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Charles University in Prague
Faculty of Science

Study program: Biology
Specialization: Microbiology

Bc. Volha Ramaniuk

Mycobacterium smegmatis growth on agar plates and agar plates covered with cellophane – morphological and proteomic study

Růst *Mycobacterium smegmatis* na agarových plotnách a agarových plotnách pokrytých celofánem – morfologická a proteomová studie

Diploma thesis

Supervisor: RNDr. Jaroslav Weiser, CSc.

Prague, 2012

Declaration:

I declare that this diploma thesis is based on my own work, and that all sources of literature are mentioned. I also declare that this thesis was not presented to obtain another academic degree.

Prague, 27.08.2012

This diploma thesis was created 2010 – 2012 at the Institute of Microbiology of the Academy of Sciences of the Czech Republic, Laboratory of Microbial Proteomics. The thesis was supported by grant IAAA500200913.

Acknowledgements:

I would like to warmly thank my supervisor RNDr. J. Weiser, CSc. for enabling me to work in the laboratory and for leading my diploma thesis. I also thank Mgr. K. Buriánková, PhD. for her help in preparing this thesis, S. Bezoušková for the two-dimensional gels, RNDr. J. Janeček, CSc. for valuable advices and RNDr. D. Petráčková, PhD. for the PDQuest course. I also thank all these people and in addition Mgr. Z. Sochorová, Mgr. Š. Bobková, PhD., Mgr. B. Sitařová and Mgr. E. Tesařová for comprehensive help, support and very friendly surroundings.

Finally I am very grateful to my parents and brother for their support, without which my study at the Charles University could not take place.

CONTENTS

ABSTRACT

Biofilm formation is one of the most common bacterial survival strategies. Majority of bacterial species are able to form these three-dimensional structures, including pathogens like *Mycobacterium tuberculosis*. Representatives of *Mycobacterium* genus widely occur in the nature, although they can cause serious problems when they appear in medical equipment and artificial replacements of the human body.

Non-pathogenic *Mycobacterium smegmatis mc^2 155* was used as a model organism in our experiments. We investigated morphology of the three- and six-day-old colonies (in fact biofilms) on agar and agar covered with cellophane using Stereo microscope and Scanning Electron Microscope. We found that a type of surface as well as a carbon source has a great influence on the morphology of the *M. smegmatis* colonies. We isolated proteomes from the agar and cellophane cultures and from planktonic culture. Two-dimensional electrophoresis was used as the main proteomic method. Proteomic data were analyzed using PDQuest software. Then the sets of proteins detected by qualitative and quantitative analyses were compared using Venn diagrams. As a result, we recognized 7 unique proteins that might be specific for recognition and adhesion of bacteria to the cellophane, no unique protein in agar proteome and 46 unique proteins that might be specific for submerged growth in liquid culture. Quantitative analysis revealed only 3 proteins with different expression in all proteomes. These results show that morphological changes are associated with changes in proteome. Expression of new unique proteins rather than the changes in expression of those present in all conditions seems to play a key role in adapting to different types of cultivation.

Key words: *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, biofilm, colony morphology, carbon source, two-dimensional electrophoresis, SEM, proteome.

ABSTRAKT

Tvorba biofilmů je jednou z nejčastěji používaných strategií umožňujících bakteriím přežít nepříznivé podmínky. Většina bakterií, včetně patogenů jako je například *Mycobacterium tuberculosis*, je schopna tvořit tyto trojrozměrné struktury. Zástupci rodu *Mycobacterium* se hojně vyskytují v přírodě, ale mohou způsobit i vážné problémy v případě výskytu ve zdravotnických zařízeních a na umělých náhradách v lidském těle.

V našich experimentech jsme použili jako modelový organismus nepatogenní kmen *Mycobacterium smegmatis mc^2 155*. Pomocí binokulární lupy a rastrovací elektronové mikroskopie jsme zkoumali morfologii tří- a šestidenních kolonií (v podstatě biofilmů) na agarových plotnách a agarových plotnách pokrytých celofánem. Zjistili jsme, že typ povrchu, stejně jako zdroj uhlíku, mají velký vliv na morfologii kolonií *M. smegmatis*. Následně jsme izolovali proteomy z kultur rostoucích na povrchu agaru a celofánu, a z planktonicky rostoucí kultury. Dvojrozměrná elektroforéza byla použita jako hlavní proteomická metoda. Proteinové profily, získané pomocí elektroforetických gelů, byly analyzovány pomocí softwaru PDQuest. Výsledky kvalitativních a kvantitativních analýz jsme porovnali pomocí Vennových diagramů. Identifikovali jsme 7 unikátních bílkovin, které by mohly být specifické pro adhezi *M. smegmatis* na povrch celofánu, a 46 unikátních bílkovin, které by mohly být důležité pro planktonický růst v tekutém mediu. Proteiny specifické pro růst na agarových plotnách nalezeny nebyly. Kvantitativní analýza odhalila pouze 3 proteiny se změnou exprese ve všech proteomech. Naše výsledky ukazují, že morfologické změny jsou spojeny se změnami v proteomu. Klíčovou úlohu při přizpůsobování se různým podmínkám hraje právě exprese nových specifických proteinů spíše než rozdíly v úrovni exprese těch společných.

Klíčová slova: *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, biofilm, morfologie kolonii, zdroj uhlíku, dvojrozměrná elektroforéza, SEM, proteom.

ABBREVIATIONS

2-DE	two-dimensional electrophoresis
A. U.	arbitrary units
AG	arabinogalactan
AHL	acyl-homoserine lactone
BCG	Bacillus Calmette-Guérin
bp	base pair
BSA	bovine serum albumin
CBB	Coomassie Brilliant Blue dye
c-di-GMP	cyclic di-guanosinmonophosphate
ddH_2O	double deionized water
dH_2O	deionized water
DNA	deoxyribonucleic acid
EPS	extracellular polymeric substance
GC	guanosine, cytosine
GTP	guanosinetriphosphate
HIV	human immunodeficiency virus
IEF	isoelectric focusing
IPG	imobilizied pH gradient
LAM	lipoarabinomannan
MS	mass spectrometry
MTC	*Mycobacterium tuberculosis* complex
NTM	nontuberculous mycobacteria
OD	optical density
pI	isoelectric point
QS	quorum sensing phenomenon
RNA	ribonucleic acid
r. t.	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
SSP	sample spot protein
UV	ultraviolet light
WHO	World Health Organisation

1. INTRODUCTION

Mycobacterium smegmatis is a widely used model system for the research analysis of other mycobacteria such as highly pathogenic species *Mycobacterium tuberculosis* and *Mycobacterium leprae*. It is commonly used in laboratory experiments due to its safeness and a relatively short doubling time.

An ability to create biofilms is one of the distinctive features of the *Mycobacterium* representatives. Biofilms usually occur in nature; however they also very easily appear on surfaces of medical equipment and artificial replacements introduced into the human body. Therefore research of mycobacterial biofilms and biofilms in general is a highly important topic of the present.

Our laboratory is involved in a project focused on investigation of mycobacterial biofilms. My colleagues have already investigated the development of *M. smegmatis* biofilms on the surface of glass and zirconium beads. The morphology and protein profiles of these biofilms were compared with these of planktonic culture.

The main **aim** of this diploma thesis was study of morphology of the *M. smegmatis* colonies grown on agar plates and agar plates covered with cellophane sheet using Stereo microscope and Scanning Electron Microscope (SEM). Cellophane was chosen as an alternative surface due to its properties and successful using in cultivation of streptomycetes, close relatives of *Mycobacterium* genus. The effect of two different carbon sources (glycerol and glucose) on the morphology of the colony was investigated. The proteomes of *M. smegmatis* obtained from the solid medium cultures and from planktonic culture were compared and the influence of these different cultivation conditions on protein profiles was analyzed. According to our **hypothesis** the type of surface and carbon could have an impact on the morphology of the colonies and these differences could be reflected by changes in the protein profiles.

Objectives of diploma thesis:

1. Comparison of *M. smegmatis* morphology grown on agar plates and agar plates covered with cellophane by Stereo microscope and SEM.
2. Cultivation of *M. smegmatis* on two different carbon sources.
3. Analysis of *M. smegmatis* proteomes isolated from solid surfaces and planktonic culture by two-dimensional gel electrophoresis.

2. LITERATURE REVIEW

2.1 Surface and planktonic growth of microorganisms

Since the establishment of microbiological science in XIX century one of the main methods for growing bacteria isolated from the nature was cultivation in liquid medium (so-called planktonic culture). Much of the knowledge in microbiology, including the knowledge about antibiotic activity, is based on work with these free-floating organisms. However, in recent years scientists investigating bacterial growth on different surfaces came to the conclusion that removing bacteria from their real environments and cultivating them as planktonic cells in single-species cultures is not the correct way to understand their roles in natural ecosystems (COSTERTON 2004). According to Gilbert et al. (2002), in the majority of natural habitats microorganisms grow and survive as adherent clusters of cells – biofilms (GILBERT *et al.* 2002). The considerable amount of evidence of substantial differences comes from comparisons of the morphology of planktonic cells from laboratory culture and sessile cells of the same species from mixed-species biofilms (COSTERTON 2004). Therefore it is possible to speculate that physiological and morphological data obtained from experiments with bacteria growing in liquid medium cannot correspond to those obtained from bacteria growing as a biofilm. Thus, in this sense, the planktonic microorganisms in the test-tube culture might be considered as an artifact (PALKOVÁ and VACHOVÁ 2010).

2.1.1 Characteristics of planktonic growth of microorganisms

There are two basic types of planktonic cultivation of microorganisms under laboratory conditions: batch and continuous cultivation.

Batch culturing method is one of the most common techniques used in microbiology. A nutrient solution is prepared at the beginning of the experiment and then inoculated with the desired organism. No additional nutrients are then supplied. The growth in a culture where no additional nutrients are supplied, planktonic bacteria typically exhibit four stages of population development that are known as lag phase, logarithmic (or exponential) phase, stationary phase, and death (or decline) phase.

It is also possible to grow planktonic culture under conditions where fresh nutrients solution is added at the same rate as the culture is removed. This method is called continuous

cultivation. Then, the bacteria grow as fast as the rate of addition of the new nutrient source and can remain in exponential phase (in "steady state") for as long as the conditions are maintained. Thus, planktonic bacteria can display a wide range of the speeds at which they can grow.

The physiological character of planktonic bacteria is very different from their adherent counterparts. They are single-celled bacteria, living as floating organisms in the respective environments. Additionally, planktonic bacteria tend to have cell surface that is relatively hydrophilic, and their pattern of gene expression is markedly different from bacteria growing on a surface. Also, planktonic bacteria tend not to have a surrounding cover so they are more susceptible to antimicrobial agents. Planktonic bacteria are also predisposed to eradication by the immune system of human and animals (HALL-STOODLEY *et al.* 2004). Examination of many pathogenic bacteria has demonstrated that in host organism they tend to adopt number of strategies to evade the host reaction. These strategies include formation of the adherent, glycocalyx enclosed populations (development of biofilm).

2.1.2 Characteristics of surface growth of microorganisms

Robert Koch was the first scientist who cultivated microorganisms, mainly bacteria, on solid culture media – at the beginning on a potato slices. Than he used a gelatin as a solidifying agent; afterwards it was improved by Walter Hesse's discovery, that agar can be used as a gelling agent for microbial culture media (MADIGAN and MARTINKO 2006). It is still used today for obtaining and maintaining pure cultures of bacteria (**Fig. 1**).

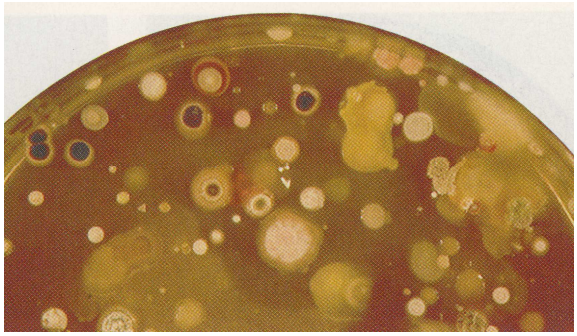

Figure 1. Casein-starch agar plate with the colonies of soil microorganisms (MADIGAN and MARTINKO 2006).

In laboratory conditions microorganisms grow on solid medium in the form of colonies. These multicellular structures have a number of unique features and capabilities that individual cells lack. For example, colonies of yeasts have an alarm system based on its own

metabolite – ammonia – which helps them to survive under conditions of starvation. Also in yeast colonies there are evolving specialized cells which are functioning for the benefit of the whole colony (PALKOVÁ and VACHOVÁ 2010).

The study of some bacterial species shows that morphology and, possibly, gene expression significantly reflects cultivation conditions. Laboratory strains of *Bacillus subtilis* form smooth colonies and sporulation takes place in random places. On the contrary, wild strains of *B. subtilis* form structured colonies and spores are found in small defined areas which are the part of the structure of the colony. Colonies of wild and laboratory strains of yeast *Saccharomyces cerevisiae* also differ morphologically (PALKOVÁ and VACHOVÁ 2010).

Bacterial colony is defined as a visible cluster of bacteria growing on the surface of or within a solid medium. Because all organisms within the colony descend from a single ancestor, they are genetically identical. Obtaining such genetically identical organisms (or pure strains) can be useful in many cases; this is done by spreading bacteria on a culture plate and starting a new strain of bacteria from a single colony. Colonies of different microorganisms have unique morphology, shape, size, texture and color, which are used as the diagnostic features of the species (**Fig. 1, 2**).

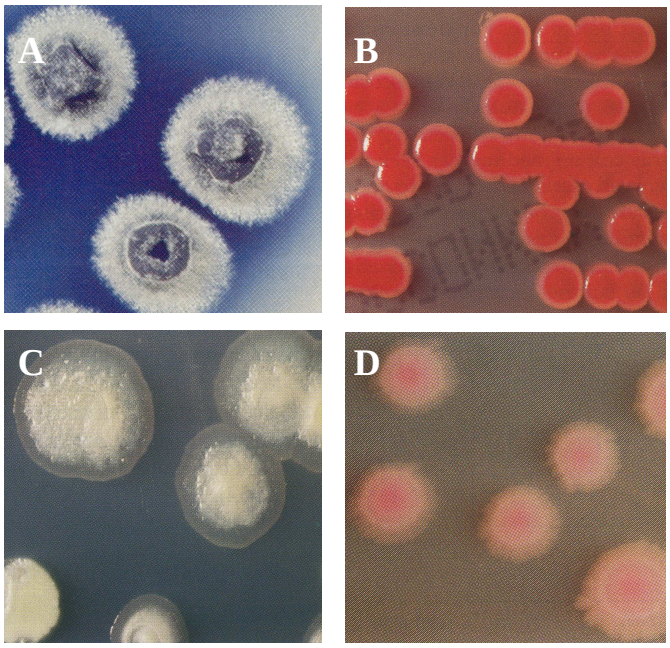

Figure 2. Examples of bacterial colonies on the agar plates: A – *Streptomyces coelicolor*; B – *Serratia marcescens*; C – *Pseudomonas aeruginosa*; D – *Shigella flexneri* (MADIGAN and MARTINKO 2006).

Characteristics of colonies correspond with the characteristics of biofilm. Therefore here and below in this work bacterial colonies are alluded as biofilms, which are described in detail in the following chapter.

2.2 Biofilms

The idea that some bacteria in natural ecosystems live preferentially on surfaces, has been presented consistently since researchers have begun to study microorganisms. Wild bacterial strains must survive in nature and host organisms exposed to various antibacterial factors. Therefore they needed to create some defensive structures to protect themselves. These structures are called biofilms.

There are several definitions of biofilms. According to McGraw-Hill Encyclopedia of Science and Technology, biofilm is an adhesive substance (the glycocalyx) and the bacterial community which it envelops at the interface of a liquid and a surface (www.accessscience.com). According to Wikipedia, biofilm is an aggregate of microorganisms in which cells adhere to each other on a surface (www.en.wikipedia.org). According to Medical Dictionary, biofilm is a very thin layer of microscopic organisms that covers the surface of an object (www.medical-dictionary.thefreedictionary.com). Common in all definitions is that biofilm cells must be adherent to the surface and to each other and create an outer coat.

By adopting sessile mode of life, biofilm-embedded microorganisms enjoy a number of advantages over the planktonic counterparts (GILBERT *et al.* 2002). For example, biofilm can promote a horizontal gene transfer (ABBASSI *et al.* 2008). It is also known that adherent microorganisms tend to be less responsive to environmental changes. Besides bacteria in biofilm can usually survive higher concentrations of antibiotics than planktonic cells of the same species. Nevertheless it was shown that oxygen is limited in deeper layers of biofilm. Therefore it has been proposed that at least some of the cells in biofilm may experience nutrient limitation and therefore exist in a slow-growing or starved state (BECKER *et al.* 2001).

2.2.1 Structure of biofilm and stages of its development

A diagnostic characteristic of biofilm communities is a close proximity of cells maintained through the presence of extracellular matrices (GILBERT *et al.* 2002). To protect themselves from a host antibacterial factors microbial community needs to have protective

outer shield. Direct light and electron microscopy show this layer – exopolymeric matrix of biofilms – as a thick coating of the community with an ordered array of fine fibers comprised of gelled and highly hydrated exopolysaccharides (EPS).

The microcolony is the basic structural unit of the biofilm. Depending on conditions of growth it may include cells of one or more species. Microcolony may be composed of 10-5 % cells and 75-90 % EPS matrix, and the matrix material often appears to be most dense in the area closest to the core of the microcolony (DOMINIAK *et al.* 2011; XIAO and KOO 2010).

The formation of bacterial biofilm must, necessarily, begin with the adhesion of a small number of bacterial cells to a surface. Thus biofilm starts when a few pioneer cells use specialized “chemical hooks” to adhere to a surface. These cells help to make a target surface more attractive to subsequent cells, which eventually mature into a complex, structured film. In motile bacteria, the presence of fimbriae and flagella also influence the attachment to surface (HAABER *et al.* 2012).

The development of a biofilm is believed to occur in a sequential process that includes following stages: stage 1) initial attachment; stage 2) irreversible attachment; stage 3) maturation I; stage 4) maturation II; stage 5) dispersion (**Fig. 3**).

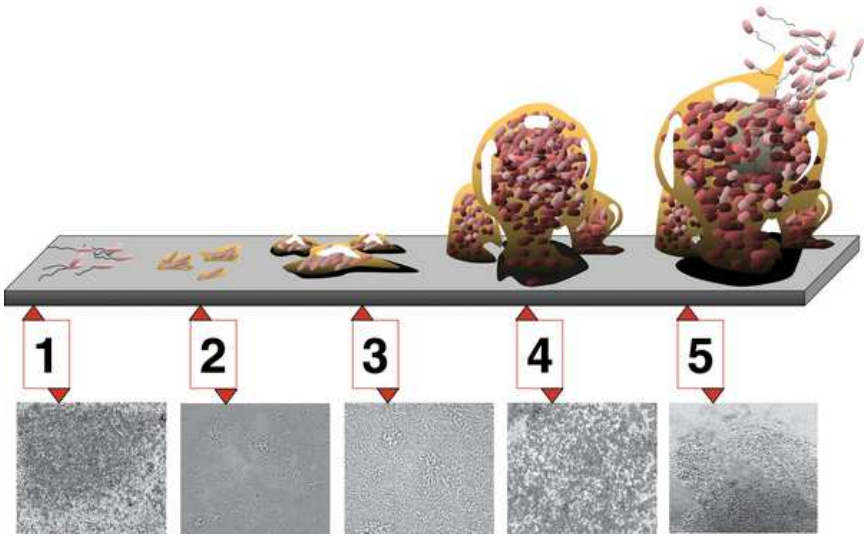

Figure 3. Biofilm maturation is a complex developmental process involving five stages. Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm. All photomicrographs are shown to same scale (MONROE 2007).

2.2.2 Occurrence of biofilms

In nature biofilms are found everywhere: they occur on rocks and pebbles, at the bottom of streams and rivers, on the surface of and inside plants, in waste-water, in hot springs. Almost without exception, surfaces within aqueous and humid environments become colonized with microorganisms growing as biofilm.

In its habitat biofilms can play both positive and negative role. For example, the waste-water biofilms are extremely important in the waste-water treatment process since they remove many key chemical contaminants, such as nitrogen-containing and phosphorous-containing compounds (NICOLELLA *et al.* 2000). On the other hand, biofilms can cause the biofouling and the corrosion of the metal pipes. Corrosion of the metal pipes is one of the significant problems caused by biofilms in the industry, where increased frictional resistance to fluid flow greatly increases energy consumption (LEE and NEWMAN 2003). Contamination and spoilage in food industry and processing represent also a serious problem.

Biofilms also occur in the human body. The well-studied model of biofilm communities in terms of human health are oral biofilms (dental plaque), in which hundreds of bacterial species have been identified. Biofilms are known to contribute to dental caries and gingivitis (KOLENBRANDER 2000).

Recently, biofilms were widely studied in connection with a variety of chronic bacterial infections (PARSEK and SINGH 2003). It is obvious that they are involved in cystic fibrosis pathogenesis (*Pseudomonas aeruginosa*), in otitis (*Haemophilus*) in osteomyelitis (*Staphylococcus aureus*). Another organism that has been implicated in biofilm infections is *Enterococcus faecalis*, which can cause endocarditis and post-operative infections (TOLEDO-ARANA *et al.* 2001). Biofilms are very easily created on surfaces of medical equipment and artificial replacements introduced into the human body such as intravenous catheters, artificial heart valves and joint replacements, surgical sutures, contact lenses, urinary catheters, etc. Producers of medical devices, such as catheters, frequently try to prepare the device surfaces to disrupt the initial adhesion, but yet without great success.

2.2.3 Quorum sensing

Quorum sensing (QS) is a term used to describe cell-to-cell signaling system. With this mechanism bacteria carry out collective behavior within a population (MORRISON 1997).

QS is generally considered to facilitate gene expression only when the population has reached a certain cell density. QS depends on the synthesis of a small signal molecules that diffuse in and out of bacterial cells, also called autoinducers. As the bacterial population density increases, so does the synthesis of QS signal molecules and consequently, their concentration in the external environment increases (MORRISON 1997). Several chemically distinct families of QS signal molecules have been described, of which the N-acylhomoserine lactone (AHL) family in Gram-negative bacteria have been the most intensively investigated (**Fig. 4**) (WILLIAMS 2007).

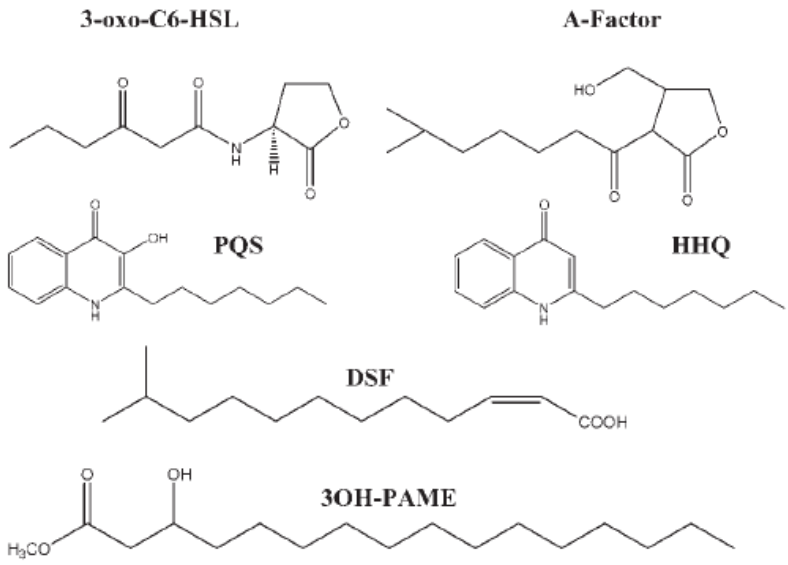

Figure 4. Examples of the structures of some representative QS signal molecules: 3-oxo-C6-HSL, N-(3-oxohexanoyl)-L-homoserine lactone; A-Factor, 2-isocapryloyl-3-hydroxy-methyl-γ-butyrolactone; PQS, pseudomonas quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone; HHQ, 2-heptyl-4(1H)-quinolone; DSF, "diffusible factor", cis-11-methyl-2-dodecenoic acid; 3OH-PAME, hydroxyl-palmitic acid methyl ester (WILLIAMS 2007).

QS occurs in bacterial biofilms created by one species of bacteria, and also in biofilms, consisting of different genera of bacteria. The latter has not yet been thoroughly explored, however it can be speculated that such different genera which nevertheless produce the same AHL molecule should be capable of cross-talk. For example, *Pseudomonas aeruginosa, Serratia liquefaciens* and *Aeromonas hydrophila* all produce N-butanoylhomoserine lactone (WILLIAMS 2007). Although bacterial QS signal molecules have largely been considered as effectors of prokaryotic gene expression, they are bioactive molecules which can modify the behavior of fungal, plant and animal cells as well (SHINER *et al.* 2005).

2.2.4 Biofilm resistance to antibiotics

Important consequences of biofilm formation with profound clinical implications include enhanced resistance to antibiotics and disinfectants and protection from host defense. Currently it is one of the main reasons for the interest in biofilms.

Recognition of phenomenon of biofilm resistance to antimicrobial agents can be dated back to at least 1684, when Antonie van Leeuwenhoek recorded his observations of the microbial flora in dental plaque. When he rinsed his mouth with vinegar, thereby exposing the intact biofilm, he found that the treated plaque continued to seethe and teem with life (COSTERTON 2004).

Figure 5 shows the modern schematic representation of the major stages of biofilm formation and some of the principal resistance mechanisms observed in mature biofilms.

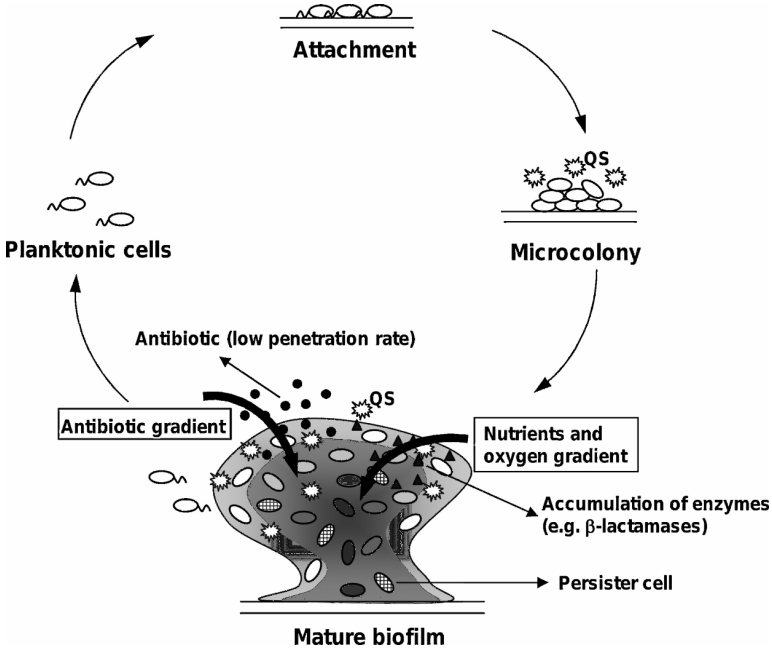

Figure 5. The major stages of biofilm formation and some of the principal resistance mechanisms. QS is the quorum sensing signal (FERNANDEZ *et al.* 2012).

The grey background represents the extracellular matrix that consists of EPS and DNA, both of which contribute to antibiotic resistance. The intensity of the color varies according to the nutrients and oxygen gradient (represented with a thick arrow), i.e. the lighter the color the greater the concentration of oxygen and nutrients and vice versa. The cells embedded in the biofilm also show a different shade on the basis of their growth and metabolic rate, ranging from the metabolically active cells, in white, to the slow growing cells, in dark grey (FERNANDEZ *et al.* 2012).

2.3 Mycobacteria

Mycobacterium is a genus of Mycobacteriaceae family, which is one of Actinobacteria phylum. Greek prefix "myco" means fungus, because it has been observed at the surface of liquid medium in the mold-like form (VOTAVA 2003).

Genus *Mycobacterium* contains more than 100 species. Their relatedness was confirmed by comparing the 16S rRNA sequence (TORTOLI 2006).

2.3.1 General characteristics of mycobacteria

Mycobacteria are obligatory aerobic or micro-aerophilic, Gram-positive and acid-fast bacteria. They are rod-shaped, 0,3 – 0,5 µm in diameter and of variable length, immobile and until recently considered as not forming spores (GHOSH *et al.* 2009; TRAAG *et al.* 2010). Some of the species (like *M. gordonae* and *M. marinum*) are able to synthesize carotenoid pigments (MADIGAN and MARTINKO 2006). Due to their acid-fast property mycobacteria significantly resist to disinfection. They displayed reliable sensitivity only to UV radiation, phenol derivatives and aldehydes.

Representatives of the genus are quite pleomorphic and may undergo branching and filamentous growth. However, in contrast to those of the Actinomycetes, filaments of the mycobacteria become fragmented into rods and true mycelium is not formed. Mycobacterial genome size is quite variable from 3268203 bp in *M. leprae* to 6988209 bp in *M. smegmatis* and is characterized by a high proportion (62 – 70 %) of guanine-cytosine (GC) base pairs (COOK *et al.* 2009).

2.3.2 Cell envelope of mycobacteria

The distinctive feature of mycobacteria that deserves a separate description is their cell envelope.

Unlike other Gram-positive bacteria, mycobacteria have evolved a very complex cell wall (BARRY *et al.* 1998). It comprises a peptidoglycan-arabinogalactan polymer with covalently bound mycolic acids of considerable size (up to 90 carbon atoms – **Fig. 6**), and a large variety of extractable lipids and pore-forming proteins. Mycolic acids are α-branched β-hydroxy fatty acids. They are the mayor components of the mycobacteria cell wall (BARRY *et al.* 1998). Mycolic acids produced by mycobacteria are the longest fatty acids identified in nature (NIEDERWEIS 2003). Due to the *M. tuberculosis* medical importance, the

ultrastructure of mycobacterial cell envelope has been intensively studied for decades by electron microscopy. According to X-ray diffraction studies the mycolic acids of mycobacterial cell wall are oriented parallel to each other and perpendicular to the plane of the cell envelope (NIKAIDO *et al.* 1993).

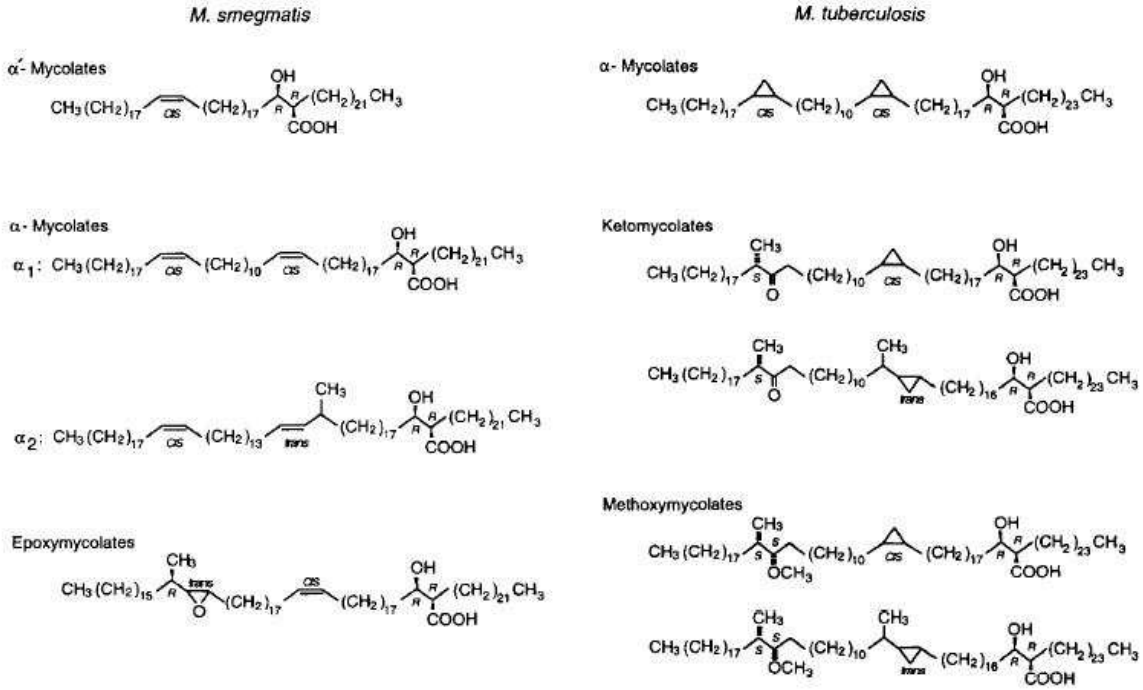

Figure 6. Structures of mycobacterial mycolic acids in *M. smegmatis* and *M. tuberculosis* (CHATTERJEE 1997).

Despite the fact that mycobacteria are classified as Gram-positive genus, during recent years cryo-electron tomography showed that they have something like "mycobacterial outer membrane" (HOFFMANN *et al.* 2008). Cryo-electron tomography and cryo-electron microscopy of ultrathin sections clearly demonstrated that the mycobacterial outer membrane is a bilayer structure (**Fig. 7**) (COOK *et al.* 2009). This unique mycolic acid bilayer is functionally analogous to the outer membrane of Gram-negative bacteria.

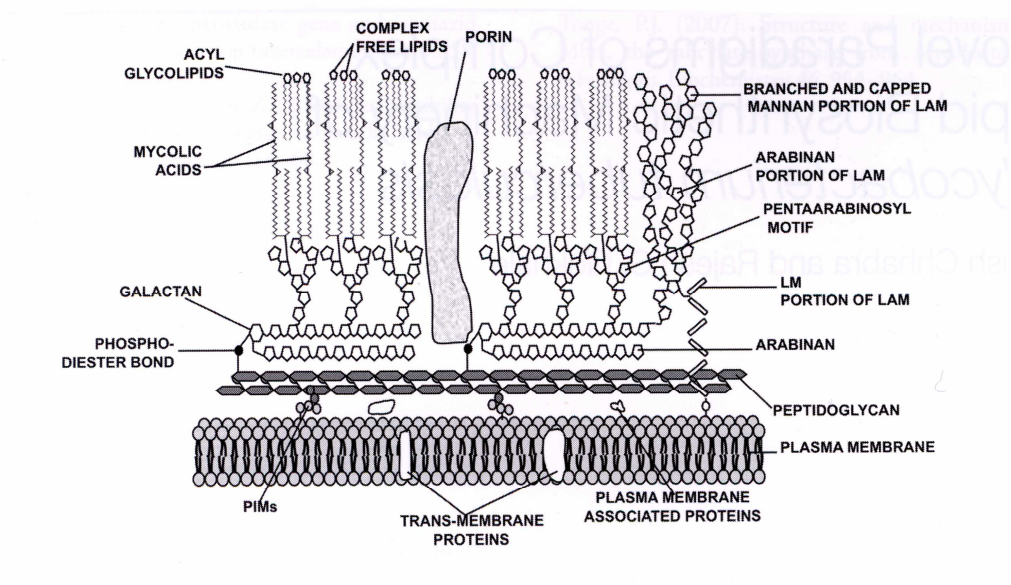

Figure 7. The structure of the mycobacterial cell wall. Lipoglycans: LM – lipomannan; LAM – lipoarabinomannan; PIM – phosphatidyl inositol mannoside. (CHHABRA and GOKHALE 2009).

Thus mycobacteria protect themselves by an outer lipid bilayer, which is one of known thickest biological membranes and has an exceptionally low permeability rendering mycobacteria intrinsically resistant to many antibiotics, chemotherapeutic agents and chemical disinfectants (BRENNAN and NIKAIDO 1995).

2.3.3 Uptake of nutrients by mycobacteria

One of the objectives of this thesis was comparison of the growth of *M. smegmatis* on glycerol and glucose as carbon sources. Therefore in this chapter the uptake and processing of nutrients into the mycobacterial cell are described in more detail.

Nutrient uptake mechanisms obviously depend on the permeability of barrier formed by the cell envelope (COOK *et al.* 2009). The presence of two lipid barriers in mycobacteria imposes considerable constraints for the transport of solutes from the exterior of the cell to the cytoplasm and has important consequences for the physiology and pathogenesis of mycobacteria (**Fig. 8)** (NIEDERWEIS 2008).

It is apparent that the uptake of nutrients by mycobacteria is not well understood, despite its importance for understanding the physiology and pathogenicity of *M. tuberculosis* and the availability of an abundance of genomic information. Not all comparisons with the saprophyte *M. smegmatis* can contribute much to understanding the physiology and virulence of *M. tuberculosis*. However, studying nutrient uptake pathways in *M. smegmatis* should

reveal the principal mechanisms by which the permeability barriers in mycobacteria can be overcome (NIEDERWEIS 2008).

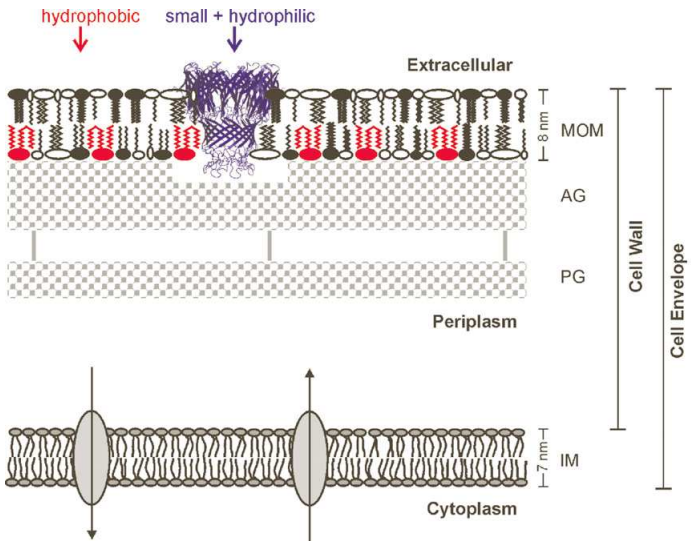

Figure 8. Transport processes across the mycobacterial cell envelope. AG – arabinogalactan, PG – peptidoglycan, OM – outer membrane. Extractable lipids are represented in black. The porin MspA mediates the uptake of small and hydrophilic nutrients such as sugars and phosphates across the OM of *M. smegmatis*. Hydrophobic compounds are assumed to diffuse directly across the OM (HOFFMANN *et al.* 2008).

2.3.3.1 Use of carbohydrates as carbon sources by mycobacteria

If more than one carbohydrate is present in the growth medium, bacteria normally take up and utilize only one carbon source at a time and leave the other substrate(s) in the medium for later use. Hierarchical sugar utilization is meant to use carbon and energy sources economically (BRUCKNER and TITGEMEYER 2002). This phenomenon is called Carbon Catabolic Repression. It means that the presence of a carbon source in the medium can repress expression of certain genes and operons, whose gene products are often concerned with the utilization of alternative carbon sources (STULKE and HILLEN 1999). In the vast majority of documented cases, the preferred carbon source for bacteria is glucose with the famous *Escherichia coli* glucose-lactose diauxia as a classical example. On the contrary, the lactic acid bacterium *Streptococcus thermophilus* prefers lactose over glucose, indicating that adaptation to special ecological niches may result in the choice of practically any carbohydrate as favored substrate (BRUCKNER and TITGEMEYER 2002).

Nutrient uptake into mycobacterial cell has its own characteristics. In experiments with *Mycobacterium phlei*, Tepper (1968) has found that quantitatively the uptake and

assimilation of glycerol was far more rapid than that of glucose for all substrate concentrations employed (TEPPER 1968). It was shown that *M. phlei* cells grown on glycerol medium and glucose medium had identical rates of growth, as judged by protein and DNA synthesis. However, glycerol use up was far more rapid than that of glucose and cell mass obtained on glycerol was consistently greater than on glucose. These quantitative differences in the utilization of glycerol and glucose and increase in cell mass was attributable to an increased content of nonessential lipids and polysaccharide storage materials found in glycerol-grown *M. phlei*. Bacilli from glucose media were found to have 60 to 80 % less lipid than glycerol-grown cells (TEPPER 1968).

For cultivation of mycobacteria, glycerol is considered the preferred carbon source under laboratory conditions, although in *M. tuberculosis, M. phlei and M. smegmatis* glycerol utilization is blocked until all other carbon sources (including glutamate and asparagine) have been consumed. Growth of most mycobacterial species with glycerol results in greater mass per unit volume of medium (TUCKMAN *et al.* 1997). Besides, glycerol and its metabolic derivatives are at the interface of catabolic and anabolic processes and therefore play important regulatory roles in cell growth. Glycerol is also an important precursor of key cell wall constituents in mycobacteria (TUCKMAN *et al.* 1997). According to Tepper (1968), the respiratory activity of *M. tuberculosis* and other mycobacteria increases with increasing glycerol concentration in medium, and glycerol also stimulates respiration to greater extent than glucose (TEPPER 1968).

It has been widely documented that *M. smegmatis* can grow on many carbon sources such as polyols, pentoses, and hexoses (EDSON 1951; IZUMORI *et al.* 1976). Nevertheless, *M. smegmatis* did not grow on lactose, maltose and sucrose as a sole carbon source. Trehalose is the only disaccharide that *M. smegmatis* can use as a sole carbon source (NIEDERWEIS 2008). The majority of sugar transport systems in *M. smegmatis* (19/28) belong to the ATP-binding cassette (ABC transporter family, **Fig. 9**). *M. smegmatis* genome further possesses one putative glycerol facilitator of the major intrinsic protein (MIP) family, four sugar permeases of the major facilitator superfamily (MFS), of which one was assigned as a glucose transporter, and one galactose permease of the sodium solute superfamily (SSS). Thus, inner-membrane transport systems for polyoles, pentoses and hexoses are predicted to exist in *M. smegmatis*.

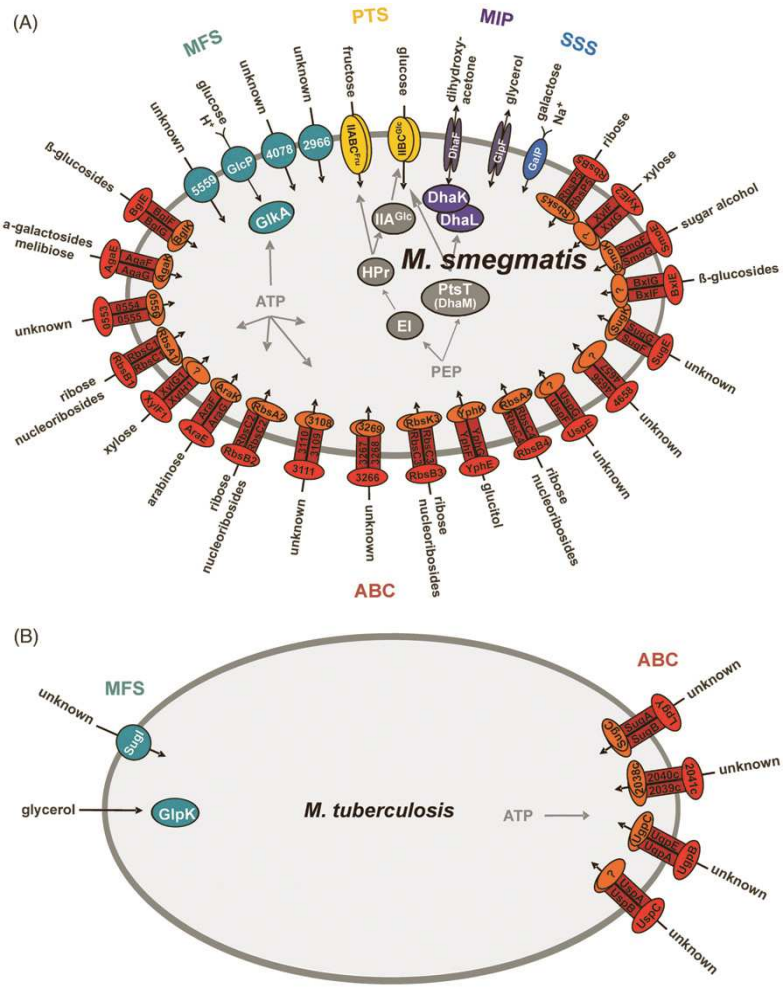

Figure 9. Transporters for uptake of carbohydrates across the inner membrane of *M. smegmatis* (A) and *M. tuberculosis* (B). The outer membranes of *M. smegmatis* and *M. tuberculosis* were omitted for clarity reasons (NIEDERWEIS 2008).

2.3.3.2 Use of another carbon sources by mycobacteria

Apart from carbohydrates (the main and preferential carbon sources) mycobacteria can use lipids as a source of carbon. Biochemical studies suggest that, in chronically infected lung tissues, fatty acids might be a major source of carbon and energy for *M. tuberculosis* (WHEELER *et al.* 1990). In another cases of saprophytic mycobacteria carbon backbones of d-amino acids can be used for this purpose.

2.3.4 Best-known members of *Mycobacterium* genus

Genus *Mycobacterium* is widely distributed in the environment. Representatives of the genus are mostly saprophytes, but there are also pathogens known to cause serious

diseases in mammals. The main interest in the genus *Mycobacterium* is due to tuberculosis causing agent *Mycobacterium tuberculosis*. It remains one of the most deadly pathogens of humans even though infections with drug-sensitive strains can be cured (NIEDERWEIS 2003).

With the exception of *M. leprae*, the mycobacteria are classified into two broad categories: members of the *Mycobacterium tuberculosis* complex (MTC) causing the tuberculosis, and nontuberculous mycobacteria (NTM, virtually all other species), which are ubiquitous in the environment, mostly non-pathogenic but some species are opportunistic pathogens. Additionally mycobacteria can be separated into two major groups, slow growers (*Mycobacterium tuberculosis, M. bovis, M. avium, M. kansasii, M. leprae*) with doubling time of up to 12-14 days (*M. leprae*), and fast growers (*Mycobacterium phlei, M. smegmatis, M. abscessus, M. chelonae, M. parafortuitum*) with doubling time of about several hours (MADIGAN and MARTINKO 2006).

2.3.4.1 *Mycobacterium tuberculosis*

M. tuberculosis, the causative agent of tuberculosis disease, was discovered in 1882 by Robert Koch. *M. tuberculosis* forms very slim rods, creating the specific cording formations, with the doubling time of 18-24 hours. Colonies on solid medium grow during 3-6 weeks in a slightly yellowish or creamy, rough cauliflower or warty type colonies with irregular edges and friable consistency (VOTAVA 2003). Like other mycobacteria, *M. tuberculosis* is highly resistant to desiccation, in the dust it withstands up to 10 days, in dry sputum to 8 months. It is transmitted by the respiratory route. Up to one-third of the world's population have been infected with these bacteria and had a latent form of tuberculosis. Most common is a lung form. In individuals with low resistance the bacteria are not controlled, and an acute pulmonary infection occurs. It is leading to the extensive destruction of lung tissue, the spread of the bacteria to other parts of the body, and death. However, in most cases of tuberculosis the acute disease does not occur, it affects only about 10 % of infected people.

According to World Health Organization (WHO) data, tuberculosis is a disease of poverty affecting mostly young adults in their most productive years. The vast majority of tuberculosis deaths are in the developing world, and more than half of all deaths occur in Asia. In 2008 there were 9.4 million new tuberculosis cases, including 1.4 million cases among people living with HIV, whose leading killer it is; 1.8 million people died, including 500 000 people with HIV. In 2009 1.7 million people died, including 380 000 people with HIV. In 2010, 8.8 million people fell ill with tuberculosis and 1.4 million died from it, despite

the fact that the tuberculosis death rate dropped 40% between years 1990 and 2010 (www.stoptb.org).

In nowadays the big medical problem is resistant strains of *M. tuberculosis*. Infectious strains that are resistant to a single anti-tuberculosis drug have been documented in every country surveyed. Multidrug-resistant tuberculosis is a form of tuberculosis caused by bacteria that do not respond to, at least, isoniazid and rifampicin (the two most powerful, first-line anti-tuberculosis drugs). There were about 650 000 cases of multidrug-resistant tuberculosis present in the world in 2010, and about 150 000 died due to this form of tuberculosis (www.stoptb.org).

2.4.3.2 *Mycobacterium bovis*

A common pathogen of dairy cattle, *Mycobacterium bovis*, is pathogenic for humans, as well as animals. *M. bovis* enters humans via the intestinal tract, typically from the ingestion of raw milk. After a localized intestinal infection, the microorganism eventually spreads to the respiratory tract and initiates the classic symptoms of pulmonary tuberculosis or tuberculosis of the cervical and mesenteric lymph nodes. Pasteurization of milk and elimination of diseased cattle have eradicated bovine-to-human transmission of tuberculosis. *M. bovis* is quite different microorganism from *M. tuberculosis*, but they are highly homologous at the DNA level (MADIGAN and MARTINKO 2006).

M. bovis BCG is a weakened strain that is used all over the world as vaccine against tuberculosis.

2.4.3.3 *Mycobacterium leprae*

Mycobacterium leprae, discovered by G. A. Hansen in 1873, is the causative agent of leprosy (Hansen's disease). *M. leprae* is the only *Mycobacterium* species that has not been grown on artificial media. The only experimental animal that has been successfully used to grow this bacillus and reproduce a similar disease is the armadillo.

M. leprae has the longest doubling time of all known bacteria – 12-14 days. It is interesting that this bacterium represents an extreme case of reductive evolution: only less than half of its genome contains functional genes (WHEELER *et al.* 1990).

In most cases the source of infection is diseased by leprosy, but scientists are still searching for natural reservoirs of this bacterium. *M. leprae* is the only bacterium that affects peripheral nerves, and then multiplies in the skin, the nasal cartilages and bones of the fingers.

2.3.5 *Mycobacterium smegmatis*

M. smegmatis is a rapidly growing, non-pathogenic saprophytic bacterium (**Fig. 10**). It commonly occurs in soil and water, on plants. The species was first isolated and discovered in 1884 by Lustgarten. By its modern name *M. smegmatis* species was called in 1899. It reveals all the essential features of the genus (see **Chapter 2.3.1**).

Systematic position of the *M. smegmatis* (www.ncbi.nlm.nih.gov):

Domain Bacteria;

Phylum Actinobacteria;

Class Actinobacteridae;

Order Actinomycetales;

Family Mycobacteriaceae;

Genus *Mycobacterium*.

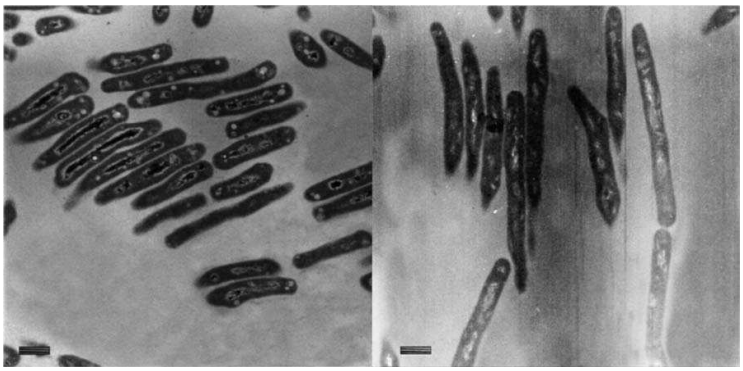

Figure 10. Electron micrograph of *M. smegmatis mc^2 155* taken by Transmission Electron Microscope (NIEDERWEIS 2003).

M. smegmatis cells creates finely wrinkled and creamy white colony while it is growing on accessible nutrients. When *M. smegmatis* has been growing for quite some time (generally after 48 hours growth) and is abundant, the color will turn from white to a non-pigmented creamy yellow. It will also be waxy because of the unique Gram-positive cell wall coated with mycolic acids. The bacterium also ranges in textures, being seen as smooth, flat and glistening or coarsely folded or finely wrinkled (www.microbewiki.kenyon.edu).

M. smegmatis does not normally reside in any animals, and does not cause dangerous or even hardly any infections. But in case of immunosuppression (like in the case of HIV-patients) *M. smegmatis*, as well as *M. wolinskyi*, *M. goodii*, *M. thermoresistible* and

M. palustre, have been reported to cause wound infections and also bacteremia (KATOCH 2004).

The strain *mc² 155* is a mutant of *M. smegmatis*, isolated in 1990 (SNAPPER *et al.* 1990). Unlike other strains of this species, *mc² 155* can be 10 to 100 thousand times more efficiently transformed with plasmid vectors than the parental strain. Thus, in contrast to other fast-growing mycobacteria, *M. smegmatis* strain *mc²155* in particular is very useful for the molecular analysis of other species in the genus *Mycobacterium* (FUJIWARA *et al.* 2012).

The genome of *M. smegmatis* is constituted by 6,988,209 base pairs. It has 67% guanine cytosine content and 33% adenosine thymine content, and is therefore classified as a high G-C content gram-positive bacterium. 90% of the genome (6716/6938 genes) represents coding regions that encode for 6716 proteins. The 6938 genes of the genome are composed circularly with an absence of any plasmids (microbewiki.kenyon.edu). *M. smegmatis* is a generally slow growing bacterium, although among mycobacteria it belongs to the fast growing group (doubling time about 2-3 hours) (DZIADEK *et al.* 2003).

2.4 *Mycobacterium* and *Streptomyces*

Mycobacterium and *Streptomyces* are two significant genera of the Actinobacteria class. The majority of their species are saprophytes usually occurring in soil and water, but the consequences of their vital activity for human beings differ greatly.

Streptomycetes and mycobacteria share a similar growth strategy. Growing evidence suggests the existence of similar developmental stages in the life cycles of mycobacteria and streptomycetes species (**Fig. 10**). Besides, mycobacterial cells associated with these developmental stages also display morphological alterations that resemble Streptomyces, although developmental stages of mycobacterial life cycle are not well defined (SCHERR and NGUYEN 2009). Species of both genera display flexible apical growth modus that is characterized by insertion of new peptidoglycan precursors exclusively at the growth apexes, the hyphal tips in *Streptomyces* or the two cell poles in *Mycobacterium* (SCHERR and NGUYEN 2009).

The *Streptomyces* genus is characterized by the ability to form spores. *Mycobacterium* genus was generally considered as non-spore-forming Actinobacteria, but recently many lines of evidence have suggested that mycobacteria undergo analogous developmental stages to its of as streptomycetes, including sporulation (GHOSH *et al.* 2009; TRAAG *et al.* 2010).

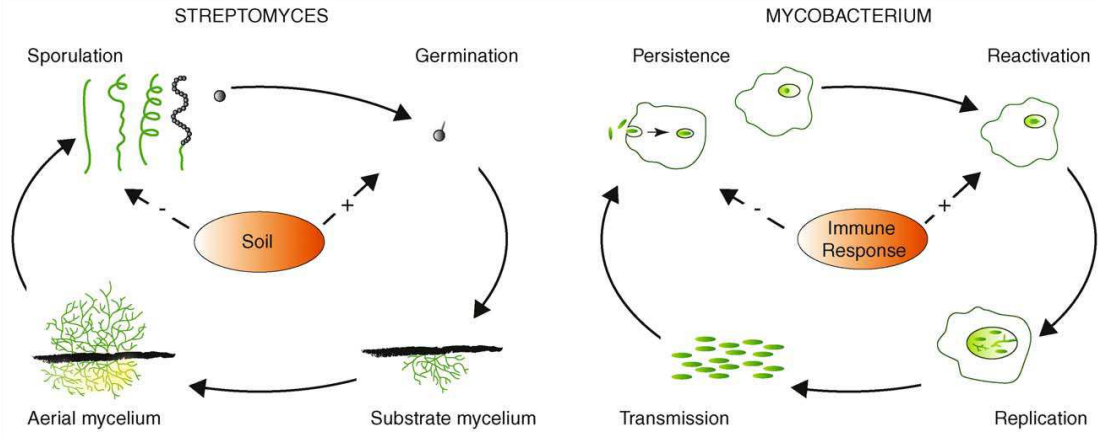

Figure 10. Developmental and morphological stages of *Streptomyces* and *Mycobacterium* life cycles. The orange ovals at the centers represent the microenvironments that trigger developmental and morphological alterations of the bacteria. Unfavorable conditions (–) induce the formation of dormant cells with increased survival capabilities, whereas the return of favorable conditions (+) triggers the exit of dormancy and reactivation of vegetative growth (SCHERR and NGUYEN 2009).

Although mycobacteria and streptomycetes have common features, their influence on human health is different. Some species of mycobacteria are pathogens that have haunted humans hundreds of years (see **Chapter 2.3.4**). In contrast, *Streptomyces* species are best known as abundant producers of antibiotics that save humans from deadly bacterial infections.

Representatives of *Streptomyces* and *Mycobacterium* genera have different morphology. Streptomycetes species have a filamentous mycelium septate in their spores at later stages of the life cycle. In contrary to this morphology of streptomycetes, mycobacteria generally display a rod-like morphology although pleomorphism has been reported for many species of this group (KIRSEBOM *et al.* 2012). Moreover, the multi-layered cell wall of mycobacteria is more similar to the cell wall of Gram-negative bacteria than to it of Gram-positive bacteria, including *Streptomyces*.

2.4.1 Cultivation of streptomycetes on solid medium covered with cellophane

Cellophane is a thin, flexible, transparent sheet made of regenerated cellulose. Its low permeability to air, oils, greases, bacteria and water makes it useful for food packaging. Chemically cellophane is a polymer of glucose. It contains the following chemical elements:

carbon, hydrogen and oxygen. Cellophane is transparent to the visible spectrum of light (www.en.wikipedia.org). Cellophane membrane is semi-permeable for nutrients contained in the underlying layer of the medium.

Cultivation of bacteria on a solid medium covered with cellophane is often exploited with *Streptomyces* genera. This technology began to be used for facilitating manipulations with colonies and harvesting spores (HOPWOOD and GLAUERT 1960). For instance, Sermonti (1966) used cellophane transfer technique to investigate genetic background of S*treptomyces coelicolor* sensitivity to streptomycin (SERMONTI *et al.* 1966).

2.4.2 Cultivation of other microorganisms on solid medium covered with cellophane

Due to its properties cellophane was widely used for studying morphology, cytology and physiology of fungi and bacteria since 40-th years of XX century. Cellophane technique was applied by Carmichael for studying hyphall fusions and for observation the growth of yeast-like fungi (CARMICHAEL 1956). The method of bacteria cultivation on the surface of cellophane sheets was developed to prepare suspensions of bacteria reasonably free from contamination by the culture medium. Reed and McKercher (1948) used procedure which consisted in spreading a sheet of cellophane over a layer of absorbent material (cotton) saturated with the desired fluid culture medium. The microorganisms were cultivated on the surface of the cellophane (REED and MCKERCHER 1950). Increased yields of staphylococcal enterotoxins have been observed and reported in the literature from cultures of *Staphylococcus aureus* grown on cellophane-over-agar media (MINOR and MARTH 1972). In Gorelick (1951) has shown that unusually high concentrations of bacteria can be obtained by cultivation in a charcoal-cellophane system (GORELICK *et al.* 1951).

Despite the fact that using of cellophane is not very common for experiments with mycobacteria, it was occasionally used for this purpose, for instance while developing a cellophane membrane mutant-screening method (VAJDA 1979).

Cellophane is still useful for investigation of bacterial and fungal biofilms (GIBBONS 2011; KIRISITS *et al.* 2005; WOZNIAK *et al.* 2003). Similarly it can be used as an *in vitro* model for replication of a helical growth of human fungal pathogens such as *Candida albicans* (BRAND 2012).

2.5 Use of proteomics in study of mycobacteria

Proteomics is the large-scale study of proteins, particularly their structures and functions (ANDERSON and ANDERSON 1998). Classical proteomics combines 2-DE for the separation and quantification of proteins and mass spectrometric identification (MS) of selected proteins. More recently the combination of liquid chromatography, stable isotope tagging and tandem MS has emerged as an alternative to quantitative proteomics technology (SCHMIDT *et al.* 2004).

The term proteome was coined by Marc Wilkins in 1994. Wilkins used this term to describe the entire set of proteins expressed by a genome, cell, tissue or organism (WILKINS *et al.* 1996). More specifically, it is the set of expressed proteins in a given type of cell or in an organism at a given time under defined conditions. The proteome is larger than the genome, in the sense that there are more proteins than genes due to alternative splicing of proteins and post-translational modifications like glycosylation, phosphorylation or protein turnover (MEHAFFY *et al.* 2012). Perhaps, protein modifications are one of the most important and yet challenging subjects in the field of functional proteomics. Due to new developments in MS instrumentation and proteomic data analysis, the characterization of post-translational modifications is possible now (MEHAFFY *et al.* 2012).

Most of the proteomic studies of the *Mycobacterium* genera have been conducted in the field of proteomics of pathogenic species, mainly with *M. tuberculosis*. The proteome of the *M. tuberculosis* cell wall, membrane, cytosol, lysate, and culture filtrate was defined and analyzed from 1997 to 2011 with a high coverage. Involved techniques included one- and 2-DE, MS, isotope-coded affinity tag technology, protein profiling, liquid chromatography, label-free proteomic method (BELL *et al.* 2012). The proteomes of *M. bovis BCG* and *M. tuberculosis* were compared in attempt to identify protein candidates for the development of vaccines, diagnostics and therapeutics (JUNGBLUT *et al.* 1999). While describing the proteome of *M. tuberculosis* there was used bioinformatics to assess the level of gene duplication undergone by *M. tuberculosis* to probe the chromosomal context of multigene families (TEKAIA *et al.* 1999). These and many other studies could result in the development of improved strategies for mycobacterial disease control and prevention through vaccination and immuno-diagnosis.

Many proteins required for pathogenicity of mycobacteria are surface proteins. Thus, cell wall proteome describing remains essential for study of mycobacteria. Representatives of *Mycobacterium* genus share much of surface-exposed proteome with related species. These

proteomic similarities prove that many of the functions that are essential to mycobacterial pathogenesis (attachment, the invasion of host cells and the manipulation of the host immune response) are common between the various species of *Mycobacterium* (MCNAMARA *et al.* 2012). Thus it is possible to say that the results of proteomic studies of non-pathogenic species can be applied to the study of *M. tuberculosis*. For instance, McNamara with colleagues in 2011 identified more than 100 putative surface proteins in *M. avium* subsp. *hominissuis*, which can cause opportunistic infections in humans (MCNAMARA *et al.* 2012). He and De Buck in 2010 obtained a comprehensive picture of the *M. smegmatis* cell wall protein repertoire, which was the first proteomic analyses of cell wall proteins of *M. smegmatis* mc^2 *155.* As a result there were identified 390 proteins divided into 21 functional groups. The methods used were one-dimension electrophoresis, shotgun liquid chromatography and tandem MS (HE and DE BUCK 2010).

3. MATERIAL AND METHODS

3.1 Material

3.1.1 Strain

The bacterium we used in our experiments was a laboratory non-pathogenic strain of *M. smegmatis mc^2 155*. It is a highly transformable mutant often used for DNA cloning (SNAPPER *et al.* 1990).

3.1.2 Culture media

7H9 broth (DIFCO, France). Base for the cultivation of mycobacteria (pH 6,6±0,2):
4,7 g of the powder is dissolved in 1 l of distilled water (dH_2O). Glycerol is added to the final concentration of 0,2% (v/v). Tween 80® is added for submerged cultivation after sterilization to the final concentration of 0,05 % (v/v).

7H10 agar (DIFCO, France). Base for the cultivation of mycobacteria (pH 6,6±0,2):
19 g of the powder is dissolved in 1 l of dH_2O. Glycerol is added to the final concentration of 0,5% (v/v).

3.1.3 Buffers and solutions

3.1.3.1 Buffers and solutions for isolation of the samples

Sonication buffer (pH 8,0):
50 mM TRIS, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, Protease Inhibitor Cocktail.

Tris is mixed with NaCl and EDTA, this solution is sterilized and stored at 4 °C. 1M DTT stock solution is stored separately at -20 °C. 100 mM PMSF stock solution is stored separately at -20 °C. PMSF is dissolved in isopropanol.

Complete^{TM} Mini – Protease Inhibitor Coctail Tablets (ROCHE, Germany):
One tablet is dissolved in 0,5 ml of deionized water (ddH_2O). Solution is stored at -20 °C.

Phosphate buffer (pH 6,8):

0,1 M $NaH_2PO_4 \cdot 2\ H_2O$, 0,1 M $Na_2HPO_4 \cdot 12\ H_2O$.

51 ml of $NaH_2PO_4 \cdot 2H_2O$ is mixed with 49 ml of $Na_2HPO_4 \cdot 12H_2O$. Store at 4 °C.

50% TCA:

25 g TCA is dissolved in distilled water to final volume of 50 ml. Stock solution is stored at 4 °C.

3.1.3.2 Buffers and solutions for 2-DE

Sample buffer:

7 M Urea, 2 M Thiourea, 4% CHAPS (w/v), 1 % Ampholyns (pH 3-10, w/v), 1 % DTT (Dithiothreitol) (w/v), Bromophenol Blue – few crystals, dH_2O. Store at -80 °C.

Tris-HCl buffer:

5 M Tris-HCl (pH 8,8, w/v)

130,8 g of Tris base and 66,3 g of Tris-HCl is dissolved in 1000 ml of ddH_2O. Store at 4 °C.

Acrylamide stock:

30% Acrylamide (w/v), 0,8% Bisacrylamide (w/v), ddH_2O.

Solution is filtered and stored in the dark at 4 °C.

Composition of polyacrylamide gels:

Acrylamide stock, 5 M Tris-HCl, 10% SDS (Sodium Dodecyl Sulfate, w/v), 10% APS (Ammonium Persulfate, w/v), TEMED, ddH_2O.

Solution must be freshly prepared.

0,5% Agarose :

0,5 g of Agarose is dissolved in 100 ml of 1x Tank Buffer. Solution must be freshly prepared.

10% SDS:

100 g of SDS is dissolved in dH_2O to final volume of 1000 ml. Store at r. t. (room temperature).

15 mM DTT:

0,024 g of DTT is dissolved in 10 ml of dH_2O. Store at -20 °C.

10% APS:

0,05 g of APS is dissolved in 450 µl of dH_2O. Solution must be freshly prepared.

10 x Tank Buffer (pH 8,3):

0,25 M Tris, 1,92 M Glycine, 1% SDS (w/v), dH_2O. Store at r. t.

Equilibrating stock solution:

0,5 M Tris-HCl pH 6.8, 6 M Urea, 30% glycerol (v/v), 2% SDS (w/v). Store at r. t.

Equilibrating solution with DTT:

033 g of DTT is added to 15 ml of equilibration stock solution. Solution must be freshly prepared.

Equilibrating solution with IAA:

0,375 g of IAA (Iodoacetamide) and a few crystals of Bromophenol Blue are added to 15 ml of equilibration stock solution. Solution must be freshly prepared.

3.1.3.3 Solutions for Colloidal Coomassie Blue staining

Fixing solution:

50% Methanol (v/v), 7%Acetic acid (v/v). Store at r. t.

Stainer A:

40% Ammonium sulphate (w/v), 8% Orthophosphoric acid (v/v), ddH_2O. Store at r. t.

Stainer B:

1,2% Coomassie Blue G-250 (w/v), ddH_2O. Store at r. t.

Final staining solution:

20% Methanol (v/v), 20% Stainer A (v/v), 5% Stainer B (v/v). Solution must be freshly prepared.

3.1.4. Chemicals and kits

Chemical	Producer	Abbreviation
Acetic acid	ACHNER	
Acetone	MERCK	
Acrylamide	SERVA	
Agar	OXOID	
Agarose	SIGMA	
Ampholine mixture (pH 3-10)	SIGMA	
Ammonium persulfate	SIGMA	APS
Benzonase	MERCK	
Bisacrylamide	SERVA	
Bromophenol blue	MERCK	
Chaps	SERVA	
Dibasic sodium phosphate	MERCK	
Dithiothreitol	SERVA	DTT
Ethylenediaminetetraacetic acid	SIGMA	EDTA
Ethanol	MERCK	
Formaldehyde	SIGMA	
Glutaraldehyde	SIGMA	
Glycerol	AMERSHAM	
Glycine	SERVA	
Hydrochloric acid	LACHEMA	
Iodoacetamide	SIGMA	IAA
Lysozyme	SERVA	
Magnesium sulfate	LACHEMA	
Methylene blue	LACHEMA	
Mineral oil	AMERSHAM	
Phenylmethylsulfonyl fluoride	SIGMA	PMSF
Potassium carbonate	FLUKA	
Potassium dihydrogen phosphate	LACHEMA	
Potassium chloride	LACHEMA	
Potassium hydrogen	LACHEMA	

Sodium acetate	SIGMA	
Sodium chloride	LACHEMA	
Sodium dihydrogen	MERCK	
Sodium dodecylsulfate	SERVA	SDS
Sodium hydroxide	MERCK	
Sodium thiosulfate	APPLICHEM	
Tetramethylethylenediamine	SERVA	TEMED
Thiourea	SIGMA	
Trichloroacetic acid	SERVA	TCA
Tris	APPLICHEM	
Tris HCl	SERVA	
Tween 80 ®	SERVA	
Urea	APPLICHEM	

Kits

Determination of protein concentration:

Pierce® BCA Protein Assay Kit	THERMO SCIENTIFIC, USA
2D Quant kit	GE HEALTHCARE, USA

Purification of protein samples for 2-DE:

ReadyPrep™ 2-D Cleanup Kit	BIO RAD, USA

3.1.5. Laboratory equipment

Laminar box

Aura Mini	BIOAIR

Centrifuges

Z160M	HERMLE
Biofuge A	HERAEUS SEPATECH
Zentrifugen universal 320R	HETTICH
MiniSpin plus	EPPENDORF

Ultrasonic homogenizer	
4710 Series	COLE PARMER
Scales	
Scales 572	KERN
Analytical SCALES A200S	SARTORIUS
Digital Dry Bath	
AccuBlock	LABNET
Vortexes, mixers, shakers	
Rocker 25	LABNET
Magnetic stirrer MM5	POLAMED
Magnetic stirrer 4802-02	COLE PARMER
Vortex Genie	BENDER HOBEIN AG
Vortex Mixer SA8	STUART
pH meter	
Seven Easy	METTLER TOLEDO
Adjustable Mechanical Pipettes	GILSON
Spectrophotometer	
DU® 730 Life Science	BECKMAN COULTER
Camera	
Power Shot A530	CANON
Stereo microscope	
National	MOTIC
Microscopes	
Optical microscope Motic BA 300	MOTIC
Scanning Electrone Microscope Aquasem	TESCAN

Softwares

Motic Images Plus 2.0, software for	
Stereo microscope and Optical microscope	BIO RAD
PDQuest™ 7.1.0, 2-D Gel Analysis Software	BIO RAD

Equipment for 2-DE

1. Dimension:	
Multiphor II Electrophoresis system	PHARMACIA BIOTECH
Electrophoresis Power Supply EPS 3501 XL	AMERSHAM BIOSCIENCES
2. Dimension:	
Investigator™ 5000	GENOMIC SOLUTIONS

3.2 Methods

3.2.1 Cultivation of the *M. smegmatis* in 7H9 broth

First, individual colonies were grown on agar plates from stock culture kept in glycerol at -80 °C. Colonies were grown for 3 days at 37 °C. Then, always one colony was transferred into the liquid medium with glycerol or glucose as carbon source containing 0,05% Tween 80. Tween 80 was added to prevent clumping of cells in the culture. This pre-culture has grown overnight (12-20 hours) to exponential phase. Then 25 µl of it were inoculated into the 25 ml of the same fresh medium to grow the culture from which the samples for electrophoresis were taken. The culture was grown on shaker for about 9 hours at 37 °C, when it reached optical density from 1 to 1,3 (exponential phase).

3.2.2 Cultivation of the *M. smegmatis* on 7H10 agar plates and agar plates covered with cellophane

Freshly grown culture (50 µl, optical density about 0,5) was inoculated onto the 7H10 plates. Cultures were grown for 3 days at 37 °C.

A wet cellophane disk was placed before inoculation on agar for cultivation on agar plates covered with cellophane. Cellophane disk was pre-sterilized in distilled water. Cultures grew for 3 days at 37 °C.

3.2.3 Microscopy

3.2.3.1 Optical microscopy

Digital Microscope Motic was used during experiments, as one of the possibilities to check the purity of the cultures. A drop of culture was put on a microscopic slide, covered with a cover glass and observed.

3.2.3.2 Stereo microscope

Digital Stereo microscope National was used for studying of the colonies morphology after the 3 and the 6 days of the cultivation using appropriate magnification (10 x and 30 x).

3.2.3.3 Scanning Electron Microscopy (SEM)

SEM was used for detailed study of *M. smegmatis* colonies after 3 and 6 days of cultivation. The procedure was conducted in the Laboratory of Molecular Structure Characterization (Institute of Microbiology, Academy of Sciences). Samples were processed according to Kofrońová et al.: they were fixed in vapor of 2% OsO_4 in desiccator for at least 1 week with subsequent alcohol dehydration (KOFROŇOVÁ *et al.* 2002). Prepared samples were observed in the Scanning Electron Microscope Aquasem (TESCAN) at 15 kV.

3.2.4 Preparation of protein samples from the planktonic culture of *M. smegmatis*

50 ml of culture grown to optical density between 1,0 to 1,3 were transferred into a sterile centrifuge tube and centrifuged 10 min at 5 000 g at 4 °C (10 x 5) to sediment the cells. The supernatant was removed and the sediment was washed with phosphate buffer to remove the rest of the medium. After short centrifugation – 5 min at 5 000 x g at 4 °C (5 x 10) and the supernatant removed the sediment was resuspended in the sonication buffer. The suspension was sonicated on ice: eight 30 sec cycles with, 1 minute pause between the cycles. Then the suspension was centrifuged 10 min at 9 000 g at 4 °C (10 x 9) and 1 volume of 50% TCA was added to 4 volumes of separated supernatant. The solution was incubated on ice for one hour and after incubation centrifuged (10 x 9). The pellet was washed with 0,5-1,0 ml of ice cold ethanol to remove remains of salts and buffer components. After 5 min incubation 0,4-1,0 ml of ice-cold acetone was added to precipitate proteins. The solution was centrifuged (10 x 9) and sediment washed with acetone/ethanol/water (2:2:1). Obtained proteins in sediment were solubilized in 0,4-1,0 ml of phosphate buffer.

3.2.5 Preparation of protein samples from the *M. smegmatis* culture grown on agar plates

Three-day-old bacterial biomass was scraped from agar plates and transferred into centrifugation tube. 1 ml of phosphate buffer was added to remove the rest of the medium. The suspension was dispersed on vortex mixer and centrifuged (5 x 5). Obtained sediment was resuspended in 5 ml of sonication buffer. Following steps were identical as described in chapter 3.2.4.

3.2.6 Protein concentration measurement by BCA protein assay kit (Pierce)

Prior starting the procedure we diluted albumin solution for standard curve and prepared working reagent (50 parts reagent A + 1 part reagent B). Then 0,05 ml samples were mixed with 1 ml of working reagent. These mixtures were incubated 30 min at 37 ºC, and their optical density (OD) measured in spectrophotometer (DU® 730 Life Science) at 562 nm. Unfortunately this method of protein concentration measurement was not accurate enough for our purposes because of special characteristics of *Mycobacterium* samples. So we had to use Pierce assay only for the first approximate measurements with subsequent use of more precise measurement by 2-D Quant kit (see below).

3.2.7 Treatment of the samples by 2-D Cleanup kit

Before the samples cleaning we needed at least 400 µg of proteins in phosphate buffer for one sample (during the cleaning process we usually lost about half of the proteins). For this purpose we calculated required amount of proteins from Pierce standard curve. This amount of proteins was then precipitated in 3 volumes of acetone overnight at -20ºC. Suspension was centrifuged (10 x 9), supernatant was removed and sediment was resuspended in 0,4-1,0 ml of phosphate buffer.

Precipitating agents 1 and 2 were added with mixing, then suspension was centrifuged (10 x 9) and the supernatant was discarded. We followed instructions of the kit producer and the final protein sediment was resuspended in 400 µl of the sample buffer. Samples were stored at -80 °C.

3.2.8 Protein concentration measurement by 2-D Quant kit

Prior starting the procedure we diluted albumin solution for standard curve and prepared working color reagent (100 parts of color reagent A + 1 part of color reagent B). Then 20 µl samples were mixed with precipitant, co-precipitant and centrifuged (10 x 12). Then copper solution and distilled water were added, and this solution was mixed with 1 ml of working color reagent. These mixtures were incubated 15 min at room temperature, and OD measured in spectrophotometer at 480 nm.

This method provided a more accurate measurement of protein concentration of our samples.

3.2.9 Two-dimensional electrophoresis of proteins

2-DE is a method used for separation of complex mixtures of soluble proteins. The first dimension is isoelectric focusing; it means the separation of proteins according to their isoelectric points (pI). The separation takes place in an electric field in presence of an ionized substance that forms a pH gradient. The second dimension is polyacrylamide gel electrophoresis in presence of SDS, in which the proteins can be separated according to their molecular weight. By combining these two effective techniques thousands of proteins can be separated.

3.2.9.1 Rehydration of the samples

Samples were diluted by a sample buffer after determination of protein concentration to get desired amount of protein (200 µg) in volume of 380 µl and applied to immobilized pH gradient (IPG) strip by process of rehydration. It allows penetration of the proteins into dehydrated gel on commercial IPG-strip. We used the IPG – strips with pH 4-7 gradient and 18 cm length. Rehydration was running over night at r. t. under a layer of inert mineral oil to prevent drying and oxidation of the strips.

3.2.9.2 Isoelectric focusing (IEF)

An IEF was performed after rehydration using devices Multiphor II (PHARMACIA BIOTECH, GB) and Electrophoresis Power Supply EPS 3501 XL (AMERSHAM BIOSCIENCES, USA) under following conditions:

Step	Voltage	Amperage	Wattage	Electric power	Time
Step 1	150 V	1 mA	5 W	1 Vh	
Step 2	150 V	1 mA	5 W	300 Vh	2 h
Step 3	300 V	1 mA	5 W	1 Vh	
Step 4	300 V	1 mA	5 W	600 Vh	2 h
Step 5	3500 V	1 mA	5 W	10 kVh	5 h
Step 6	3500 V	1 mA	5 W	60 kVh	17.1 h
Total				70900 Vh	26.1 h

After IEF the strips were removed from the device and equilibrated in buffer as described further.

2.2.9.3 Equilibration of the strips

Each IPG strip was equilibrated for 10 min in 6 ml of equilibration solution with dithiothreitol (DTT) and 10 min in 6 ml of equilibrating solution with iodoacetamide (IAA) and bromophenol blue before the second dimension of electrophoresis. The same procedure was applied when frozen (-20 °C) IPG strips, prepared in previous IEF runs, were used. After equilibration the strips were fixed in pre-prepared gels slabs.

2.2.9.4 SDS PAGE

The following solutions were mixed in a vacuum flask: acrylamide + deionized water + Tris buffer. Then the mixture was de-gassed for 10 minutes by applying vacuum. After that the appropriate volume of SDS stock solution was added to the mixture. Polymerization of gels was initiated by adding TEMED and APS solutions and immediately mixed. Prepared gels were put in glass slabs.

Strips attached to gel in slabs were overlaid with melted 0,5% agarose solution. After solidification glass slabs with gels were transferred into the Investigator 5000 electrophoresis unit (GENOMIC SOLUTIONS, USA) which was filled with running buffer. The electrophoresis was run at max voltage setting to 500 V, max power setting to 20000 mW per gel, at 15 °C using Programmable Power Supply electrophoresis device (GENOMIC SOLUTIONS, USA). The running time was about 4 h 20 min. After the procedure the slabs were disassembled and the upper corner of each gel was marked for easy determination of the orientation of the gel.

2.2.9.5 Fixation and staining of the gels

Gels were placed into fixing solution for 45 min and then transferred into a Colloidal Coomassie Blue staining solution and kept there overnight.

Next day the stain from the background of the gels was removed by multiple rinsing with deionized water.

3.2.10 Digitalization of gel images

Coomassie blue-stained gels were scanned on the scanner Epson Expression 10000 x (Epson Scan), at resolution 400. Due to the expected identification of proteins using MS gels were scanned in clear plastic pockets. The images were stored in a special data format that is

compatible with PDQuest software. The PDQuest software is then able to convert files into common formats such as "TIFF" or "JPG".

3.2.11 Computer analysis of gel images

Analysis of the proteome was carried out in PDQuest software (BIO RAD) version 7.1.0. Individual groups of protein spots were compared with each other in "Matchset" program. Matchset enabled the creation of a "Mastergels". Mastergel is imaginary gel consisting of all the protein spots found on individual gels from the set. Each protein spot had a program assigned number thus can easily be compared on all the gels where they occurred. Comparisons were made with two types of analyzes. Qualitative analysis showed whether the protein is present or absent in samples from different conditions. Quantitative analysis selected proteins occurring in both of experimental conditions but with different concentrations (expression or density spots). For all quantitative analysis we used the cut off limit 8-fold change of spot density and selection was based on a comparison of 3-5 parallel gels from each sample.

4. RESULTS

4.1 Basic physiological characteristic of the *M. smegmatis* mc^2 *155* growth

4.1.1 Growth curves of the *M. smegmatis* planktonic culture in liquid 7H9 medium

Figure 11 shows the growth curves of *M. smegmatis* in the liquid medium with glycerol and glucose as carbon sources, respectively. Exponential growth on glycerol ceased after 15 hours at optical density (OD) value about 2,0, while on glucose the culture reached stationary phase after 30 hours at OD around 6,0. Semi-logarithmic plot was then used for the calculation of *M. smegmatis* doubling time (**Fig. 12**). Calculated values were 3,267 hours for culture grown on glycerol and 4,883 hours for culture grown on glucose.

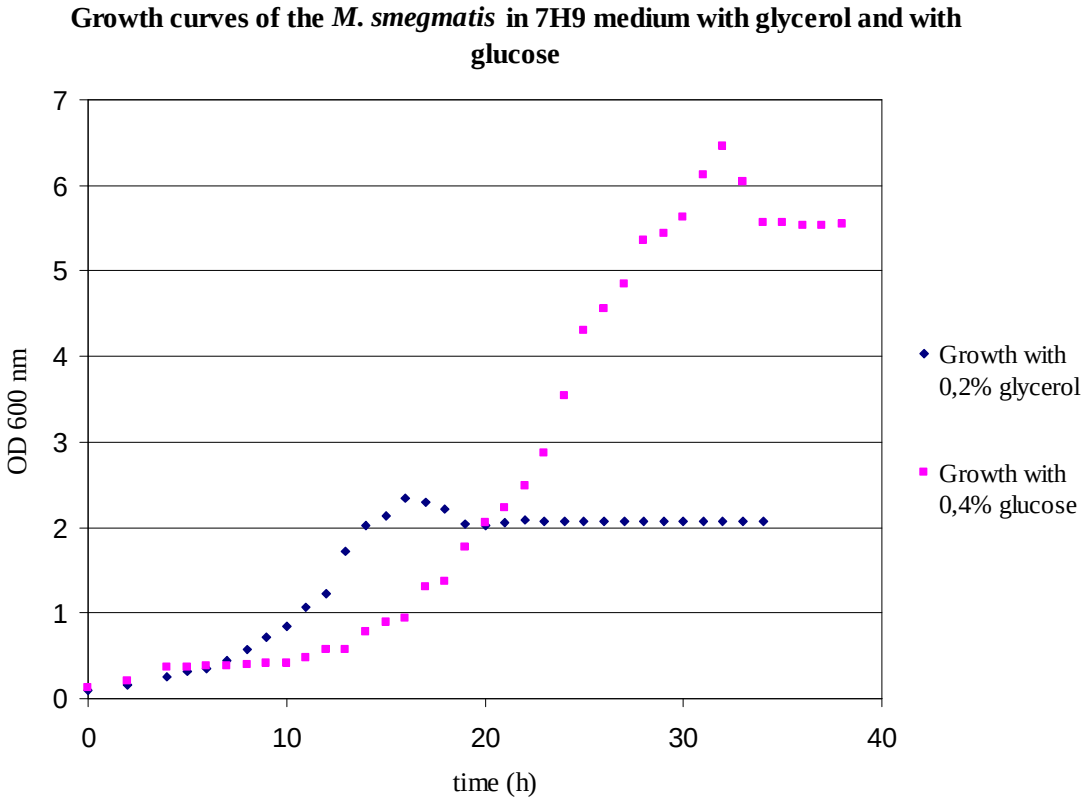

Figure 11. *M. smegmatis* growth curves in 7H9 medium with glycerol and glucose, respectively.

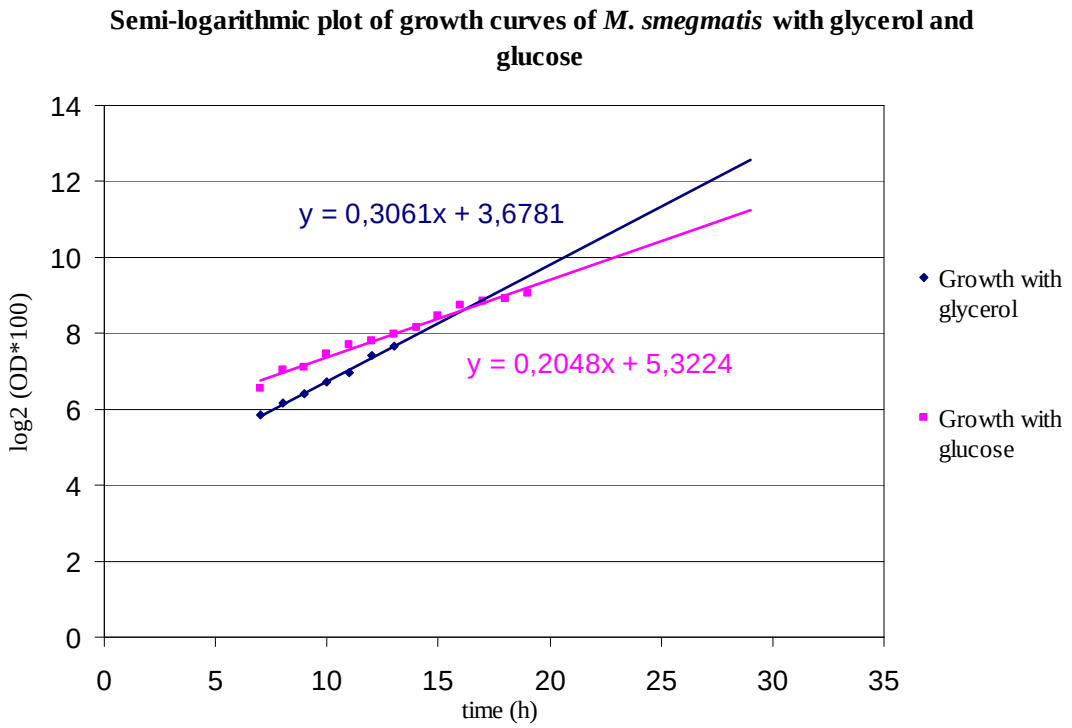

Figure 12. Semi-logarithmic plot of the *M. smegmatis* growth curves in 7H9 medium with glycerol and glucose, respectively.

4.1.2 *M. smegmatis* growth on solid surfaces

Generally colonies of *M. smegmatis* appear on solid Middlebrook 7H10 medium on the second day of the cultivation at 37 °C in the form of dots. On the third day of the cultivation they already have a distinguishable morphology, which can be registered under Stereo microscope.

4.2 Morphological study of *M. smegmatis* growth on agar plates and agar plates covered with cellophane

Agar plates and agar plates covered with cellophane were used as solid surfaces. Glycerol and glucose were used respectively as carbon sources in 7H10 medium.

The first series of pictures under Stereo microscope were taken always on the third day of incubation at 37 °C. Samples for proteome analysis were also prepared from three-day-old cultures. The plates were incubated for another three days and colonies with more pronounced morphological changes were observed.

4.2.1 *M. smegmatis* growth on agar plates

4.2.1.1 *M. smegmatis* growth on agar plates with glycerol

Figures 13 and **14** represent *M. smegmatis* colonies after 3 and 6 days of the cultivation on agar plates, respectively. Glycerol was used as a carbon source. The first photo in the series was taken by camera, the second and the third under Stereo microscope (at 10 x and/or 30 x magnification (in accordance with colony size).

The size of the colonies was 3 – 5 mm after 3 days and the morphology was already visibly wrinkled (**Fig. 13**). The size of the colonies after 6 days was about 10 mm. The morphology was typical – it had the “cauliflower” (“C”) structure (**Fig. 14**).

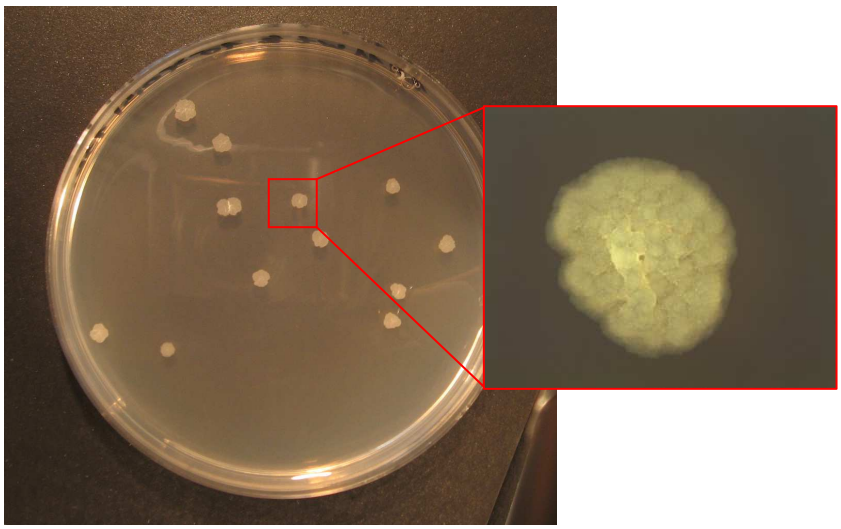

Figure 13. *M. smegmatis* colonies after 3 days of the cultivation on agar surface. Agar contained 0,5% glycerol. The magnification of the individual colony is 30 x under Stereo microscope.

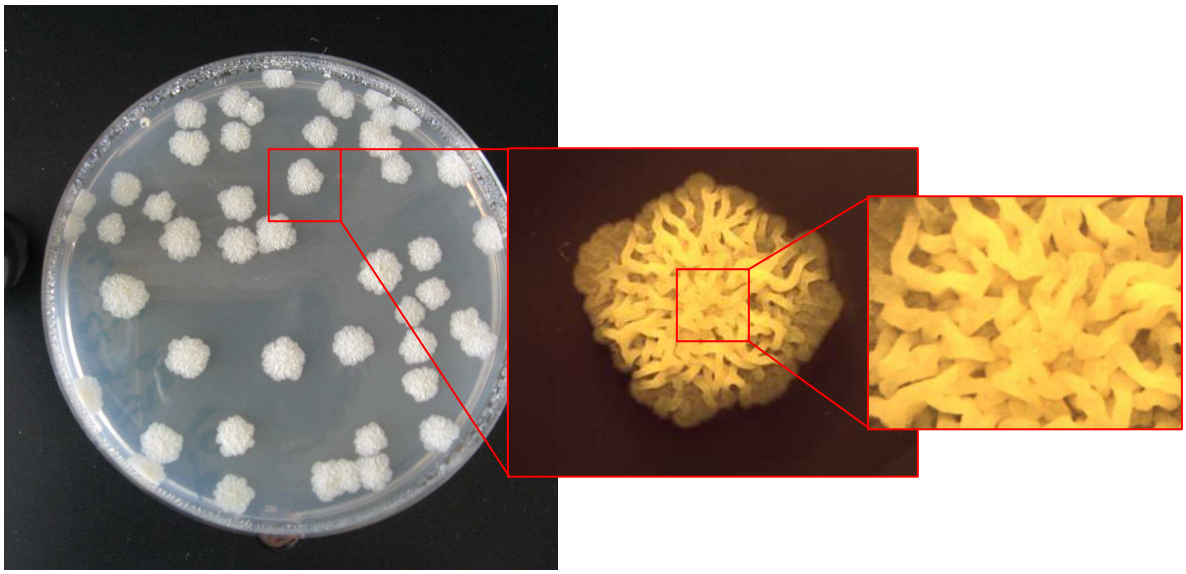

Figure 14. *M. smegmatis* colonies after 6 days of the cultivation on agar surface. Agar contained 0,5% glycerol. The magnification of the individual colony is 10 x, the magnification of colony center is 30 x under Stereo microscope.

4.2.1.2 *M. smegmatis* growth on agar plates with glucose

Figures 15 and **16** represent *M. smegmatis* colonies after 3 and 6 days of the cultivation on agar plates, respectively. Glucose was used as a carbon source. The size of the colonies was 3 – 4 mm after 3 days. The colonies were much less wrinkled than on glycerol (**Fig. 15**). The size of the colonies after the 6 days was about 10 mm, and their morphology was evidently different from the colonies grown on glycerol – they had a granular structure (**Fig. 16**).

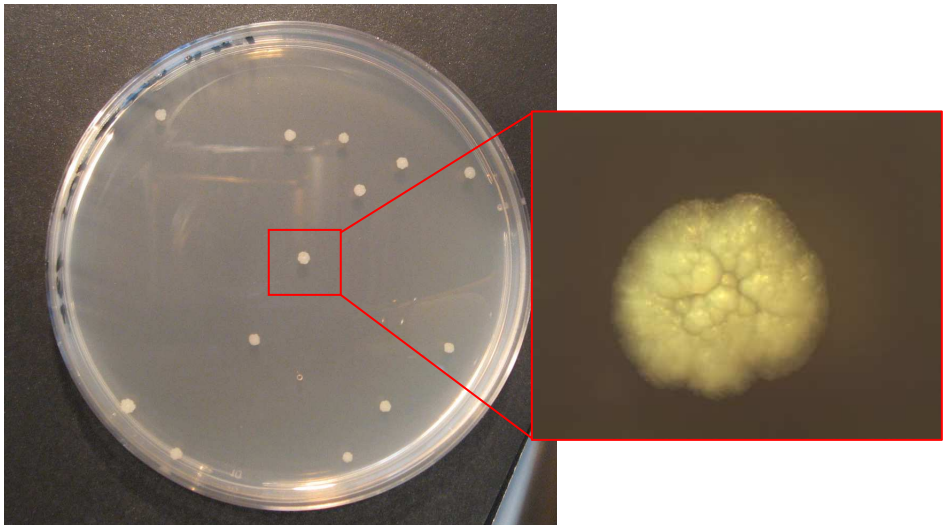

Figure 15. *M. smegmatis* colonies after 3 days of the cultivation on agar surface. Agar contained 1% glucose. The magnification of the individual colony is 30 x under Stereo microscope.

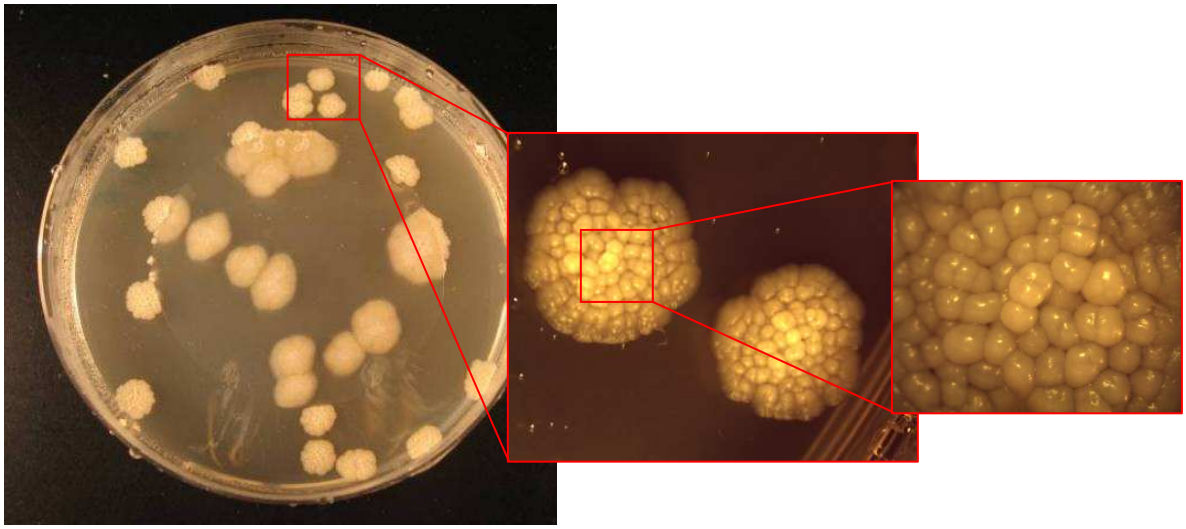

Figure 16. *M. smegmatis* colonies after 6 days of the cultivation on agar surface. Agar contained 1% glucose. The magnification of the individual colonies is 10 x, the magnification of colony center is 30 x under Stereo microscope.

4.2.2 *M. smegmatis* growth on agar plates covered with cellophane

4.2.2.1 *M. smegmatis* growth on agar plates with glycerol covered with cellophane

Figures 17 and **18** represent *M. smegmatis* colonies after 3 and 6 days of cultivation on agar plates covered with cellophane, respectively. Glycerol was used as a carbon source.

The size of the colonies was 5 – 6 mm after 3 days, their morphology was rather amorphous (**Fig. 17**). The size of the colonies after the 6 days was up to 15 mm. The colonies were flat and they had a slightly wrinkled structure (**Fig. 18**).

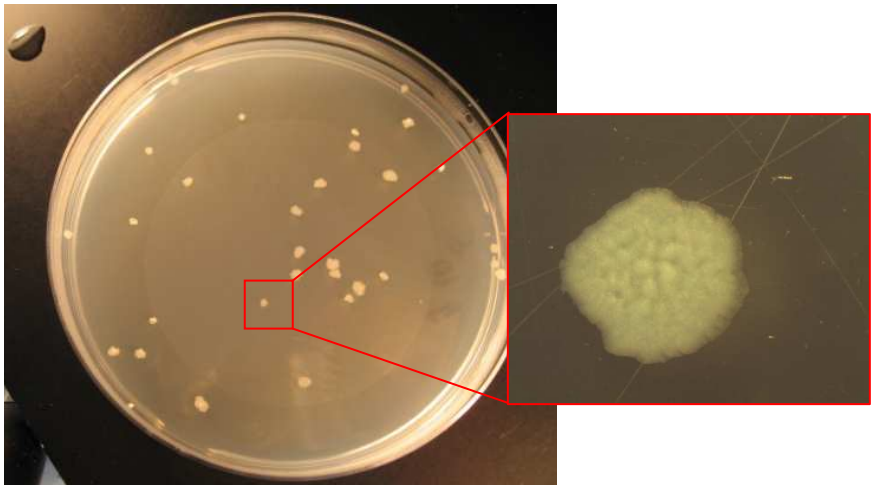

Figure 17. *M. smegmatis* colonies after 3 days of the cultivation on cellophane surface. Agar contained 0,5% glycerol. The magnification of the individual colony is 30 x under Stereo microscope.

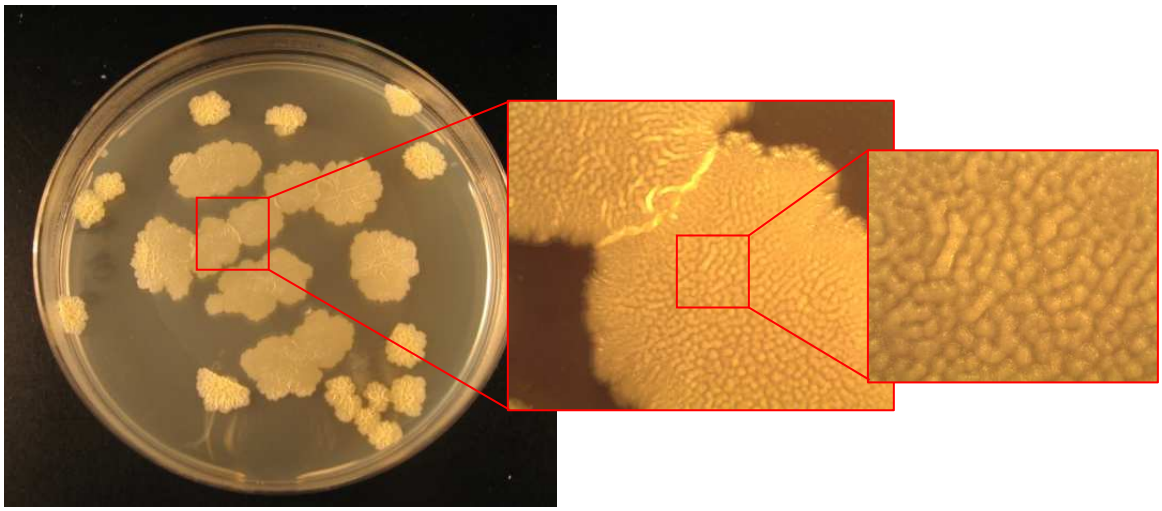

Figure 18. *M. smegmatis* colonies after 6 days of the cultivation on cellophane surface. Agar contained 0,5% glycerol. The magnification of the individual colony is 10 x, the magnification of colony center is 30 x under Stereo microscope.

4.2.2.2 *M. smegmatis* growth on agar plates with glucose covered with cellophane

Figures 19 and **20** represent *M. smegmatis* colonies after 3 and 6 days of cultivation on agar plates covered with cellophane, respectively. Glucose was used as a carbon source.

The size of the colonies was 5 – 6 mm after 3 days, their structure was shapeless like in case of the glycerol colonies (**Fig. 19**, compared with **Fig. 17**). The size of the colonies

after 6 days was up to 15 mm. The colonies were flat and they had an unusual and a slightly wrinkled structure (**Fig. 20**, compared with **Fig. 18**).

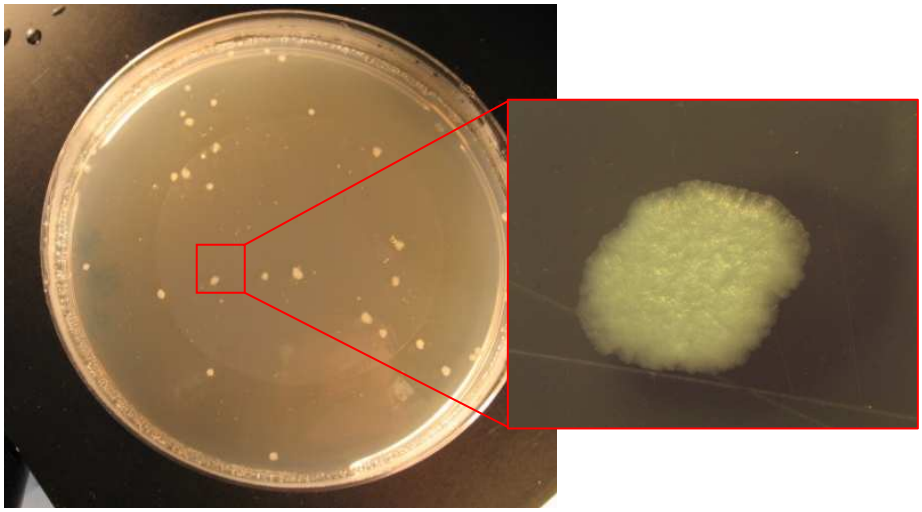

Figure 19. *M. smegmatis* colonies after 3 days of the cultivation on cellophane surface. Agar contained 1% glucose. The magnification of the individual colony is 30 x under Stereo microscope.

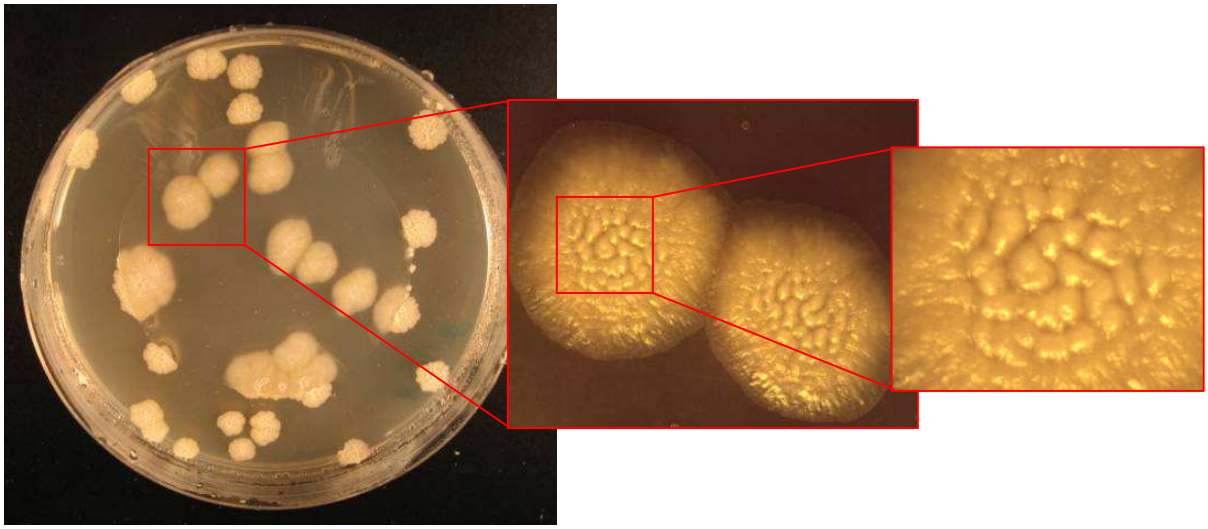

Figure 20. *M. smegmatis* colonies after 6 days of the cultivation on cellophane surface. Agar contained 1% glucose. The magnification of the individual colonies is 10 x, the magnification of colony center is 30 x under Stereo microscope.

4.2.3 Colonies on the border between agar and cellophane

4.2.3.1 Border between agar and cellophane on plates with glycerol

Colonies growing on the edge of the cellophane sheet covering agar were observed in order to see more clearly the differences in morphology of colonies growing on different surfaces and carbon sources. **Figures 21** and **22** represent *M. smegmatis* colonies growing on the border between agar and cellophane after 3 and 6 days of the cultivation. Glycerol was used as a carbon source.

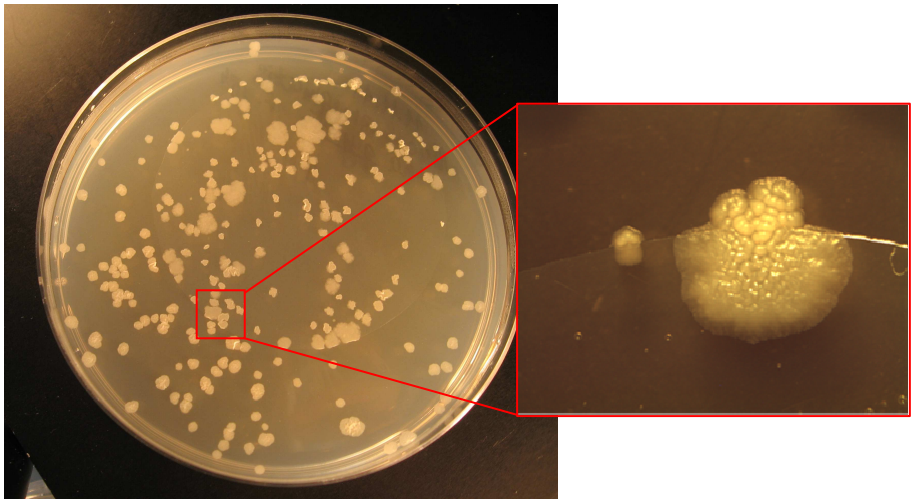

Figure 21. The border between cellophane and agar on the plate with glycerol after 3 days of the cultivation. The magnification of the individual colonies is 30 x under Stereo microscope.

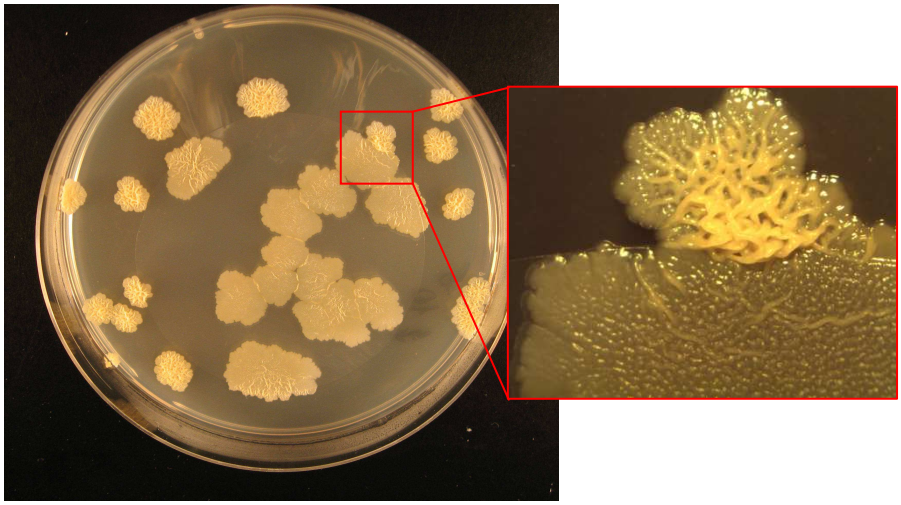

Figure 22. The border between cellophane and agar on the plate with glycerol after 6 days of the cultivation. The magnification of the individual colonies is 10 x under Stereo microscope.

4.2.3.2 Border between agar and cellophane on plates with glucose

Figures 23 and **24** represent *M. smegmatis* colonies growing on the border between agar and cellophane after 3 and 6 days of the cultivation on agar plates covered with cellophane, respectively. Glucose was used as a carbon source.

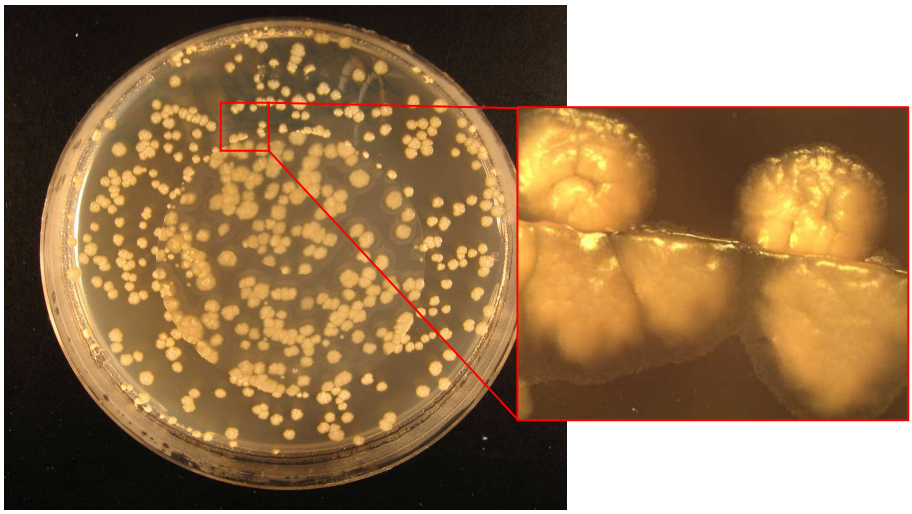

Figure 23. Border between cellophane and agar on the plate with glucose after 3 days of the cultivation. The magnification of the individual colonies is 30 x under Stereo microscope.

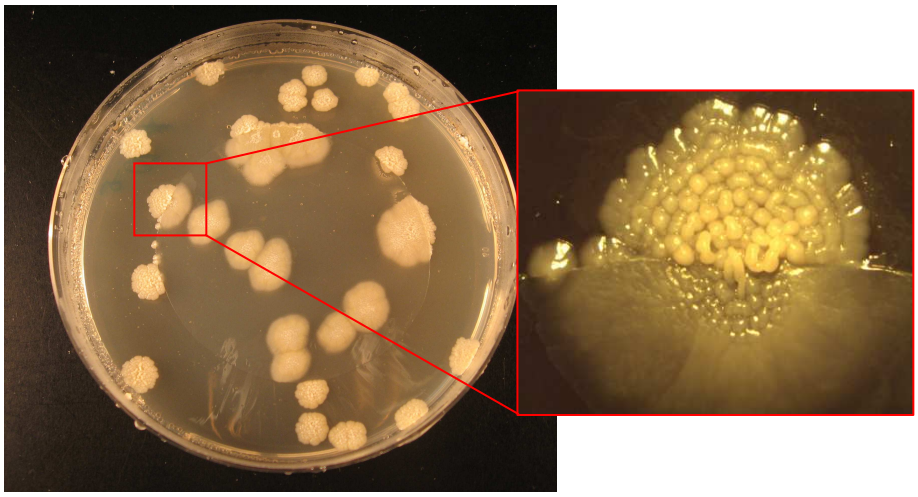

Figure 24. Border between cellophane and agar on the plate with glucose after 6 days of the cultivation. The magnification of the individual colonies is 10 x under Stereo microscope.

4.2.4 Scanning Electron Microscopy

Figure 25 represents the morphology of *M. smegmatis* "C" colonies after 3 days of cultivation on agar plate with glycerol using Stereo microscope and SEM.

Figure 26 illustrates morphology of the colony after 3 days of the cultivation on agar plate with glycerol covered with cellophane. This colony is visibly amorphous and spread on the cellophane sheet. The cellophane has absolutely smooth surface in contrast to the agar.

Figure 27 represents an arrangement of individual cells at highest magnification using SEM. It illustrates the arrangement of cells on the agar plate with glycerol (A), on the agar plate with glycerol covered with cellophane (B), on the agar plate with glucose (C), on the agar plate with glucose covered with cellophane (D).

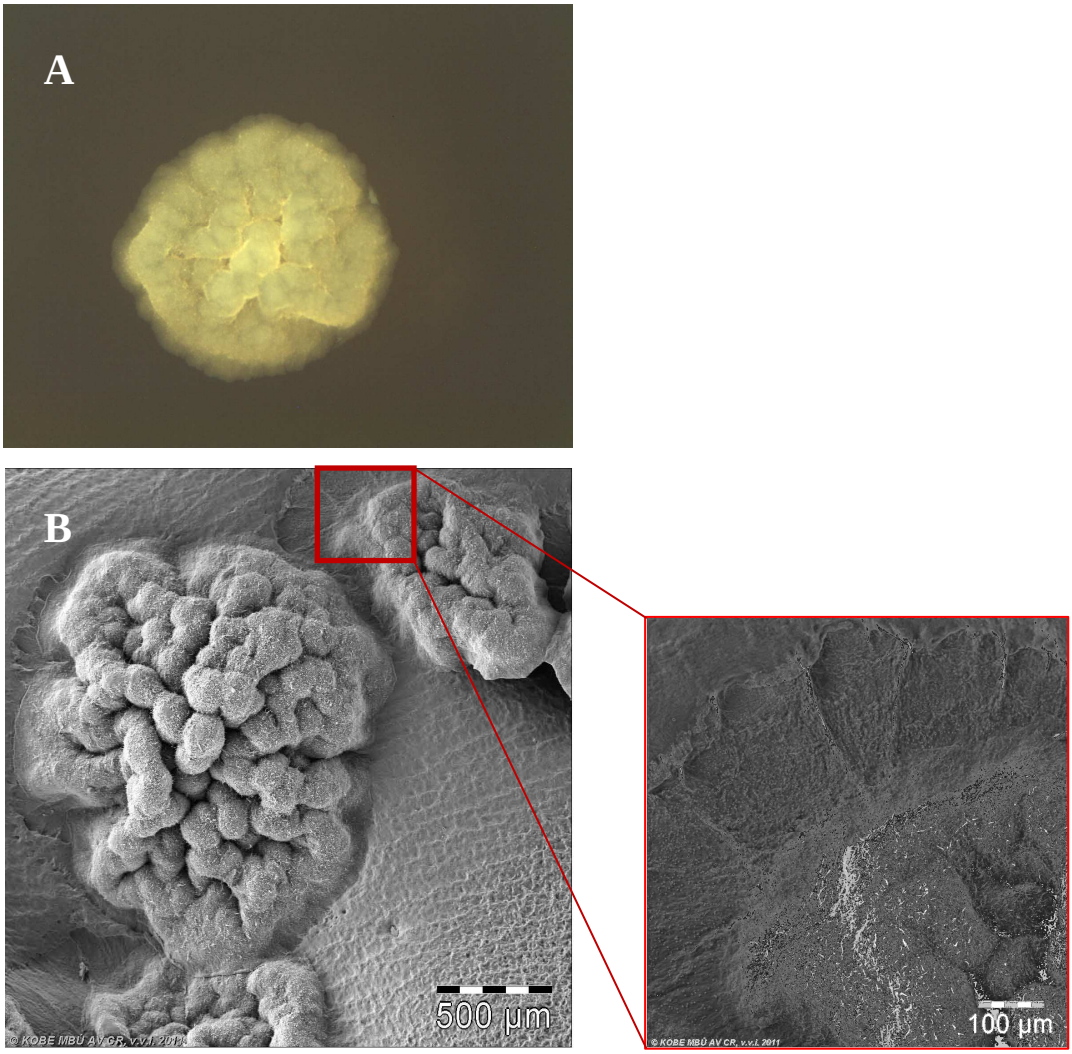

Figure 25. *M. smegmatis* colony after 3 days of the cultivation on agar plate with glycerol. A – the colony under Stereo microscope, magnification 30 x; B – SEM.

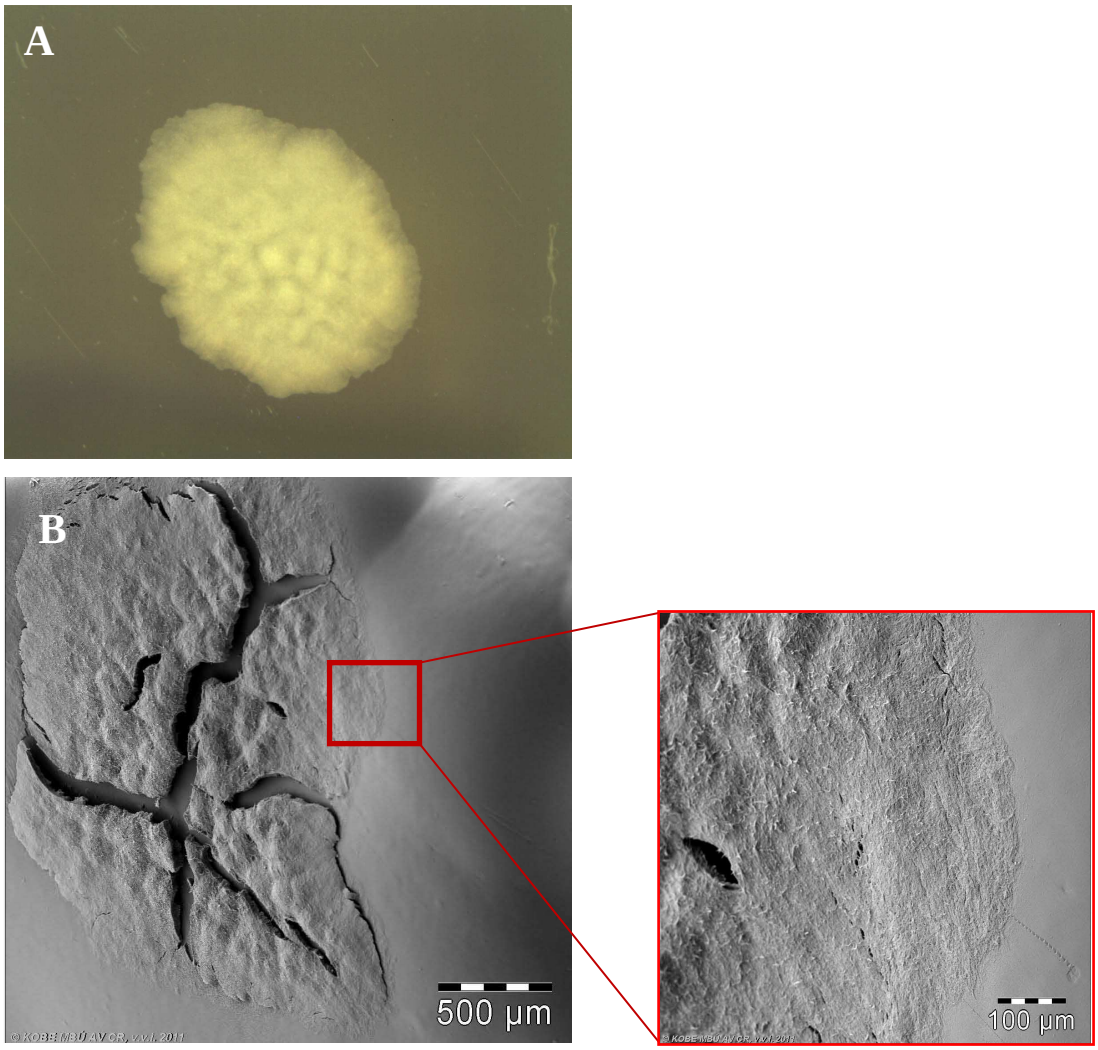

Figure 26. *M. smegmatis* colony after 3 days of the cultivation on agar plate with glycerol covered with cellophane. A – the colony under Stereo microscope, magnification 30 x; B – SEM.

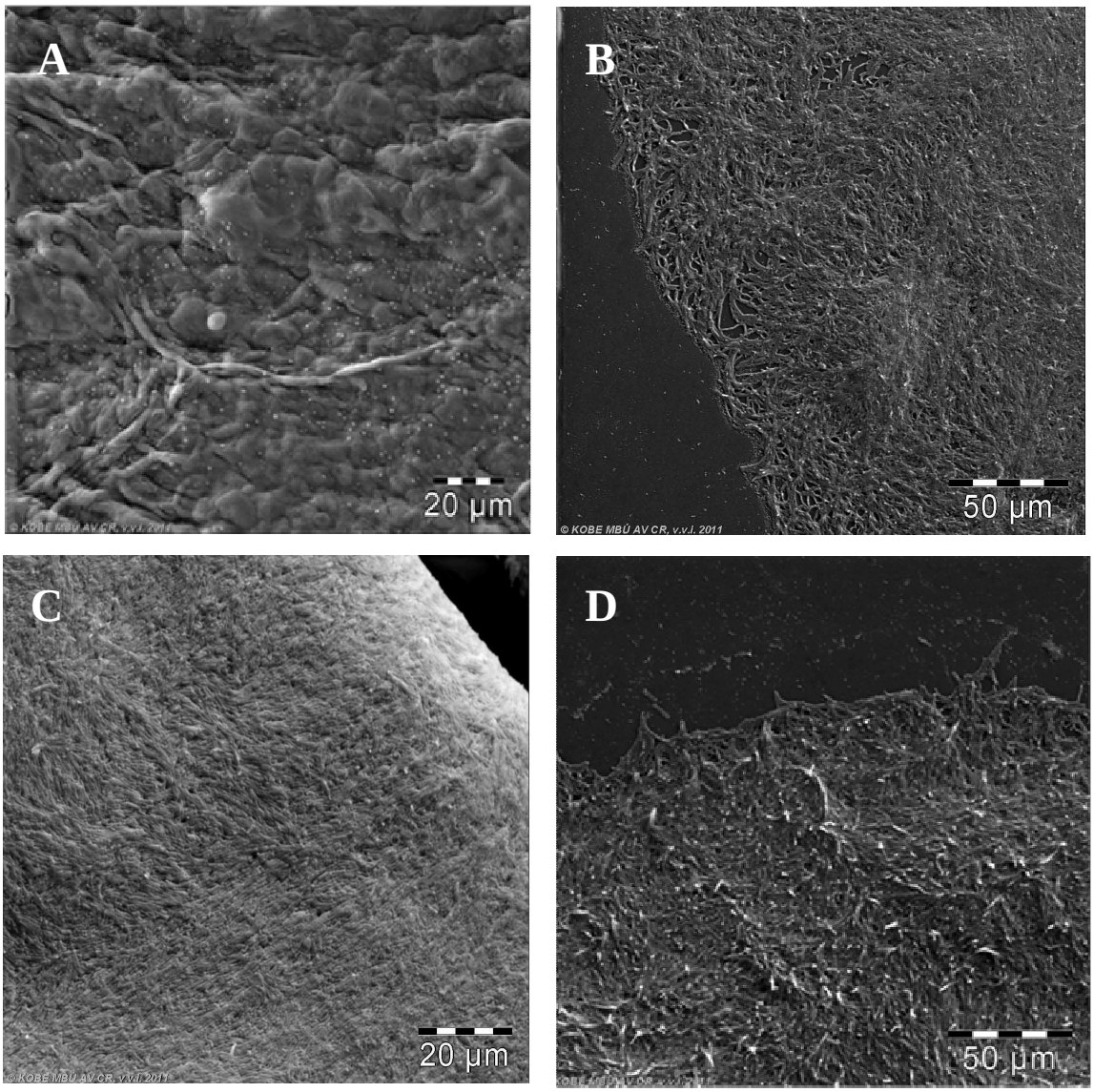

Figure 27. Scanning Electron Microscopy of individual colonies. A – the colony on agar plate with glycerol**, B –** the colony on agar plate with glycerol covered with cellophane**, C –** the colony on agar plate with glucose**, D –** the colony on agar plate with glucose covered with cellophane. All colonies were 3 days old.

4.3 Comparison of proteomes of *M. smegmatis* grown under different cultivation conditions

Protein samples for 2-DE were isolated from 3 days old cells grown on respective solid surfaces, and the planktonic culture from the middle of the exponential phase (OD at 600 nm 1,0 – 1,3). Glycerol was used as the source of carbon in all experiments.

4.3.1 Experiment A – comparison of *M. smegmatis* proteome from agar plates with proteome from agar plates covered with cellophane

The aim of the Experiment A was a qualitative and quantitative comparison of *M. smegmatis* proteomes obtained from agar plates and agar plates covered with cellophane after 3 days of cultivation.

Qualitative analysis

Figure 28 represents Master gel (**Chapter 3.2.11**) with highlighted unique proteins (in the form of spots) occurring in cells cultivated on agar surface (green crosses) and on cellophane surface (blue crosses). Qualitative analysis revealed 3 unique proteins in proteome from agar surface and 11 unique proteins in proteome from cellophane surface.

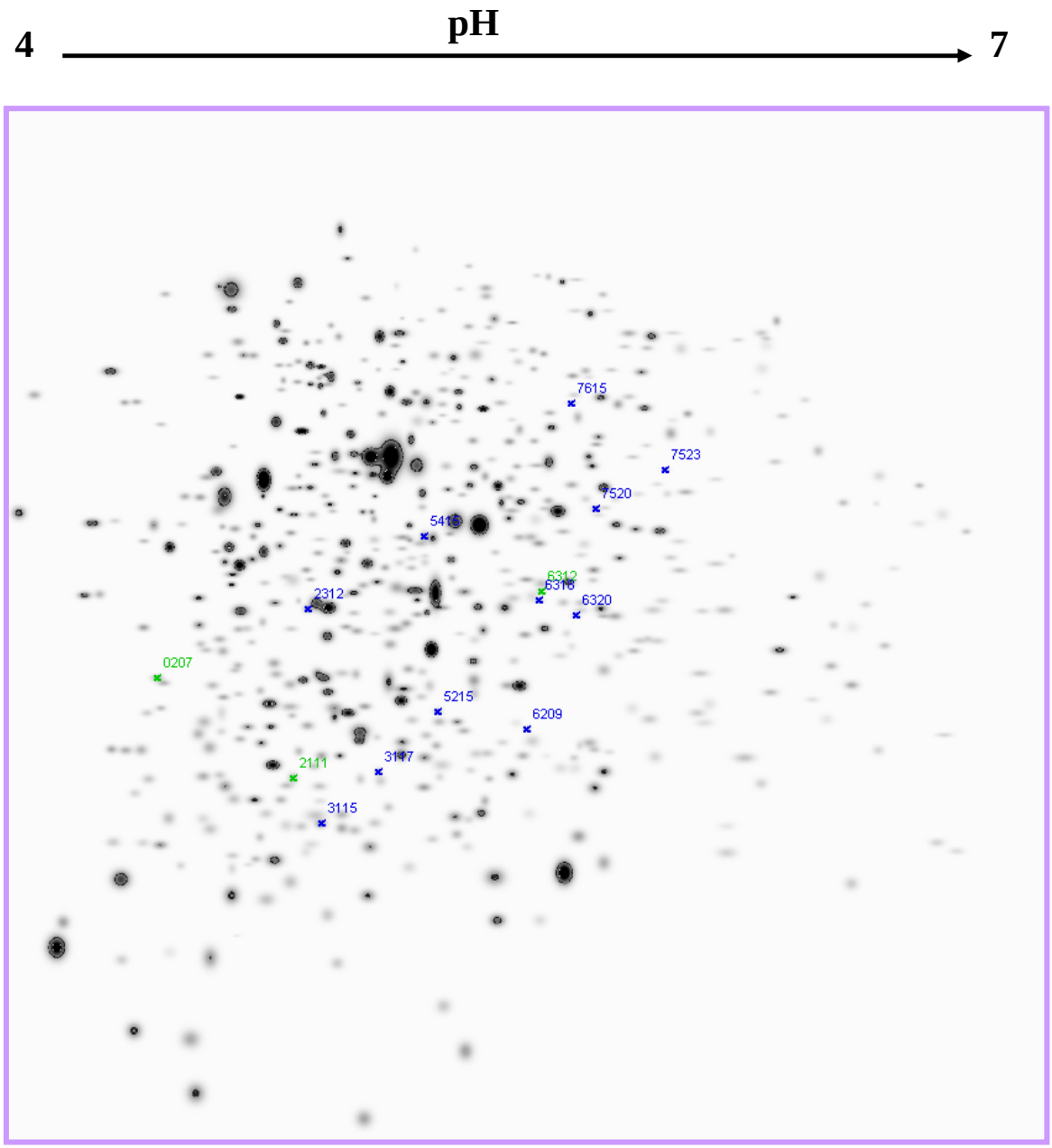

Figure 28. Master gel with highlighted unique proteins occurring in *M. smegmatis* proteomes from agar surface and from cellophane surface. Green crosses – unique proteins occurring in the cells growing on agar surface; blue crosses – unique proteins occurring in the cells growing on cellophane surface. Green and blue numbers reflect the appropriate numbers of spots assigned in PDQuest software.

Quantitative analysis

Quantitative analysis revealed no differences in protein profiles between agar surface and cellophane surface in the comparison using 8 x cut of limit (all above 8 fold difference).

4.3.2 Experiment B – comparison of *M. smegmatis* proteome from agar plates with proteome from planktonic culture

The aim of the Experiment B was a qualitative and quantitative comparison of *M. smegmatis* proteomes obtained from culture grown on agar plates for 3 days and culture grown in liquid medium to the middle exponential phase.

Qualitative analysis

Figure 29 represents Master gel with highlighted unique proteins (in the form of spots) occurring in the cells cultivated on agar surface (green crosses) and in liquid medium (red squares). Qualitative analysis revealed 69 unique spots in proteome from agar surface culture and 53 unique spots in proteome from liquid culture.

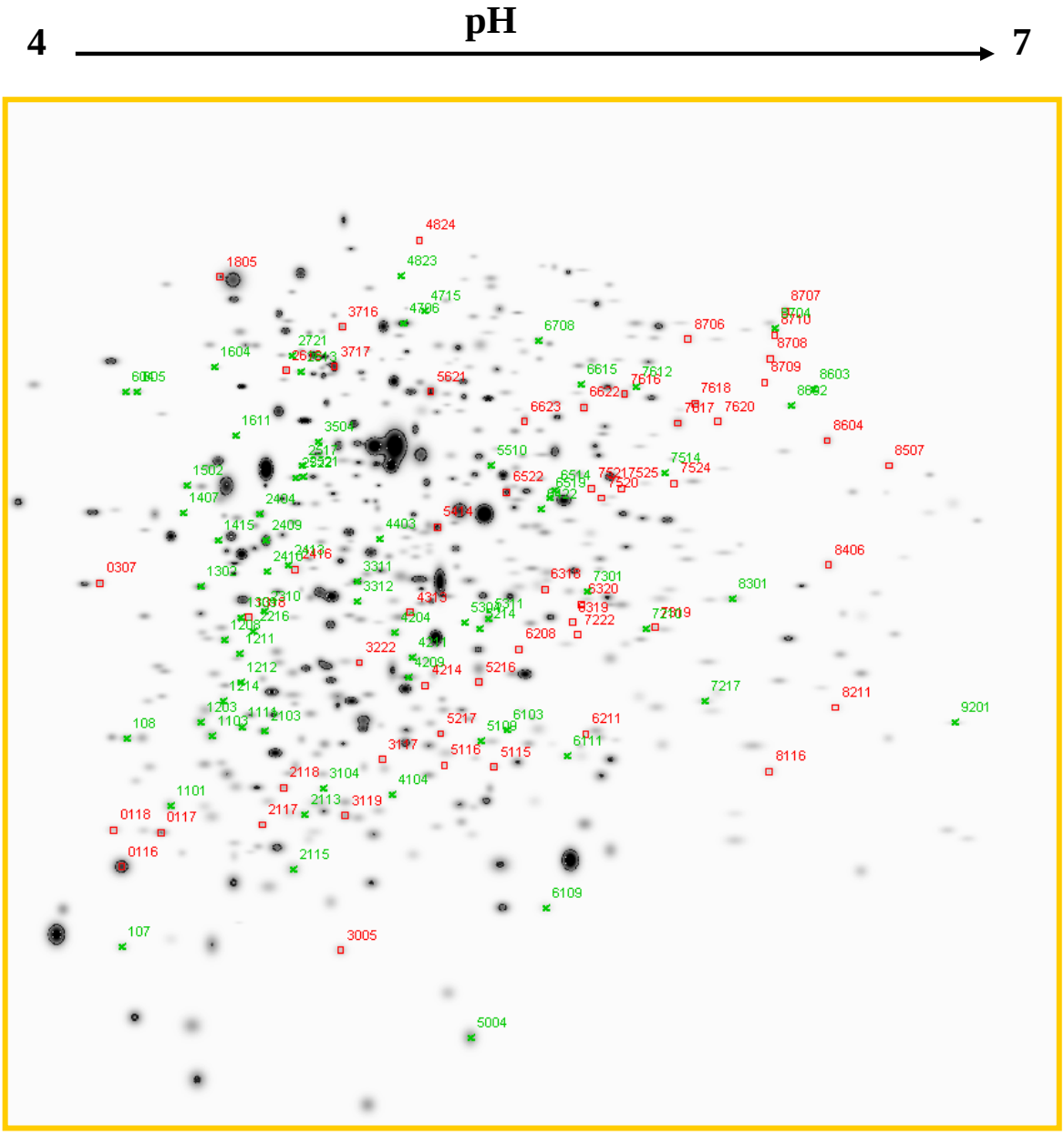

Figure 29. Master gel with highlighted unique proteins occurring in *M. smegmatis* proteomes from agar surface culture and from planktonic culture. Green crosses – unique proteins occurring in the cells growing on agar surface; red squares – unique proteins occurring in the cells growing in liquid medium. Green and red numbers reflect the appropriate numbers of spots assigned in PDQuest software.

Quantitative analysis

Figure 30 shows on 2-D electrophoretic gel image 5 proteins (spots) with different expression as a result of comparison of *M. smegmatis* proteomes isolated from the agar surface culture and the planktonic culture, respectively. **Figure 31** represents the comparison of expression profiles of these proteins.

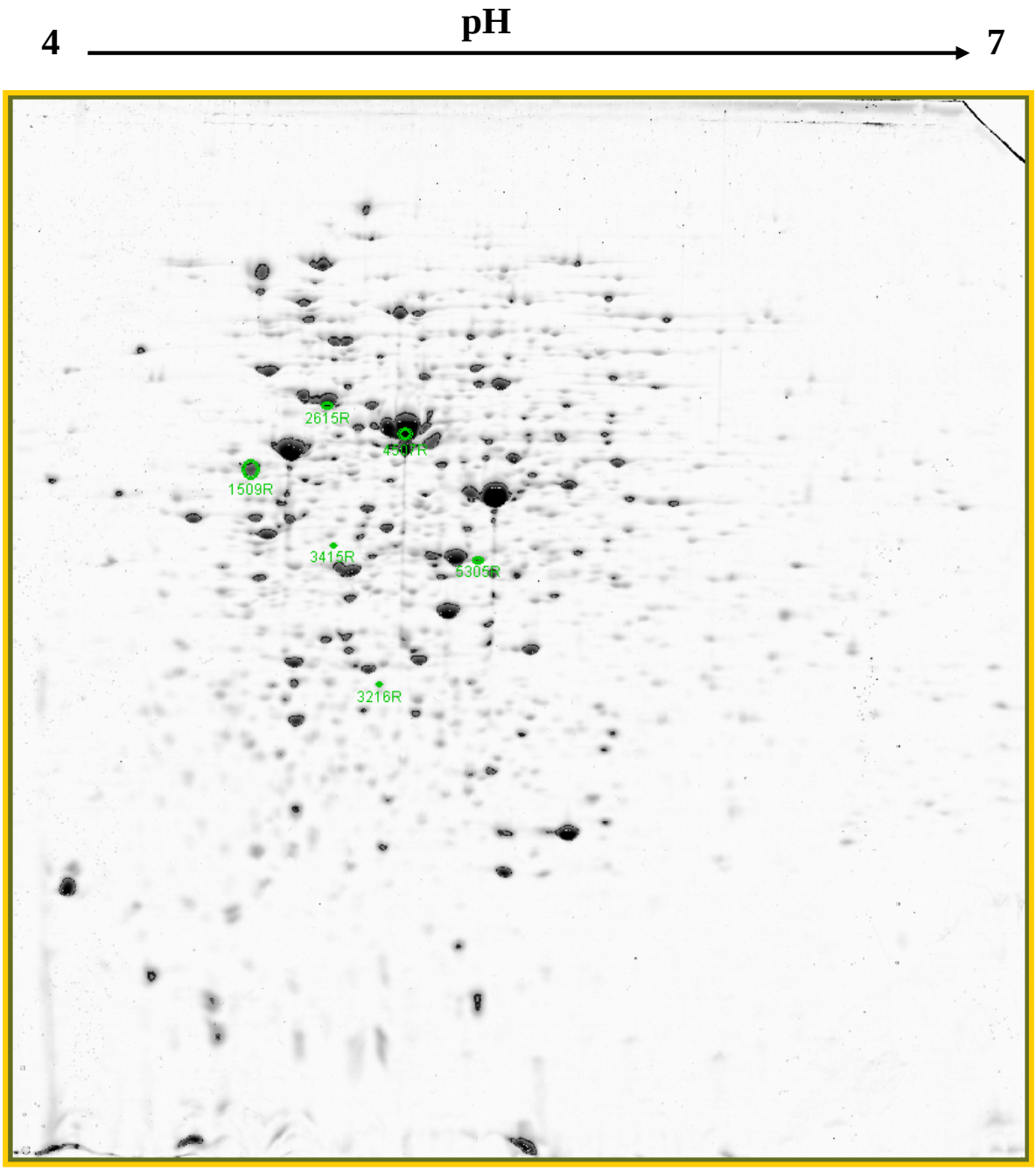

Figure 30. 2-D electrophoretic gel indicating proteins with different expression obtained from the comparison of the *M. smegmatis* proteomes from agar surface culture and from planktonic culture. The gel was obtained from agar surface culture and it was stained using Colloidal Coomassie Blue dye.

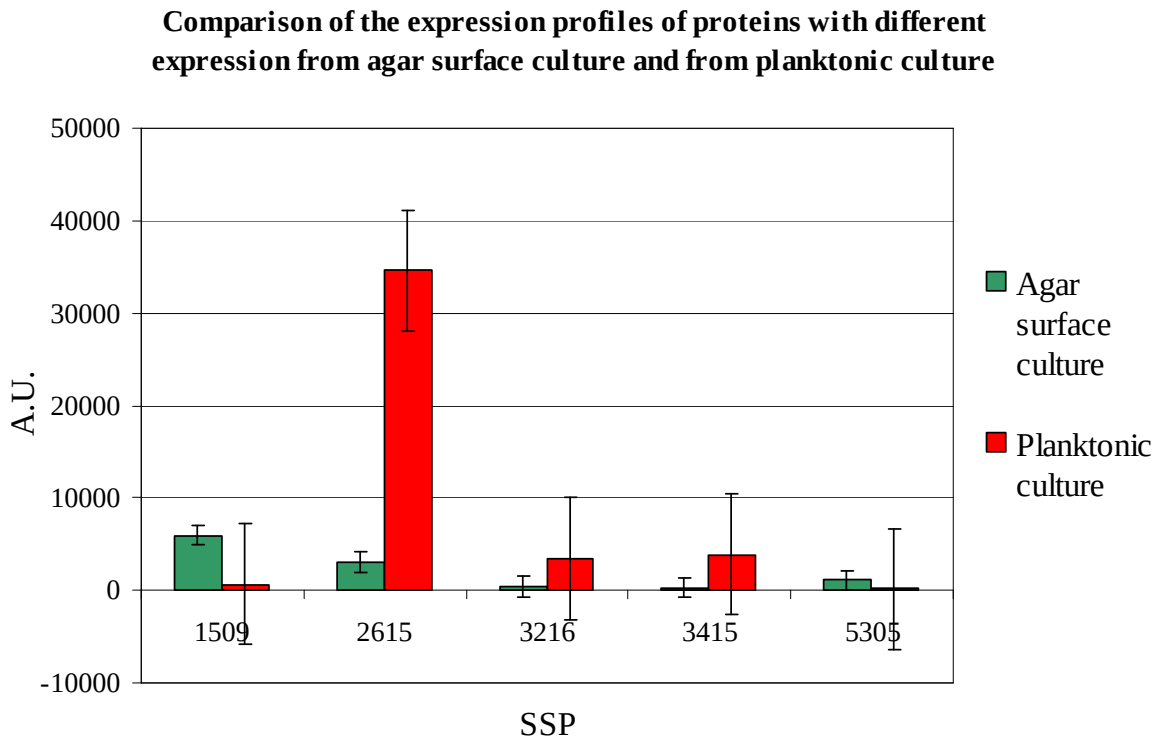

Figure 31. Comparison of the expression profiles of proteins with different expression revealed during quantitative analysis of *M. smegmatis* proteomes from agar surface culture (green columns) and from planktonic culture (red columns). SSP – protein serial numbers; A.U. – arbitrary units indicating spots density.

4.3.3 Experiment C – comparison of *M. smegmatis* proteome from agar plates covered with cellophane with proteome from planktonic culture

The aim of the Experiment C was qualitative and quantitative comparison of *M. smegmatis* proteomes obtained from culture grown on agar plates covered for 3 days and the culture grown in liquid medium to the middle exponential phase.

Qualitative analysis

Figure 32 represents Master gel with highlighted unique proteins (in the form of spots) occurring in the cells cultivated on cellophane surface (blue crosses) and in liquid medium (red squares). Qualitative analysis revealed 80 unique spots in proteome from the agar surface and 50 unique spots in proteome from the planktonic culture.

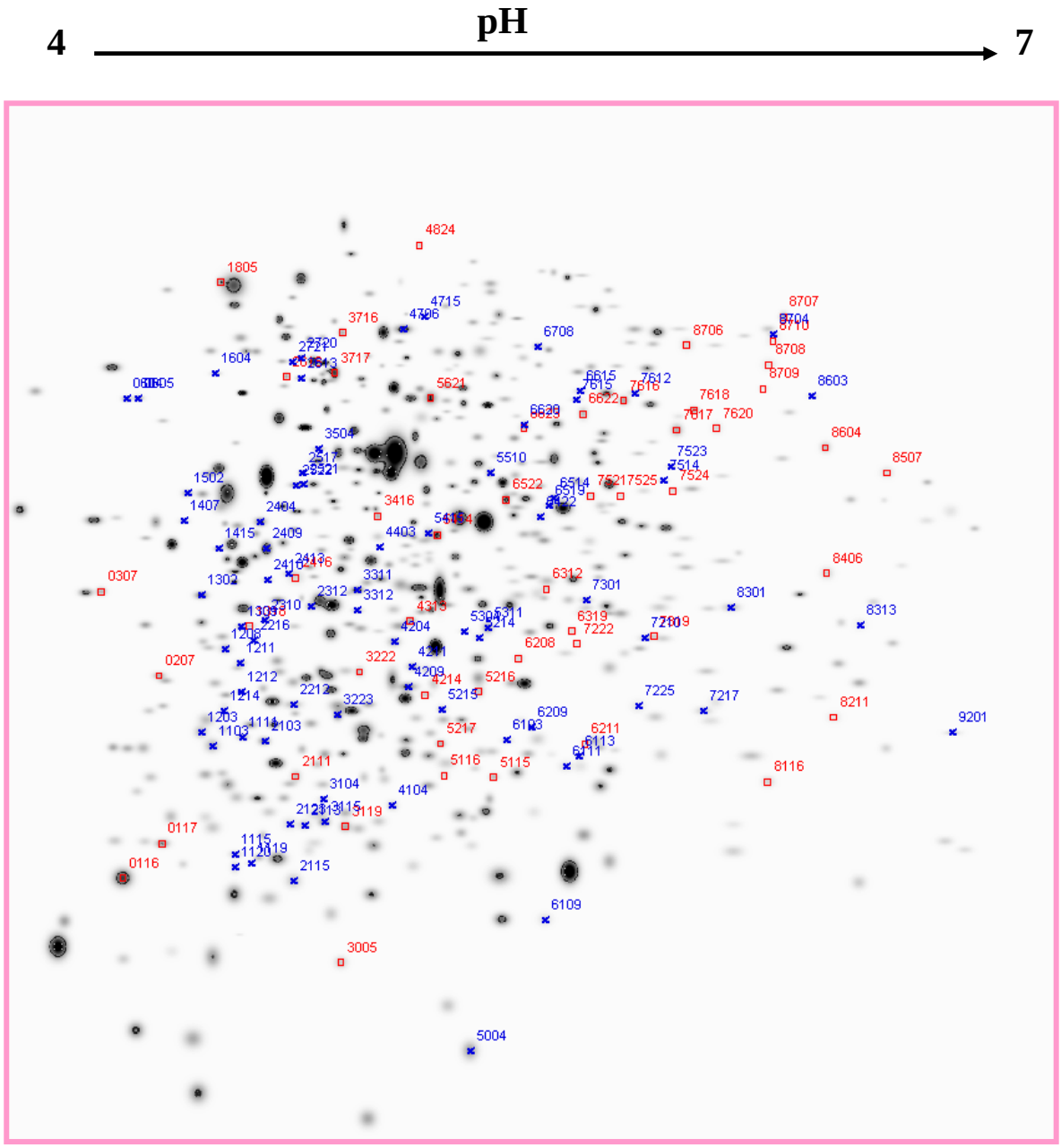

Figure 32. Master gel with highlighted unique proteins occurring in *M. smegmatis* proteomes from cellophane surface culture and from planktonic culture. Blue crosses – unique proteins occurring in cells growing on cellophane surface; red squares – unique proteins occurring in cells growing in liquid medium. Blue and red numbers reflect appropriate numbers of spots assigned in PDQuest software.

Quantitative analysis

Figure 33 shows on 2-D electrophoretic gel image 5 proteins (spots) with different expression as a result of comparison of *M. smegmatis* proteomes isolated from the cellophane surface culture and the planktonic culture, respectively. **Figure 34** represents the comparison of expression profiles of these proteins.

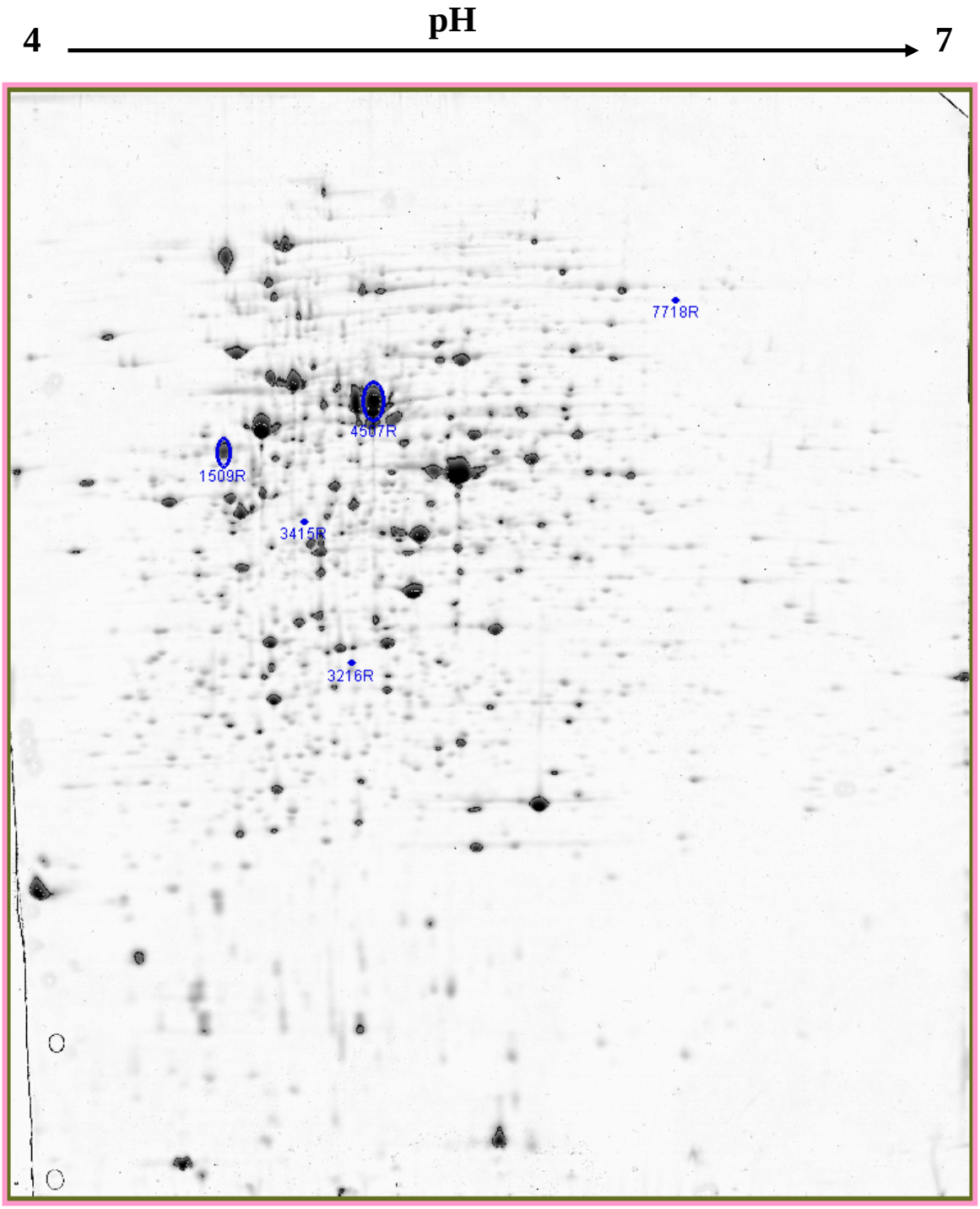

Figure 33. 2-D electrophoretic gel indicating proteins with different expression obtained from the comparison of *M. smegmatis* proteomes from cellophane surface culture and from planktonic culture. The gel was obtained from cellophane surface culture and it was stained using Colloidal Coomassie Blue dye.

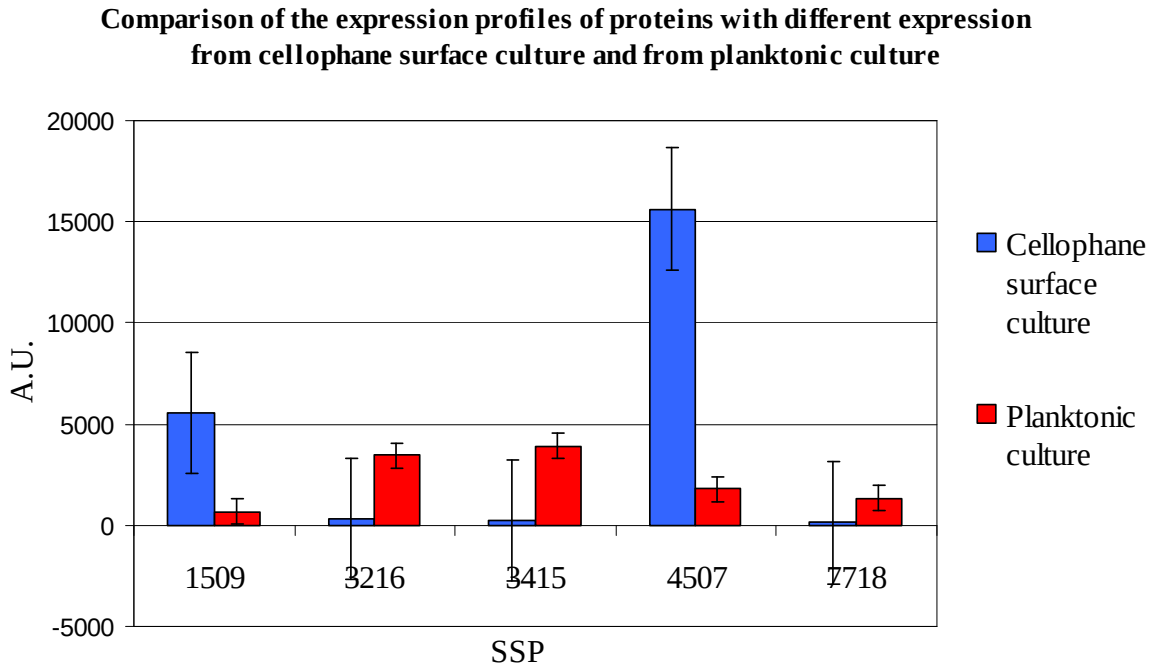

Figure 34. Comparison of the expression profiles of proteins with different expression identified by quantitative analysis of *M. smegmatis* proteomes from cellophane surface culture (blue columns) and from planktonic culture (red columns). SSP – protein serial numbers; A.U. – arbitrary units indicating spots density.

4.3.4 Overall analysis of proteomic data

Experiments A, B and C were carried out to describe differences in proteomes characterizing planktonic growth and growth in biofilm on two different solid surfaces (agar and cellophane). As a result sets of proteins were identified that reflect the qualitative and quantitative differences between the proteomes obtained from these three types of cultivation.

The revealed sets of proteins were subjected to analysis using Venn diagrams (GRUNBAUM 1984). In such a way their intersections were found, i.e. the groups of proteins that appeared simultaneously in individual comparisons. Unique proteins and quantitatively different proteins were evaluated separately.

4.3.4.1 Qualitative analysis

This chapter summarizes data from qualitative analyses in experiments A, B and C. These revealed sets of unique proteins reflecting differences in gene expression associated with planktonic growth and growth on agar or cellophane overlaying agar.

Table 1 shows the unique proteins and their amount detected in all experiments. **Figure 35** shows Venn diagrams with intersections of sets of unique proteins occurring in proteomes from agar surface culture, cellophane surface culture and planktonic culture in Experiments A, B and C. Comparison of unique proteins occurring in agar proteome (from Experiments A and B) revealed no proteins important for *M. smegmatis* growth on agar surface.

Analogous intersection of sets of unique proteins occurring in cellophane proteome (from Experiments A and C) revealed 7 proteins that apparently could be important for *M. smegmatis* growth on cellophane surface. **Table 2** lists spot numbers and densities designating these proteins.

Intersection of sets of unique proteins in the proteome from planktonic culture (from Experiments B and C) is a group of 46 proteins which are evidently important for *M. smegmatis* planktonic growth. **Table 3** lists spot numbers and densities designating these proteins.

Cultivation	**Unique proteins**		
	Exp. A **Ag. x Cel.**	**Exp. B** **Ag x Pl.**	**Exp. C** **Cel. x Pl.**
Agar surface	**3**	**69**	**-**
Cellophane surface	**11**	**-**	**80**
Liquid medium	**-**	**53**	**50**

Table 1. General view on qualitative differences between proteomes from agar surface culture, cellophane surface culture and planktonic culture in Experiments A, B and C. Ag. x Cel. – compared proteomes from agar surface culture and cellophane surface culture in Experiment A; Ag. x Pl. – compared proteomes from agar surface culture and planktonic culture in Experiment B; Cel. x Pl. – compared proteomes from cellophane surface culture and planktonic culture in Experiment C.

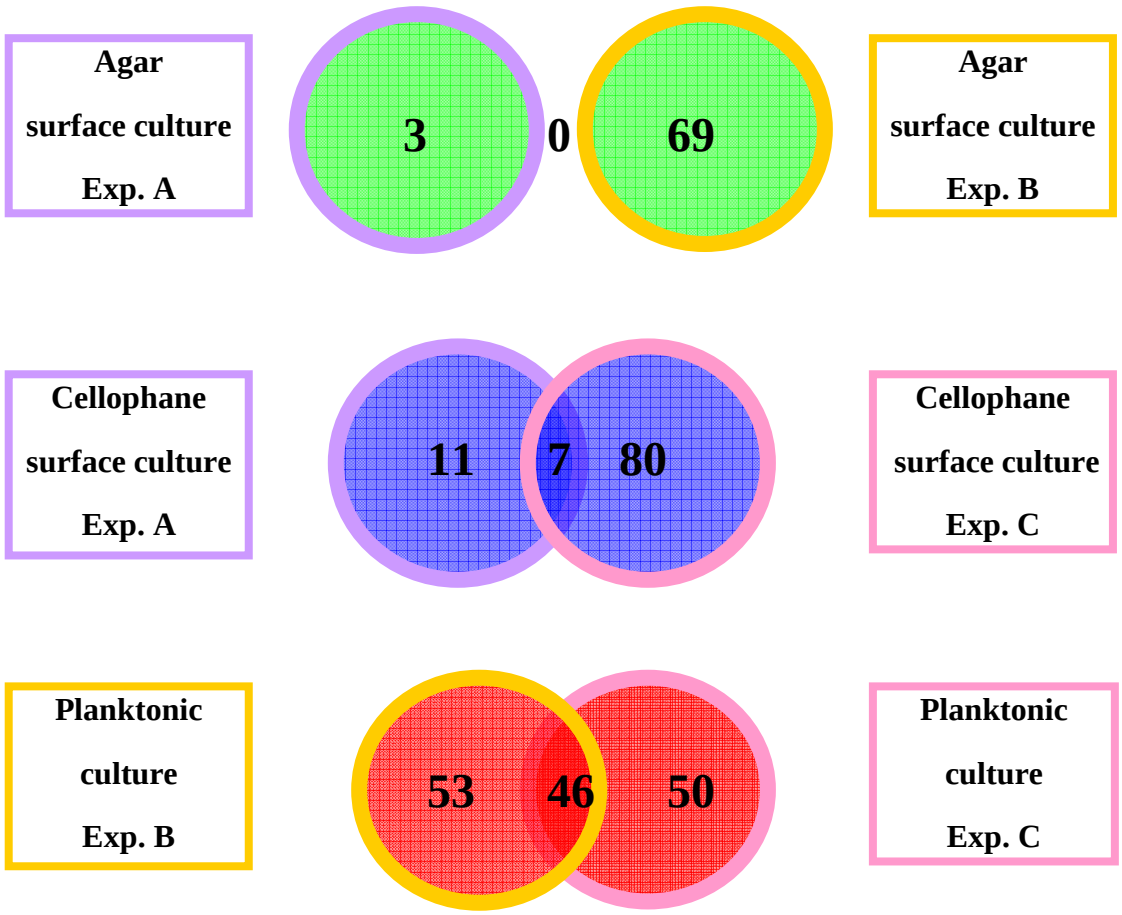

Figure 35. The intersections of sets of unique proteins revealed in Experiments A, B and C in individual types of cultivation – on agar surface, on cellophane surface, in liquid medium (according chapters 4.3.1 – 4.3.3). Green, blue and red colors of circles represented protein profiles from agar surface culture, cellophane surface culture and planktonic culture, respectively. Lilac, yellow and pink colors of edging represented Experiments A, B and C, respectively.

SSP	Average density of the spots of the cellophane surface culture proteome	SD
2312	452,07	91,72
3115	635,93	12,05
5215	325,67	7,47
5415	388,40	64,39
6209	175,17	43,55
7523	121,00	32,16
7615	122,63	29,04

Table 2. Unique proteins (7) occurring in both A and C Experiments in proteome from cellophane surface culture (according to Fig. 35). The values indicate the average density of spots in appropriate proteome. SSP – protein serial number; SD – standard deviation.

SSP	Average density of the spots of the planktonic culture proteome	SD	SSP	Average density of the spots of the planktonic culture proteome	SD
116	4176,78	520,96	6319	232,75	45,69
117	767,68	131,90	6522	1457,90	228,49
307	533,13	53,32	6622	629,68	87,77
1318	1693,63	339,24	6623	1612,45	144,09
1805	364,30	83,42	7222	205,98	51,04
2416	469,98	107,98	7319	246,05	60,21
2616	382,13	41,75	7521	217,90	34,36
3005	722,95	181,83	7524	208,68	30,28
3119	750,00	88,35	7525	169,55	10,11
3222	342,00	65,38	7616	810,88	133,15
3716	551,10	92,56	7617	982,75	116,00
3717	1412,55	500,44	7618	489,78	34,92
4214	289,25	43,76	7620	303,15	48,38
4313	664,38	62,91	8116	435,05	59,19
4824	202,90	26,14	8211	229,38	44,86
5115	278,75	22,79	8406	437,23	104,22
5116	244,80	64,86	8507	322,68	40,72
5216	451,95	70,51	8604	562,80	198,12
5217	414,60	26,01	8706	391,85	110,32
5414	1103,85	129,15	8707	370,18	96,93
5621	1864,78	319,42	8708	251,88	32,31
6208	274,98	31,12	8709	135,83	23,81
6211	228,90	56,65	8710	343,53	66,31

Table 3. Unique proteins (46) occurring in both B and C Experiments in proteome from planktonic culture (according to Fig. 35). The values indicate the average density of corresponding spots in appropriate proteome. SSP – protein serial number; SD – standard deviation.

4.3.4.2 Quantitative analysis

This chapter summarizes data from quantitative analyses in experiments A, B and C. These revealed sets of proteins differing in amount which is reflecting differences in gene expression associated with planktonic growth and growth on agar or cellophane overlaying agar.

Table 4 shows the amount of proteins with different expression detected in all experiments.

Analysis of intersection of sets of quantitatively different proteins is graphically demonstrated in **Figure 36**. The upper circles compare the results of Experiments A and B. Experiment A compared proteomes from agar surface culture and from cellophane surface culture; Experiment B compared proteomes from agar surface culture and from planktonic culture. There was no intersection between sets from these two experiments. Comparison of sets of proteins with different expression in Experiments A and C (**Fig. 36**, the middle circles) shows the same result – without any intersection.

The intersection of sets of proteins with different expression in Experiments B and C (**Fig. 36**, the lower circles) shows 3 proteins, which are apparently important for *M. smegmatis* growth in liquid medium during submerged cultivation. Experiment B compared proteomes from agar surface culture and from planktonic culture; Experiment C compared proteomes from cellophane surface culture and from planktonic culture). **Table 5** lists spot numbers and densities designating these proteins.

Changes in expression	**Compared proteomes**		
	Exp. A **Ag. x Cel.**	**Exp. B** **Ag x Pl.**	**Exp. C** **Cel. x Pl.**
Increasing	**0**	**3**	**3**
Decreasing	**0**	**2**	**2**
Total changes	**0**	**5**	**5**

Table 4. General view on quantitative differences in experiments A, B and C.
Ag. x Cel. – compared proteomes from agar surface culture and cellophane surface culture in Experiment A; Ag. x Pl. – compared proteomes from agar surface culture and planktonic culture in Experiment B; Cel. x Pl. – compared proteomes from cellophane surface culture and from planktonic culture in Experiment C.

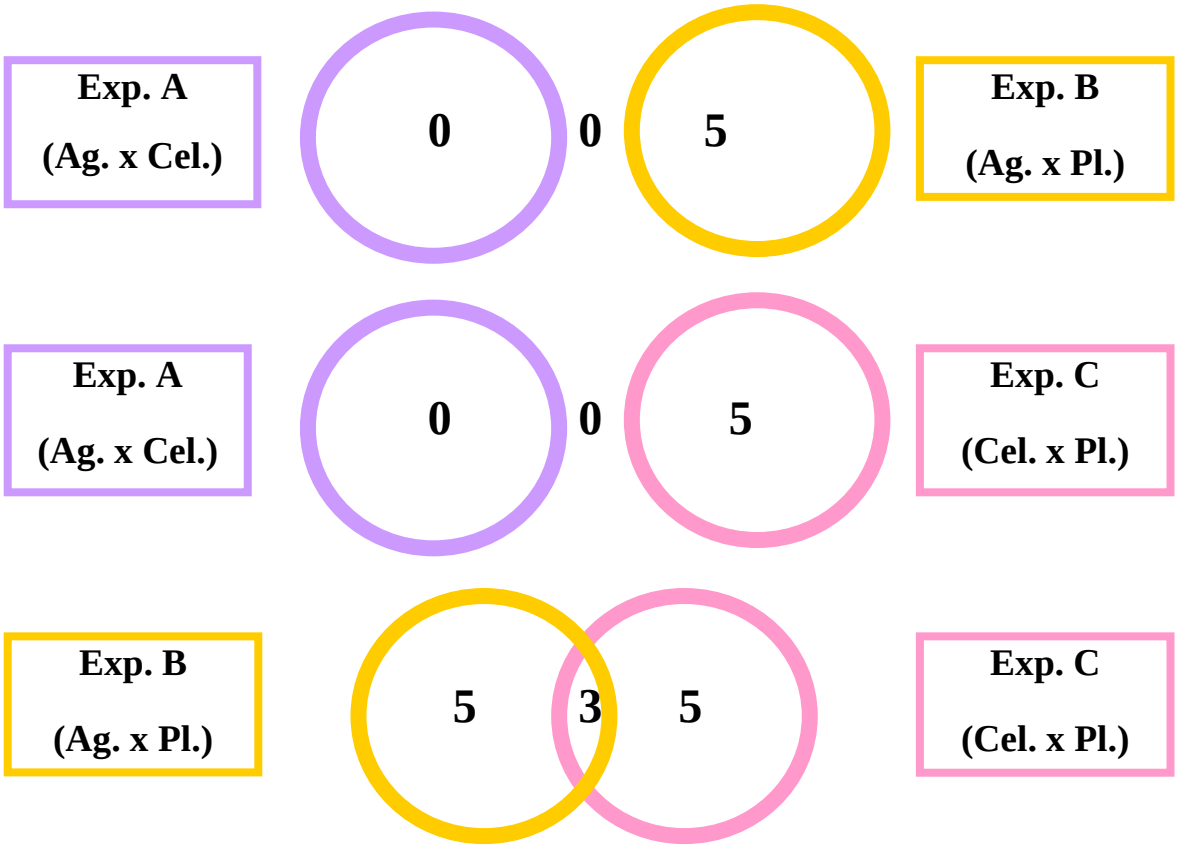

Figure 36. The intersections of sets of quantitatively different proteins in comparative Experiments A, B and C (according to Chapters 4.3.1 – 4.3.3). Lilac, yellow and pink colors of edging represented Experiments A, B and C, respectively.

SSP	Average density of the spots of the agar surface culture proteome	SD	Average density of the spots of the cellophane surface culture proteome	SD	Average density of the spots of the planktonic culture proteome	SD
1509	**5972,67**	**387,61**	**5558,23**	**1248,03**	**653,20**	**136,25**
3216	**421,13**	**19,47**	**305,60**	**49,79**	**3437,23**	**165,15**
3415	**281,27**	**14,66**	**247,23**	**20,20**	**3895,80**	**383,29**

Table 5. Proteins with different expression occurring in both B and C Experiments. Experiment B compared proteomes from the agar surface culture and from the planktonic culture; Experiment C compared proteomes from the cellophane surface culture and from the planktonic culture. The values indicate the average density of corresponding spots in appropriate proteomes. SSP – protein serial number; SD – standard deviation.

5. DISCUSSION

Mycobacterium smegmatis is a non-pathogenic and fast-growing soil bacterium. It shares many features with the pathogenic slow-growing *Mycobacterium tuberculosis.* Therefore *M. smegmatis* is widely used as a mycobacterial model system.

In this thesis we describe morphological and proteomic characteristics of *M. smegmatis mc*^{2} *155* strain during its cultivation on two kinds of solid surfaces (agar, cellophane) with two kinds of carbon sources (glycerol, glucose respectively). Many species of mycobacteria, including pathogens like *M. tuberculosis*, form structured biofilm communities at liquid–air interfaces and on solid surfaces. The physiology of biofilm cells is very different from the physiology of planktonic cells. The understanding of biofilm development and growth of *Mycobacterium* representatives is therefore very important for the successful struggle with diseases caused by pathogens of this genus and the damage they might cause in food industry.

Since agar cultivation media are the most commonly used in laboratory experiments and they resemble number of organic surfaces on which mycobacteria can grow, we compared morphology of biofilm structures and proteome profiles growing on plain agar and agar covered with cellophane which offers much finer yet still hydrophilic surface. *M. smegmatis* cultures growing on these surfaces fit to number of broad definitions of biofilms in the literature (HALL-STOODLEY 2004; TORTORA, 2009).

To identify proteins important for biofilm formation and development we compared proteomes from cultures grown on solid surfaces with proteome from planktonic culture. When looking for optimal cultivation conditions glycerol and glucose were used as a carbon sources in 7H9 broth and 7H10 solid medium. Doubling time in liquid culture with glycerol was 3,2 hours while with glucose – 4,8 hours. Exponential growth on glycerol ceased after 15 hours at OD 2,0, while on glucose the culture reached stationary phase after 30 hours at OD around 6,0 (see **Fig. 11** and **12**) . The lag phase on glucose was also longer. A larger range of values in the exponential phase of growth could be of a significant advantage for some types of experiments; however for the purpose of this thesis the shorter doubling time is more important. Besides, the size of the colonies on the plates with glucose was smaller. Therefore glycerol was used as the carbon source in our experiments.

The studies of *M. smegmatis* colonies morphology on agar plates and agar plates covered with cellophane sheets were constituent part of this thesis. *M. smegmatis* creates "C"

colonies ("cauliflower" colonies) during growth on agar plates. This term reflects wrinkled structure of the colonies which also have a shiny, waxy surface and creamy color (GORDON and SMITH 1953). Our hypothesis was that there might be a direct influence of the surface quality and type of carbon source used in culture medium on *M. smegmatis* colonies morphology.

The morphology of the colonies was studied after the 3 days and the 6 days of the cultivation. Colonies on cellophane surface were flat and had an amorphous structure, (see **Chapters 4.2.1** and **4.2.2**), regardless what carbon source was used. Colonies on agar with glycerol had a typical cauliflower shape ("C" colonies), while colonies on the agar with glucose had a granular structure (see **Chapters 4.2.1** and **4.2.2**).

Interesting are images of the colonies found on the edge of cellophane on agar. This method gave an opportunity to observe changes in morphology of colonies directly as a consequence of surface quality change under identical nutrient and cultivation conditions. The difference in morphology of the colonies at the border of two solid surfaces after the 3 days of the cultivation was less pronounced (see **Fig. 21** and **23**) than it was after the 6 days of growth (see **Fig. 22** and **24**).

Detailed structure of *M. smegmatis* colonies on agar and on agar covered with cellophane was investigated by SEM. Unfortunately majority of taken images were impossible to use due to difficulties in preparation of the samples. Nevertheless morphology of "C" type colony is clearly visible in the **Figure 25**. The agar surface on the image has a rough structure. It is remarkable that *M. smegmatis* cells visibly grow sort of submerged into the agar surface at the edge of the colony and the colony has well organized structure. On the other hand, the colony on cellophane surface is visibly amorphous and spreads on the cellophane sheet. The cellophane has absolutely smooth surface in contrast to the agar (see **Fig. 26**). In the **Figure 27** the morphology of individual cells seems to be similar, but the cells on the agar surface (see **Fig. 27 A** and **C**) give the impression of a higher content of material keeping the cells together than in case of cells on the cellophane surface (see **Fig. 27 B** and **D**). The comparison of carbon sources showed that on glycerol containing agar the cells formed ligament-like structures (see **Fig. 27 A**), while on cellophane and in case of glucose on both surfaces the cells formed shorter and less compact structures (see **Fig. 27 B, C, D**) . Obtained results confirm the initial hypothesis and show that both – the surface structure, as well as the carbon source in the medium, greatly influence the morphology of the colonies.

In our experiments no genetic manipulations with the strain were carried out: we studied *M. smegmatis mc^2 155* strain morphology and proteomes on different surfaces and

two carbon sources in cultivation medium. The variations in the morphology of the *Mycobacterium* colonies are often mentioned in connection with the genetic manipulations with the strain. Scientific literature contains descriptions of the experiments with *M. smegmatis* mutants that have colony morphology variants different from the wild-type. These morphology variants were obtained by various ways. For example, Smeulders et al. studied stationary-phase cultures of *M. smegmatis*. Flat colony variant was seen in 75% of all long-term-stationary-phase cultures on agar plates (SMEULDERS *et al.* 1999). Cultures were grown on either Hartmans-de Bont minimal medium plates or Lab-lemco medium plates. Cryo scanning electron microscopy showed that the colony organization was different in flat colony strains; flat colonies appeared less well organized than wild-type wrinkled colonies. Competition experiments with an exponential-phase-adapted wild-type strain showed that the flat strain had a competitive advantage over wild type in stationary phase (SMEULDERS *et al.* 1999).

Changes in the morphology of the colonies may be associated with changes in mycobacterial pathogenicity and virulence. According to Delogu et al. proteins that contribute to mycobacterial pathogenicity, like Erp family, may also change the colony morphology on solid medium (DELOGU *et al.* 2008). The Erp family is an expanding group of proteins originally identified and characterized from mycobacteria causing tuberculosis and leprosy. It has been demonstrated that the absence of Erp in *M. smegmatis* and *M. bovis BCG* strains transforms the spreading wild-type colony phenotype toward smaller and raised colony morphology. The loss of wild-type colony morphology correlates positively with loss of residual virulence (DELOGU *et al*. 2008).

Similarly, in experiments carried out by Dhouib et al., *M. smegmatis* MSMEG_0220 strain with disrupted gene responsible for synthesis of monoacylglycerol lipase, developed colonies with very smooth, wet, and round appearance. Monoacylglycerol lipase is the enzyme which has homologues in *M. tuberculosis* and *M. leprae*, and seems to be connected with their pathogenicity (DHOUIB *et al.* 2010). Cultures were grown on 7H11 agar plates. This phenotype was in stark contrast to the dry, rough, and irregular morphology of the colonies of the *mc^2 155* strain. It is interesting that when four strains of *M. smegmatis* were grown in 7H9 broth without Tween 80, the MSMEG_0220 strain showed a homogeneous suspension culture with individualized cells, whereas the *mc^2 155*, and two other strains with another genomic changes showed a strong pattern of cell aggregation. This fact presents strong evidence that cell aggregation was impaired upon MSMEG_0220 gene disruption (DHOUIB *et al.* 2010).

In studies carried out by Chen et al. Lsr2 protein was investigated. First this protein was described in *M. leprae* as an immunodominant T-cell antigen. Lsr2 orthologs are present in all sequenced mycobacterial genomes, including pathogens of the *M. tuberculosis* complex. They affect diverse mycobacterial phenotypes including colony morphology and biofilm formation (CHEN *et al.* 2006). A transposon insertion mutation into the *lsr2* gene of *M. smegmatis* mc^2 *155* was performed in these experiments. *lsr2* mutants exhibited altered smooth colony morphology and was defective in pellicle and biofilm formation (CHEN *et al.* 2006).

According to Kumar and Chatterji the overexpression of gene *MSDGC-1* involved in synthesis of cyclic di-GMP activity in *M. smegmatis* may also change the morphology of the colonies (KUMAR and CHATTERJI 2008). On solid agar medium, the MSDGC-1-overproducing strain showed a different colony morphology in comparison to the control, which was the wild-type strain containing the vector only. The wild-type strain colonies of *M. smegmatis* had non-uniform rough surface and were large, whereas the MSDGC-1 overexpressing strain produced smaller colonies with uniform margins, smooth surface and round shape (KUMAR and CHATTERJI 2008).

WhmD and WhiB2 proteins are an orthologs and functional equivalents in *M. smegmatis* and *M. tuberculosis*, respectively. Their genes are essential and involved in cell division of these bacteria (RAGHUNAND and BISHAI 2006). The phenotypic effects of *M. smegmatis* and *M. tuberculosis* overexpression WhmD/WhiB2 levels by transformation were reflected in small colony phenotype. It was found that these colonies were glossy and spherical, whereas the corresponding controls were rough and flat (RAGHUNAND and BISHAI 2006).

The morphological results from the last two citations are quite close to those we obtained on plates covered with cellophane and with glucose as a source of carbon. Therefore *MSDGC-1* might be a good inspiration for subsequent detailed analysis and interpreting of our results.

The objective of the proteomic part of this thesis was to obtain and compare the total cell proteomes of *M. smegmatis* cultivated on two different solid surfaces (agar and cellophane) and in the liquid medium for subsequent qualitative and quantitative comparison. 2-DE was used as the main proteomic method. In Experiments A, B and C we compared in pairwise manner proteomes of *M. smegmatis* obtained from the agar surface biofilm, cellophane surface biofilm and planktonic culture, respectively. All these cultivations were conducted in the medium with glycerol. Data from qualitative and quantitative analyzes were

summarized and logical relationships between sets of obtained proteins were demonstrated in Venn diagrams (GRUNBAUM 1984).

Comparison of proteomes from cultures grown on agar and cellophane covering agar surface revealed 3 unique proteins on agar and 11 on cellophane and there were not identified any proteins with different expression (see **Fig. 35**). This reflects the fact that the only difference between two cultivations was, in one of them, the cellophane, as fully permeable barrier, separating bacteria from agar cultivation medium. The surface characteristics of agar and cellophane are very different and morphology of colonies growing on them as well, as we demonstrated by Scanning Electron Microscopy analysis of colonies on agar and cellophane (see **Fig. 25** and **26**). It is very likely that at least some of unique proteins found in proteomes from agar and cellophane cultivations are responsible for recognition and attachment to these two different surfaces.

Comparisons of proteomes from planktonic culture and agar or cellophane cultures, respectively, revealed very extensive differences in qualitative analysis. There were 69 unique proteins in agar proteome and 80 unique proteins in cellophane proteome. The number of unique proteins present in planktonic proteome was higher, 53 proteins and 50 proteins in agar and cellophane (B and C) experiments, respectively. Venn diagram then identified 46 proteins in intersection of these two sets. In intersections of sets of unique proteins from agar and cellophane proteomes from the two above mentioned experiments we found 7 proteins common in cellophane proteomes and none in agar proteomes (see **Fig. 35**). These 7 proteins out of 11 making the difference between agar and cellophane proteomes might be specifically important for recognition and attachment of bacteria to the cellophane.

Quantitative analysis showed only 5 different proteins in experiments comparing proteomes from two solid surfaces and planktonic culture, respectively. The 3 out of them were in the intersection of these two sets (from the Experiments B and C), and they are very likely associated to planktonic growth physiology (see **Fig. 36**).

Physiological requirements of biofilm and planktonic culture are evidently different. It is interesting that as a result of proteomic analysis we obtained a large amount of qualitative differences (unique proteins) in contrast to only few proteins with different level of expression. Possibly it means that growth on solid surface requires a lot of proteins that are absent in planktonic culture and vice versa, whereas changing of expression levels is not so important. However there were two proteins which differences in their expression levels were very high, 10 fold higher expression of the protein in the spot 2615 in planktonic culture in Experiment B (agar culture x planktonic culture) and 8 fold higher expression of protein in

the spot 4507 in cellophane culture in Experiment C (cellophane culture x planktonic culture) (see **Fig. 31** and **34**). Both of them seem to be of a high importance for the respective type of cultivation. Identification of these proteins by MS might contribute to understanding of their role. These experiments are under way in our laboratory.

Still much remains unknown about the mechanisms controlling growth in mycobacteria. The large-scale study of the proteins produced by *M. smegmatis* in different growth stages has the potential to generate information about the mechanisms of cell growth, division and adaptation, as well as inform about mycobacterial proteomes in general (WANG *et al.* 2005).

Many features of the mycobacterial physiology were revealed due to use of proteomic techniques. For example, using the 2-DE-based proteomics approach helped to identify a Dps protein with non-specific DNA-binding ability (GUPTA *et al.* 2002). Dps was found to be overexpressed preferentially under carbon starvation conditions as a model for mycobacterial persistence and its function probably is to protect DNA under various kinds of environmental stresses (GUPTA *et al.* 2002).

Many proteins responsible for pathogenicity of mycobacteria are surface proteins (MCNAMARA *et al.* 2012). Thus, the cell wall proteome description is essential for the study of mycobacteria. He et al. obtained a comprehensive picture of the *M. smegmatis* cell wall protein repertoire, which was the first proteomic analysis of cell wall proteins of *M. smegmatis mc*^{2} *155.* As a result there were identified 390 proteins divided into 21 functional groups. In their work they used one-dimensional electrophoresis, shotgun liquid chromatography and tandem MS methods (HE and DE BUCK 2010). McNamara et al. identified more than 100 putative surface proteins in *M. avium* subsp. *hominissuis*, strain which can cause opportunistic infections in humans (MCNAMARA *et al.* 2012).

Until recently, the principal method used for investigation of a complete proteome was 2-DE coupled with MS. For example, using this approach 263 proteins were identified in *M. tuberculosis* and *M. bovis BCG* strains, the proteome of *M. tuberculosis* H37Rv was compared with that of *M. bovis BCG Chicago*, and 25 proteins differing in position or intensity were identified (JUNGBLUT *et al.* 1999). Similarly, 137 proteins were detected in *M. tuberculosis* H37Rv culture supernatant and 27 unique proteins were identified in *M. tuberculosis* H37Rv by comparing proteins in the culture supernatant of virulent *M. tuberculosis* H37Rv to that of attenuated *M. bovis BCG Copenhagen* (MATTOW *et al.* 2003).

2-DE approach coupled with MS is also used in this thesis. However, recent advances in multidimensional liquid chromatography coupled with tandem MS have produced a

technology capable of direct analysis of the composition of protein mixtures as a complex of cell lysates. Wang and colleagues in 2005 used multidimensional chromatography and tandem MS in combination with the partially completed genome sequence to detect and identify a total of 901 distinct proteins from *M. smegmatis* over the course of 25 growth conditions (WANG *et al.* 2005). They observed numerous proteins involved in energy production (9,8 % of expressed proteins), protein translation (8,7 %), and lipid biosynthesis (5,4 %); 33 % of the 901 proteins were of unknown function. Protein expression levels were estimated from the number of observations of each protein, allowing measurement of differential expression of complete operons, and the comparison of the stationary and exponential phase proteomes (WANG *et al.* 2005).

The results of proteomic part of our experiments should be a contribution to the study of mycobacterial biofilms and physiology as well. Therefore we confirmed our hypothesis, achieved the main aim of diploma thesis and have made contribution in the field of the study of mycobacterial biofilms.

This thesis is part of a larger project which is carried out in our laboratory. The project is focused on development and growth of biofilms on various types of surfaces with the aim to compare morphology of biofilm structures and proteome differences reflecting observed changes. Results of this work bring interesting information about importance of recognition and attachment to a surface differing in a fine structure under certain nutrient and cultivation conditions.

6. SUMMARY

1. The morphology of the *Mycobacterium smegmatis* colonies growing on agar and cellophane covered agar with different carbon sources in the medium was investigated under Stereo microscope after 3 and 6 days. We showed that both a surface quality and the type of carbon source in medium influence the morphology of the colonies. The colonies on the plates with glycerol had a "cauliflower" appearance, whereas on those with glucose they had a granular structure. The colonies on cellophane had a similar amorphous structure, regardless of what carbon source was used. Investigation of the colonies morphology using SEM confirmed the data from Stereo microscope and revealed ligamentous cell structures in case of colonies growing on agar with glycerol.

2. The proteomes of *M. smegmatis* were isolated from the agar cultures, cellophane cultures and planktonic cells and analyzed using a 2-DE. All these cultivations were carried out with the glycerol as the carbon source.

3. The gel images of above mentioned proteomes were compared and evaluated by qualitative and quantitative analyses using PDQuest software. Comparison of proteomes from agar and cellophane revealed 3 unique proteins on agar and 11 on cellophane and there were not identified any proteins with different expression. Comparisons of proteomes from planktonic culture and agar or cellophane, respectively revealed very extensive difference in qualitative analysis. It was 69 unique protein in agar proteome and 80 unique proteins in cellophane proteome. The number of unique proteins presents in planktonic proteome was 50 and 53 proteins in agar and cellophane experiments, respectively. Quantitative analysis showed only 5 different proteins in experiments comparing proteomes from respective two solid surfaces and planktonic culture.

4. Venn diagrams showing logical relations between set of selected proteins revealed 46 proteins in intersection of the two sets of proteins from planktonic proteomes. In intersections of sets of unique proteins from agar and cellophane proteomes we found 7 proteins common in cellophane proteomes and none in agar proteomes. These 7 proteins out of 11 making the difference between agar and cellophane proteomes might be specifically important for recognition and attachment of bacteria to the cellophane.

5. The detected proteins will be identified by mass spectrometry in the future. The results may lead to better understanding of mycobacterial biofilms development and physiology.

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