture communicated by small diffusible molecules. However, the clinical streptomycete strains enhanced the beta-hemolysis of both pathogens in co-cultivations (especially T-tests) more often than the environmental strains. This indicates a possible adaptation of the clinical streptomycetes to the same habitat with human pathogens. On the one hand, they may have developed similar nutrient requirements as the

pathogens and hence the competition for nutrients is higher than with environmental *Streptomyces*. On the other hand, the molecules which are responsible for the beta-hemolysis may act in synergy with those of the model bacteria.

In particular, nearly half of the streptomycete strains induced in T-type co-cultivation a gradual increase in beta-hemolysis of *P. aeruginosa* and moreover, two of them showed large activation zones (BCCO 10_numbers 1499, TR42). *S. aureus* beta-hemolysis was also activated by streptomycetes, as the parallel co-cultivation led to a double zone of beta-hemolysis in between the two bacteria in many cases. The T-type co-cultivation of streptomycetes with *S. aureus* led to weak activation and rarely to an activation zone of the pathogen (BCCO 10_numbers 1499, 1552, 2155, 1598, 1615), but also to a weak deactivation in several cases. To conclude, T-type co-cultivation and hence close proximity often led to activations of the beta-hemolysis of the pathogens. However, the interactions between *Streptomyces* and the pathogens were very strain specific and thus no clear difference in case of activation or inhibition of the beta-hemolysis of the pathogen was observed.

By evaluating the T-type co-cultivation, the growth of the pathogens was decreased by both, environmental and clinical streptomycete strains, except the combination of *P. aeruginosa* with clinical isolates. Partly, this was because streptomycetes apparently activated the motility of *P. aeruginosa* along their culture in over three quarter of the cases, and partly because the inhibitory activity of streptomycetes against *P. aeruginosa* occurred rarely. This is not so typical as only 30 % of known antibiotics inhibit Gram-negative bacteria (66 % against Gram-positive, 5 % against mycobacteria, (Bérdy, 2005)). Moreover, *P. aeruginosa* is very resistant to antibiotic treatments, for instance due as acquirement of plasmid or chromosomally encoding resistance genes, expression of multiple antibiotic modifying enzymes and lower membrane permeability for the antibiotics (Chatterjee, et al., 2016). Hence the retrieval of a potentially new inhibitory compound against Gram-negative bacteria can be important for the development of novel drugs against other Gram-negative respiratory pathogens (for example: *Haemophilus influenza, Moraxella catarrhalis, Neisseria meningitides, Klebsiella pneumonia, Chlamydophila pneumonia* (Brealey, et al., 2015)).

The increase in motility of *S. aureus* was less common in T-type co-cultivation with streptomycetes than with *P. aeruginosa*, because *staphylococci* are not typical motile bacteria; however, it can spread through "comets" under certain conditions (Pollitt, et al., 2015). Moreover, inhibitions of *S. aureus* growth by streptomycetes was more frequent and occurred in one quarter of the co-cultivations (> 1 cm: BCCO 10_numbers 491, 1543, 1606, 1632, TR42). These findings were not surprising, due to the usual action of secondary metabolites with antiobiotic activities as inhibitory compounds of Gram-positive bacteria (Bérdy, 2005). However, the produced metabolites need to be investigated in further studies.

The presence of genes encoding streptomycete hemolysin was evaluated in a set of beta-hemolytic strains (of environmental and clinical origin) as these proteins as one of the main virulence factors in pathogenic bacteria may be responsible for beta-hemolysis. The presence of four different hemolysin genes (SCO1782, SCO2534, SCO3882 (Rajesh, et al., 2013) and SCO4978. located on *S. coelicolor* A3(2) as well as *Streptomyces* sp. TR42 (genome data not available yet) (Petříčková, et al., in prep.)) was evaluated by PCR. Nine of the tested 67 strains possessed the hemolysin gene homologous to SCO1782 (BCCO 10_numbers 75, 169, 170, 670, 673, 1747, 1748, 1749, 1752). The only gene homologous to SCO2534 was detected in the strain BCCO 10_1598. A fourth gene homologous to SCO4978 was not found in any tested strain. The PCR for the third hemolysin gene did not work and very likely it needs to be re-designed. The gene SCO1782 produces S-hemolysin responsible for hemolytic activity in *S. coelicolor* (Rajesh, et al., 2013). The presence of this gene in selected strains cannot serve as proof for the hemolytic activity, thus the putative expression of the hemolysins have to be examined in a future research.

7 Conclusion

Many *Streptomyces* strains show beta-hemolysis. More than half (51.5 %) of the environmental and about three quarter (75.5 %) of the clinical isolates have this ability as the effect of diverse types of molecules. The higher frequency of the beta-hemolytic activity in clinical strains may be due to adaptations to their habitat.

However, the hemolytic zones of *Streptomyces* of clinical origin were smaller than in case of environmental isolates. This might be due to the production of different types of molecules, which are responsible for beta-hemolysis. The co-cultivation of streptomycetes with pathogens (*P. aeruginosa* and *S. aureus*) enhanced the beta-hemolytic activity of *Streptomyces* maybe due to competition for nutrients.

The pathogens were also influenced by *Streptomyces* (especially by clinical isolates) as both showed an increase in beta-hemolytic activity in closer proximity to *Streptomyces*, however, this activation was more pronounced in *P. aeruginosa*. The influence on growth of the pathogens differed as *P. aeruginosa* showed more activations of motility and *S. aureus* growth was more inhibited by the streptomycete strains. The strains with strong inhibitory activities against both pathogens, *S. aureus* in particular, are of large concern as they can be further tested to inhibit multi-resistant clinical strains, such as MRSA or VRSA. However, the interactions between streptomycetes and selected pathogen are very strain specific and there were always strains which showed opposite effects than the generally observed trend.

Genes encoding for hemolysins were present in selected strains, mainly the one showing homology to SCO1782. However, this is no proof for the activity and the putative expression of the hemolysins.

Further research is needed to investigate the beta-hemolytic activity, inhibitory/activating actions and responsible compounds, such as secondary metabolites of selected streptomycete of hemolysins strains in more detail.

8 List of Abbreviations

AIP	Autoinducing peptide
DNA.	Deoxyribonucleic acid
G+C	Guanine + Cytosine
MRSA	Methicillin-resistant *S. aureus*
MS	Mannitol-Soya
NC	Non-template control
OSMAC	One strain many compounds
pAf	Primer forward
PC	Positive control
PCR	Polymerase chain reaction
pHr	Primer reverse
PSAE	*Pseudomonas aeruginosa*
QS	Quorum sensing
rDNA	Ribosomal DNA
STAU	*Staphylococcus aureus*
STR	*Streptomyces*
VRSA	Vancomycin-resistant *S. aureus*

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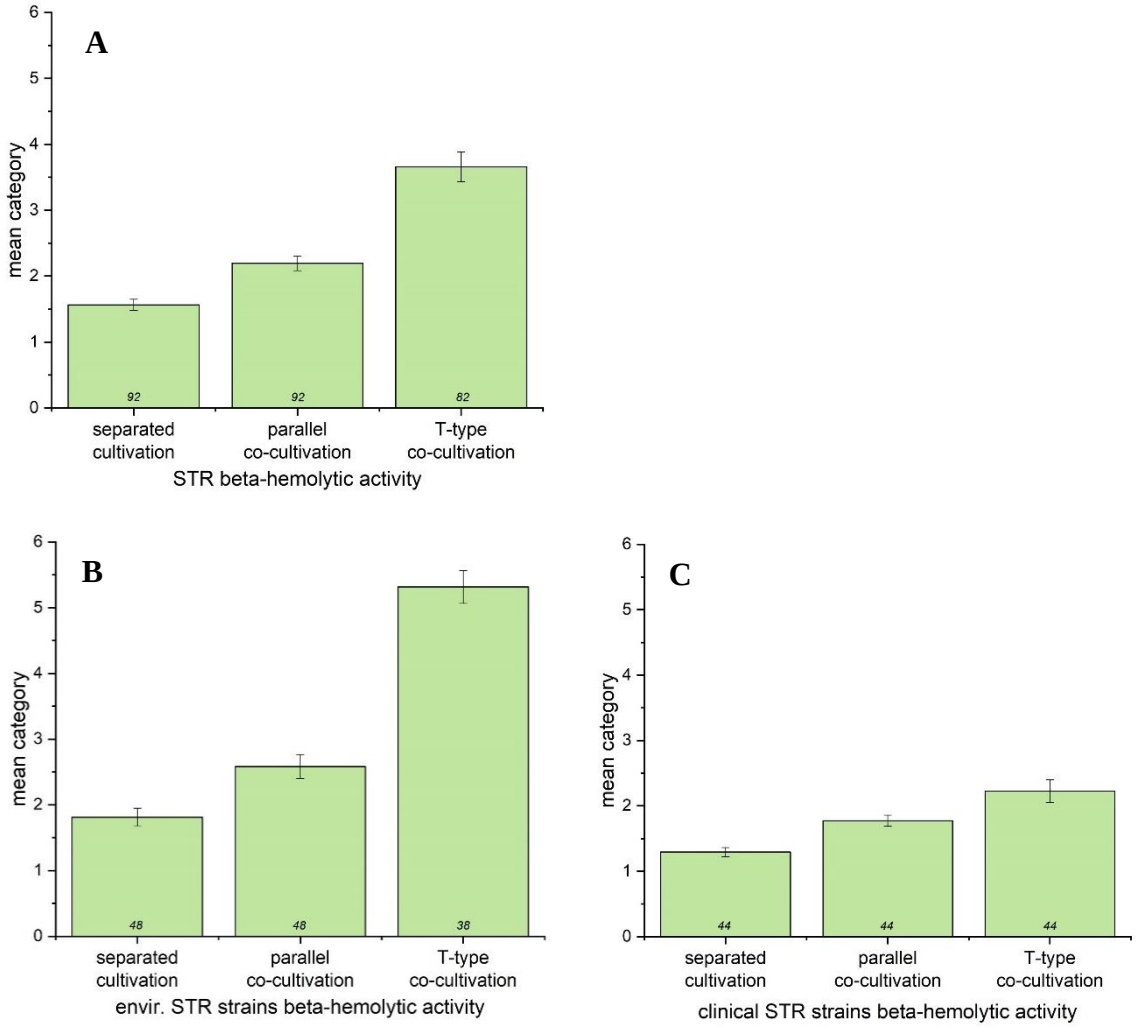
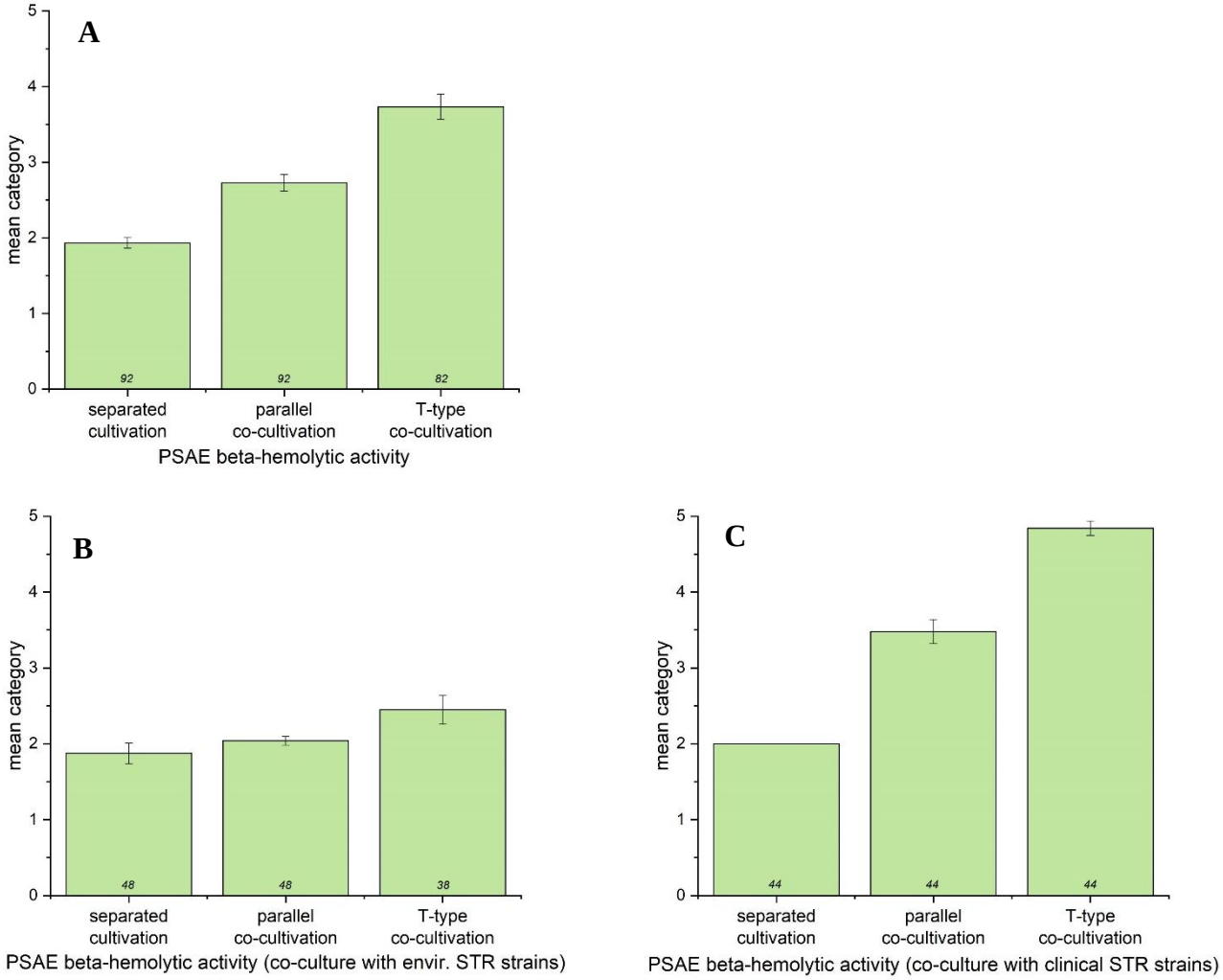
10 List of Figures

11 List of Tables

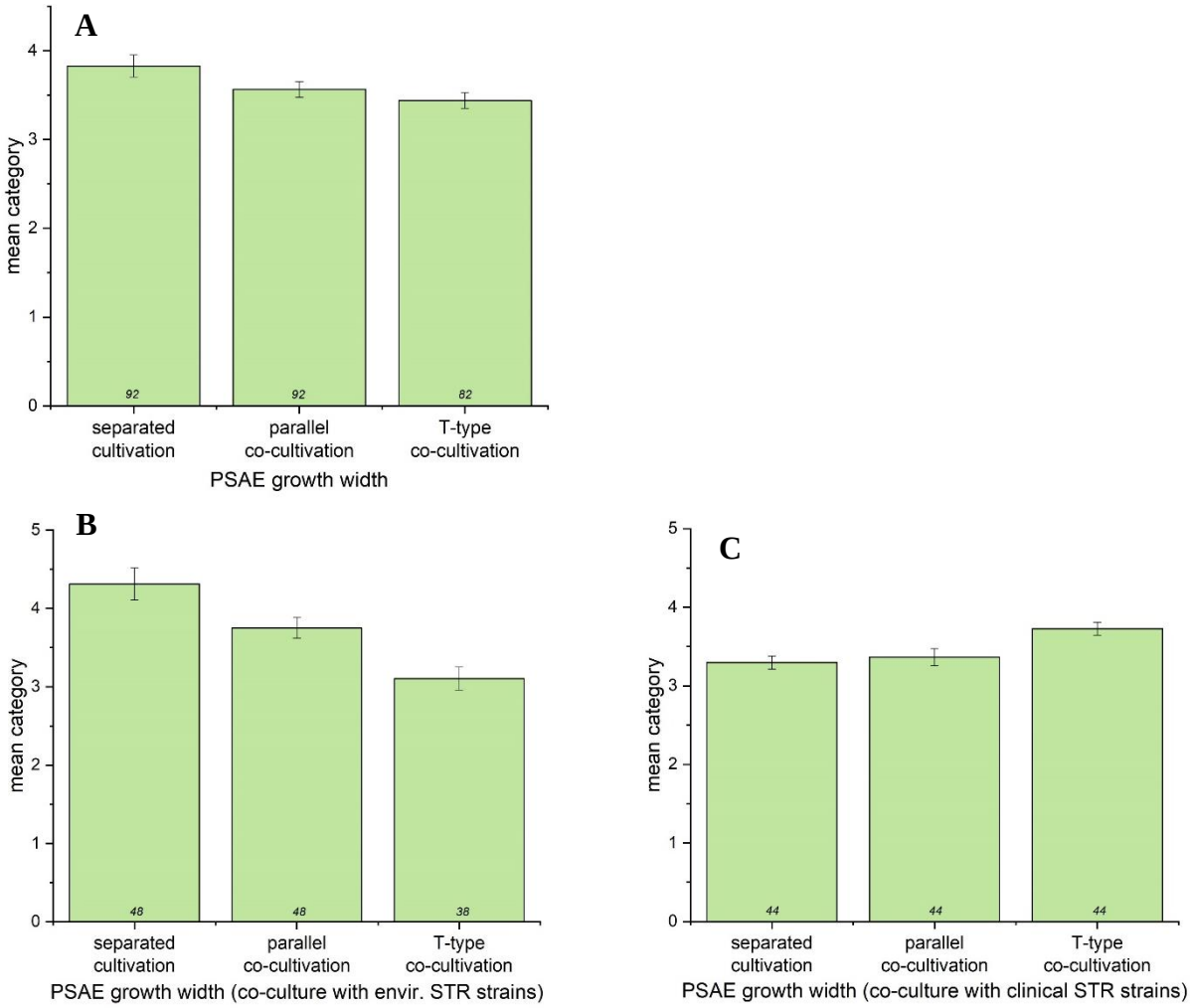
12 Appendix



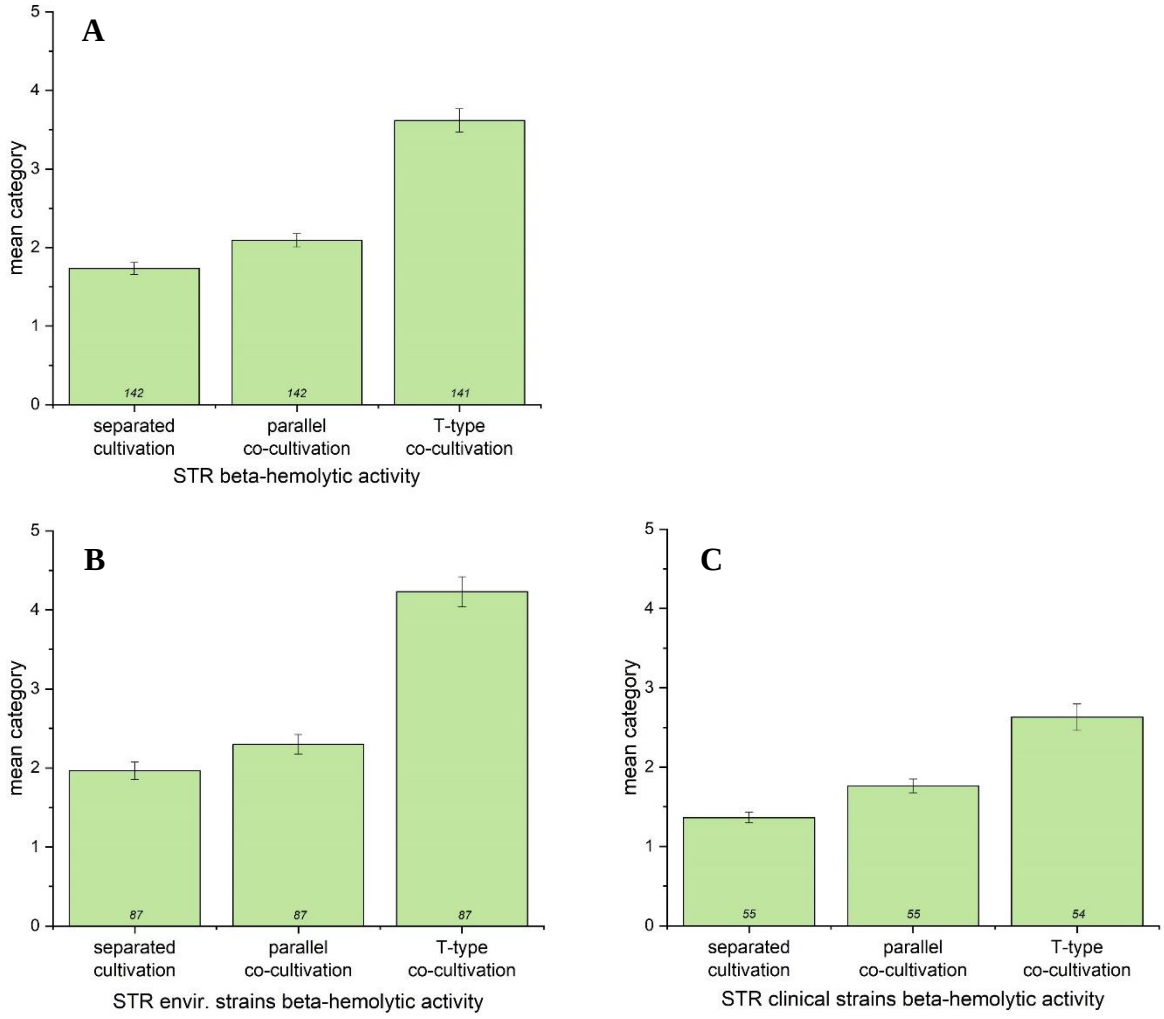
Supplementary Figure 1. Beta-hemolytic activity of *Streptomyces* (STR) in co-cultivation with *P. aeruginosa* - mean value of categories in parallel tests (separated cultivation and parallel co-cultivation) and T-type co-cultivation. The numbers inside the columns indicate the total number of evaluated tests. **A:** All tested *Streptomyces* strains. **B:** *Streptomyces* strains which originate from the environment. **C:** Clinical *Streptomyces* strains.



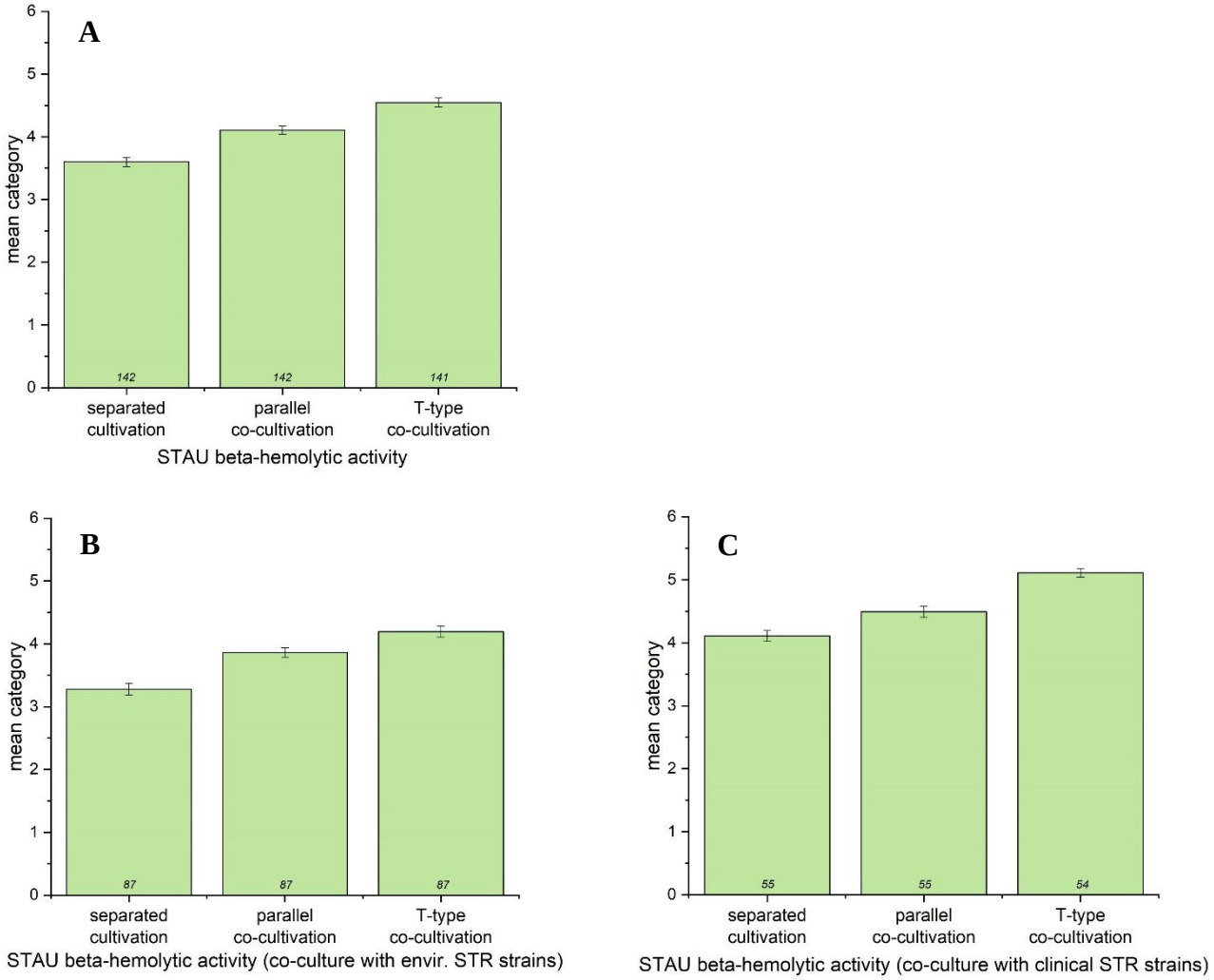
Supplementary Figure 2. Beta-hemolytic activity of *P. aeruginosa* (PSAE) in co-cultivation with *Streptomyces* (STR) - mean value of categories in parallel tests (separated cultivation and parallel co-cultivation) and T-type co-cultivation. The numbers inside the columns indicate the total number of evaluated tests. **A:** Co-cultivated with all *Streptomyces* strains. **B:** Co-cultivation with *Streptomyces* strains which originate from the environment. **C:** Co-cultivated with clinical *Streptomyces* strains.



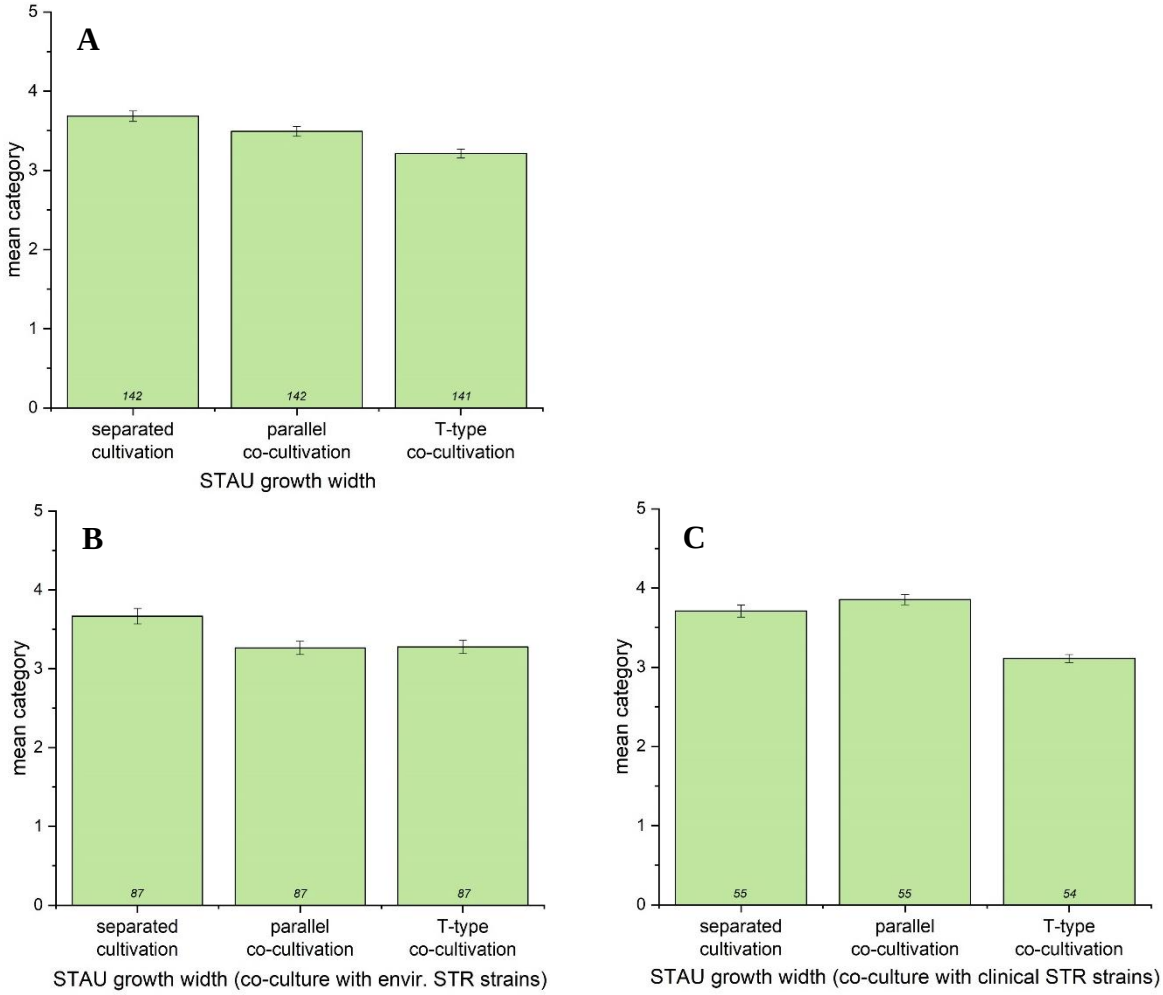
Supplementary Figure 3. Growth width of *P. aeruginosa* (PSAE) in co-cultivation with *Streptomyces* (STR) - mean value of categories in parallel tests (separated cultivation and parallel co-cultivation) and T-type co-cultivation. The numbers inside the columns indicate the total number of evaluated tests. **A:** Co-cultivated with all *Streptomyces* strains. **B:** Co-cultivation with *Streptomyces* strains which originate from environment. **C:** Co-cultivated with clinical *Streptomyces* strains.



Supplementary Figure 4. Beta-hemolytic activity of *Streptomyces* (STR) in co-cultivation with *S. aureus* - mean value of categories in parallel tests (separated cultivation and parallel co-cultivation) and T-type co-cultivation. The numbers inside the columns indicate the total number of evaluated tests. **A:** All tested *Streptomyces* strains. **B:** *Streptomyces* strains which originate from environment. **C:** Clinical *Streptomyces* strains.



Supplementary Figure 5. Beta-hemolytic activity of *S. aureus* (STAU) in co-cultivation with *Streptomyces* (STR) - mean value of categories in parallel tests (separated cultivation and parallel co-cultivation) and T-type co-cultivation. The numbers inside the columns indicate the total number of evaluated tests. **A:** Co-cultivated with all *Streptomyces* strains. **B:** Co-cultivation with *Streptomyces* strains which originate from environment. **C:** Co-cultivated with clinical *Streptomyces* strains.



Supplementary Figure 6. Growth width of *S. aureus* (STAU) in co-cultivation with *Streptomyces* (STR) - mean value of categories in parallel tests (separated cultivation and parallel co-cultivation) and T-type co-cultivation. The numbers inside the columns indicate the total number of evaluated tests. **A:** Co-cultivated with all *Streptomyces* strains. **B:** Co-cultivation with *Streptomyces* strains which originate from environment. **C:** Co-cultivated with clinical *Streptomyces* strains.