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Mycobacterium tuberculosis culturomics: Hunting for the key to more rapid and sensitive cultivation and rescue

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SAMMENDRAG

Tuberkulose (TB) er et stort folkehelseproblem som forårsakes av bakteriene tilhørende *M. tuberculosis*- komplekset (MTBC). Påvisning av mykobakterier i sputumprøver fra TB-pasienter krever tid, spesielt hvis bakteriene er i en ikke-replikerende eller sovende tilstand. Det er derfor svært viktig å optimalisere kulturmediene slik at bakteriene kan restituere raskere. De fleste medier inneholder en kompleks aminosyreblanding. Basert på dette ønsket vi å undersøke om de enkelte aminosyrene hadde en vekstfremmende effekt på *M. tuberculosis* kulturer med redusert metabolisme. Effekten av de ulike aminosyrene under dyrkning kan gi kunnskap som kan bidra til å designe en aminosyre-cocktail som bakteriene kan utnytte mer effektivt.

Formålet med denne oppgaven var å identifisere hvorvidt enkelte aminosyre tilsatt til kulturmedier kan kutte deteksjonstiden for M. tuberculosis kulturer. Vi var spesielt interesserte i den mulige effekten de ulike aminosyrene har på kulturer med redusert metabolisme. L-lysin monohydroklorid hadde de mest lovene funnene sett i denne konteksten.

Ytterligere validering med bruk av villtype og mutante *M. tuberculosis*-stammer i veldefinerte medier og vekstbetingelser er i midlertid viktig for å kunne påvise virkningsmekanismen til den vekstfremmende effekten av L- lysin.

Funnene er svært relevante for å belyse tilstanden til bakterier som er «dormant» i latent TB og for å designe og optimalisere kulturmedier for isolering av mykobakterier fra kliniske prøver.

ABSTRACT

Tuberculosis (TB) is a major public health problem caused by the infectious agents of the *M. tuberculosis complex* (MTBC). Detecting mycobacteria in sputum samples from TB patients requires time, especially if the bacteria is in a non-replicative and dormant state. Implementing the culture media to recover cells faster is therefore very important. Most media contain a complex amino acid mix. Based on this, we wanted to investigate if there was any amino acid that exhibited a growth promoting effect on *Mycobacterium tuberculosis* cultures with reduced metabolism. Learning about the independent contribution of each amino acid during culturing, could help to design an amino acid cocktail that the bacteria could use more efficiently.

The main aim of this thesis was to identify whether any amino acid added to culture media could shorten the detection time for growth of *Mycobacterium tuberculosis* cultures. In particular we were interested in the possible effect on cultures with reduced metabolism. Especially L- Lysine monohydrochloride exhibited the most promising findings in this context.

However, further validation of wildtype and mutant M. tuberculosis strains in well- defined media and growth conditions are required to discover the mechanism of action of the Lysine- induced growth enhancement.

These findings are highly relevant for elucidating the state of dormancy in latent TB and for designing and optimized culture medium for isolation of mycobacterial isolates from clinical specimens.

ABBREVIATIONS

ADC	Bovine serum albumin, dextrose and catalase
BSA	Bovine serum albumin
DC	Dextrose and catalase
DOT	Directly observed therapy
DMN-Tre	4-N, N-dimethylamino1,8-naphthalimide–conjugated trehalose
HIV	Human immunodeficiency virus
IFN- γ	Interferon-gamma
IGRA	Interferon-gamma release assay
KatG	Catalase peroxidase
L1-L7	Lineage 1-7
LTBI	Latent TB-infection
MGIT	Mycobacteria growth indicator tube
MTBC	Mycobacterium tuberculosis complex
MDR-TB	Multidrug resistant tuberculosis
NTM	Non-tuberculous mycobacteria
PZA	Pyrazinamide
TB	Tuberculosis
TST	Tuberculin skin test
WHO	World health organization
XDR-TB	Extensively drug-resistant tuberculosis

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INTRODUCTION

1. Tuberculosis

1.1 Epidemiology

Tuberculosis (TB) is an infectious disease caused by members of the *Mycobacterium tuberculosis* complex (MTBC). Studies of ancient human remains confirm that TB has existed for thousands of years. DNA from the MTBC has been detected in both Egyptian (Zink et al., 2003) and Peruvian mummies (Salo et al., 1994), as well as in bone remains from the Neolithic period (Hershkovitz et al., 2015). In the late 19th and early 20th centuries, TB was one of the leading causes of death in several European counties. However, thanks to increased socioeconomic conditions, the prevalence of TB in Western countries declined (Grange et al., 2001). The development of the Bacillus Calmette-Guérin (BCG) vaccine in 1921 (Luca and Mihaescu, 2013)and the discovery of the first effective antimycobacterial drugs in 1945, further accelerated the decline of TB-infections (Nguyen, 2016). At some point, TB was no longer considered a public health problem (Perkins, 1963). From the 1980s, however, a setback emerged in association with the epidemic of the acquired immune deficiency syndrome (AIDS) (Nguyen, 2016). Unfortunately, TB is still a major public health problem in many countries. It is also considered to be a serious global threat due to its high incidence combined with the rise in drug resistance. *M. tuberculosis* is one of the most successful pathogens of our time and is today the leading cause of death from a single infectious agent (WHO, 2018).

1.1.1 TB prevalence and mortality

According to WHO (2018), in 2017, 10 million people became sick and 1.6 million people were killed by TB. All countries had reported cases; however, India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%) had the highest prevalence and accounted for around two thirds of the cases. Europe and America have the lowest burden, covering approximately 6 % of the incidences combined. Moreover, it is estimated that 23% of the global population have a latent TB- infection (LTBI) (Houben and Dodd, 2016). Latent infection is a term referred to as a clinical state characterized by the absence of apparent disease (Young et al., 2009).

Vynnycky and Fine (2000) have estimated that in potentially 5-15% of the LTBI cases, reactivation and development of active disease will occur. Further, the probability of reactivation is much higher among immunocompromised people. Among risk factors for developing TB, human immunodeficiency virus (HIV) infection is the strongest. It has been estimated that people infected with HIV, have 20 times higher risk of developing TB compared to the rest of the world's population (WHO, 2018).

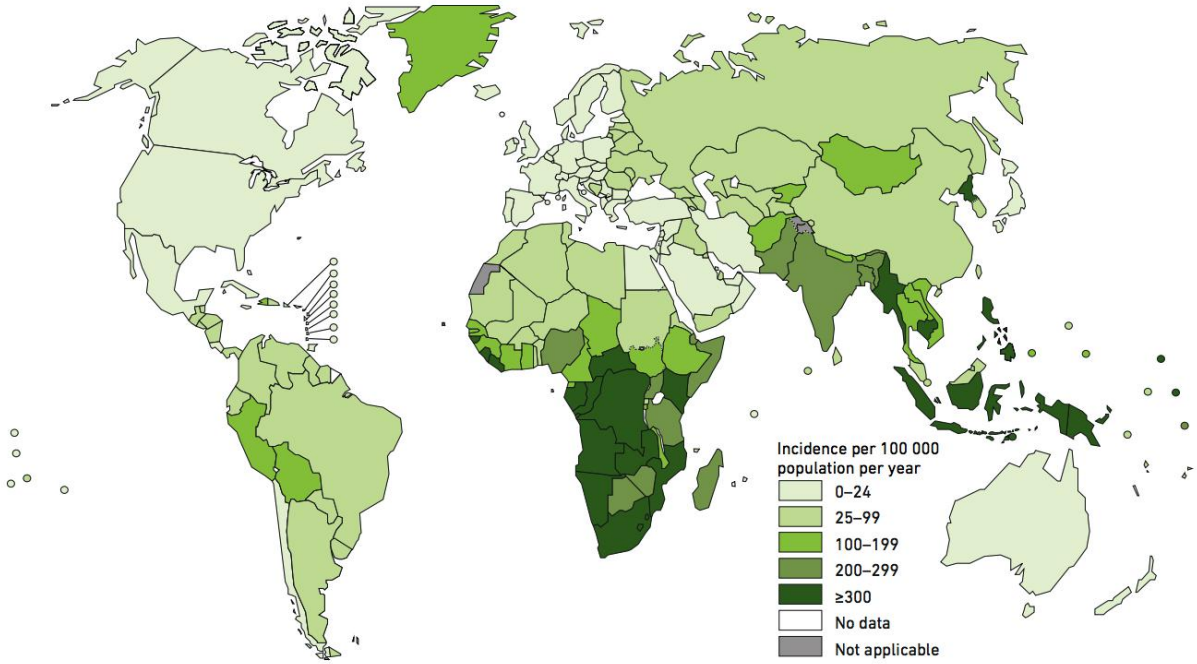

Figure 1. Estimated global TB incidence rates, 2017 (WHO, 2018)

1.1.2 Drug resistant TB

An estimated 45 million deaths have been prevented between 2000 and 2017 due to TB treatment. Unfortunately, drug resistant TB is emerging. Multidrug resistant (MDR)-TB, defined by being resistant to the two most powerful antimycobacterial drugs, rifampicin and isoniazid, represented an estimated 3.5% of new incidences and 18% of previously treated cases. China, India and the Russian Federation had the highest prevalence and accounted for around 47% of the total. Extensively drug-resistant (XDR)-TB, defined by being resistant to rifampin, isoniazid, one fluoroquinolone drug and at least one of the injectable antibiotics (amikacin, capreomycin or kanamycin), counted for 8.5% of the MDR-TB cases. Currently, a total of nearly 500 000 cases of MDR-/XDR-TB are predicted to arise globally per year (WHO, 2018).

1.2 Symptoms

The general symptoms of active TB are fever, fatigue, reduced appetite, weight loss and night sweats. Individuals with pulmonary disease may also have a long-lasting cough. In advanced disease, the sputum may be admixed with blood. This condition is referred to as haemoptysis. The infectious agent can disseminate throughout the body causing extrapulmonary TB. In this case, the spectrum of symptoms is broad. However, infected individuals with active disease may be asymptomatic, referred to as subclinical TB. Further, LTBI is both asymptomatic and a non-transmissible state (Pai et al., 2016).

1.3 Immuno-pathogenesis and development of TB disease

TB is mainly transmitted by inhalation of aerosols containing infectious bacilli, produced by individuals that are infected and contagious. The droplets are expelled through coughing and other forceful expiratory manoeuvres (Churchyard et al., 2017). Infectious droplets are 1-5 µm in diameter, can contain 1-10 TB bacteria and are small enough to reach the alveoli. Moreover, they can endure in the air for hours. Respiratory droplets that are over 100 µm will however not reach the alveoli, nor will they endure in the air, but instead fall to the ground within one meter from the origin (Lee, 2016). Infectiousness depends on bacillary load, duration of exposure, host and bacterial factors (Churchyard et al., 2017). Under 10% of infected individuals will develop disease during their lifetime. However, the risk is increased for individuals that for different reasons are undernourished or immunocompromised. Risk factors include HIV/AIDS, anti-tumor necrosis factor (TNF) therapy, mal- nutrition, diabetes, smoking and alcohol abuse. The pathogenesis of TB is a result of interaction between both, the pathogen and the host defence. Barry 3rd et al. (2009) have proposed a model for different outcomes after exposure; 1) elimination of the pathogen with innate immune responses 2) elimination of the pathogen with acquired immune response 3) latent /quiescent infection 4) subclinical infection and 5) active TB. Active TB is characterized by tissue destruction due to the massive influx of inflammatory cells. Moreover, MTBC can disseminate from the lungs, which are the main entrance into the body, causing infections in other organs.

1.3.1 Elimination of the pathogen with innate immune responses

After inhalation *M. tuberculosis* is transported through the airways. The first line of defence is the respiratory mucosa consisting of airway epithelial cells (AECs), the lamina propria; a layer of connective tissue and immune cells, and airway surface liquid, consisting of mucus and immunoglobulin A. The AECs use membrane associated pattern recognition receptors (PRRs) to recognize structures present on the mycobacteria, pathogen associated molecular patterns (PAMPs), mediating the production of proinflammatory factors that recruit and activate phagocytes, including macrophages, neutrophils, dendritic cells (DCs) and natural killer cells (NKs) (Lerner et al., 2015). Also, the phagocytes express an array of PRRs. Upon binding to the macrophages, the pathogen is ingested by receptor mediated phagocytosis, where the bacteria are exposed to the hostile environment of the phagolysosomes. Reactive oxygen and nitrogen species (ROS and RNS), antimicrobial peptides, acidification and hydrolytic enzymes may eventually destroy the bacteria (Gengenbacher and Kaufmann, 2012). Further the infected host cell can undergo apoptosis, innate programmed cell death, that maintains an intact cell membrane, enabling control over the infection (Lerner et al., 2015). It has been shown that a proportion of TB-exposed healthy individuals never covert to a positive TST or IGRA test, indicating that the innate immune system may have eliminated the intruders without the need for an acquired immune response (Morrison et al., 2008, Verrall et al., 2014). However, pointed out by Young et al. (2009), the lack of conversion to positive TST and IGRA may also reflect restrictions of the tests.

1.3.2 Elimination of the pathogen with acquired immune response

The acquired immune responses mediated by B- and T-lymphocytes is important for the elimination of M. tuberculosis. However, the bacterial antigens and the host response involved is not fully understood. Residence lung DCs are together with the macrophages of the first cell types to encounter the bacteria. After phagocytosis, they present the pathogens antigens to T-lymphocytes in the local lymph nodes. DCs are currently considered to be the most efficient inducers, thus representing the link between the innate and acquired immunity. The T- cell response in TB infection is slow, taking place 15-18 days after infection. Various T -cell types release an array of cytokines that stimulate both pro- and anti-inflammation (Cooper, 2009). Interferon gamma (INF-γ), one of the cytokines

produced by T- lymphocytes is important for macrophage activation and controlling of mycobacteria (Flynn et al., 1993).

1.3.3 Latent infection (LTBI)

A latent TB infection refers to a clinical manifestation without apparent disease or symptoms. Evidence of such infection can be detected by the positive conversion of TST and IGRA (Barry 3rd et al., 2009). Moreover, latently infected individuals are not contagious, and do not transmit the bacteria to others (Pai et al., 2016).

Alveolar macrophages phagocyte the bacteria. However, *M. tuberculosis* manages to escape eradication and survive inside the cells. For instance; the glycolipid lipoarabinomannan (LAM) found in the cell wall of *M. tuberculosis*, inhibit the phagosome-lysosome fusion (Hmama et al., 2004). Also, it has been suggested that trehalose dimycolate (TDM), another abundant cell wall glycolipid, may delay or block phagosomal maturation (Axelrod et al., 2008) (Katti et al., 2008). Tyrosine phosphatase (PtpA), a secreted protein of unknown function acts on the phagosome maturation by inhibiting the acidification (Bach et al., 2008). Inside the phagolysosome, *M. tuberculosis* are able to neutralize ROS by expressing the "enhanced intracellular survival" protein (Shin et al., 2010).

The infected cells further migrate from the airways into the pulmonary tissue producing chemokines recruiting more immune cells, including more macrophages, neutrophils, NKs and T-cells, creating lesions referred to as granulomas (Ehlers and Schaible, 2013). Granulomas are focal accumulations of bacteria, in different metabolically states, and immune cells in different states of differentiation (Ehlers, 2010).
At this stage, most individuals manage to control the pathogen, preventing it from spreading further; and a LTBI is established. Granulomas are versatile and can be differentiated into types, more and less abundant according to different states of the disease. In LTBI, solid granulomas dominate. In such granulomas, the central parts consist of phagocytes, DCs and fibroblasts, whereas lymphocytes form an outer ring. Further, a fibrotic wall distinguishes the granuloma from the surrounding tissue. The bacteria inside the granulomas are most likely in a dormant state, futured by a non- or low- replicative state with low metabolic activity (Gengenbacher and Kaufmann, 2012). In the granuloma, *M. tuberculosis* encounter various stresses, including nutrition depletion, low pH, ROS, RNS

and hypoxia (Dutta and Karakousis, 2014). Hypoxia may be the predominant trigger for the downshift into a reduced metabolic state seen in LTBI (Via et al., 2008) (Boshoff and Barry 3rd, 2005) .Especially T-lymphocytes are important for sustaining the latent infection (Gengenbacher and Kaufmann, 2012).

1.3.4 Subclinical infection

Achkar and Jenny-Avital (2011) suggest defining "subclinical TB" as an asymptomatic infection in an immunocompromised host, with evidence of pulmonary TB. By evidence, referring to microbiology or radiographic diagnostics. The state is associated with ineffective containment of the bacteria by the immune system, transitioning from LTBI to active TB. Moreover, an asymptomatic infection in a relatively immunocompetent host, in early, contained disease, can be referred to as "incipient TB".

1.3.5 Active TB

Active TB is characterised by clinical symptoms caused by the tissue damage generated by the high bacterial burden and the host's response to the intruders (Gengenbacher and Kaufmann, 2012). The initial phase, defined as primary infection, occur in individuals which have not been exposed to *M. tuberculosis* earlier. The disease develops within five years after the initial infection and generally affect individuals who are immunocompromised, children and young adults (Milburn, 2001). Typically, the disease develops and spread as caseous granulomas to lymph nodes and other organs in the weeks before immunity develops (Hunter, 2011). Necrotic or caseous granulomas have decreased structure compared to the solid granulomas seen in latent infections. Further, the cavity centre is liquefied due to lipid debris from dead host cells (Gengenbacher and Kaufmann, 2012).

Secondary infection, also referred to as post-primary infection occur in individuals as a result of reinfection of primary infection or reactivation of a latent infection. It differs from primary infection; secondary infection is not due to inadequate immunity like in primary infection. Typically, adults are affected, and the infection does not disseminate to other organs, but are localized in the upper lung lobes. Also, secondary infected individuals are more contagious than those suffering from primary infection (Hunter, 2011). When a latent infection reactivates, the solid granuloma becomes increasingly necrotic, providing conditions compatible for *M. tuberculosis* to be resuscitated from the dormant state. They

start replicating again and increase their metabolism as the granuloma matures into becoming caseous. The caseous granulomas often rupture and release bacteria, which further may invade new host cells (Lerner et al., 2015).

How host cells die after being infected with M. tuberculosis is central for the outcome of the disease. Normally, infected host cells undergo apoptosis, which is controlled and considered to be beneficial for the organism. In contrast, necrosis is an uncontrolled, traumatic cell death resulting in cellular damage. *M. tuberculosis* have the ability to manipulate human cells and both inhibit apoptosis and induce necrosis.

In patients with active TB, neutrophils are the most abundant cell population (Eum et al., 2010). Whether infected neutrophils undergo apoptosis or necrosis is also of importance for the outcome of the disease. Dallenga et al. (2017) demonstrated that *M. tuberculosis* ESAT-6 induced necrosis in neutrophils and promoted mycobacterial survival and proliferation inside macrophages that had phagocytized the cells due to the necrosis induced production of ROS by the neutrophils.

1.4 TB diagnostics

The diagnosis of active TB disease is based on clinical suspicion, which further can be investigated through radiology, histopathology and microbiology techniques. The diagnosis of LTBI is based on immunological testing (Norbis et al., 2013).

1.4.1 Immunological tests

There are two available immunological tests for the detection of LTBI, the tuberculin skin test (TST) and the IFN-γ release assay (IGRA). The principle of both tests is based on the lasting immune response against M. tuberculosis antigens. However, these tests have limitations. They cannot differentiate LTBI from active TB disease or distinguish between new or re-infection. Further, they have reduced sensitivity in immunocompromised individuals and can at different extents give a positive result after exposure to other species within the *Mycobacterium* genus (Pai et al., 2016).

1.4.2 Microbiological tests

Microbiological diagnostics are mainly based on microscopy, culture-based methods and molecular techniques.

1.4.2.1 Microscopy

Microscopy of sputum smear is a widely used method, especially in low- and middle-income countries. However, as other rapid molecular tests increase, the use of microscopy for diagnostic purposes is phased out due to low sensitivity (Pai et al., 2016).The method still remains valuable for monitoring of the disease and the contagiousness in smear- positive patients. Staining and microscopy are also used to detect mycobacteria in cultures. Because of their complex cell wall, mycobacteria are resistant to Gram- staining. Further, the cell wall is resistant to decolorization with acid alcohol, thus given the name "acid- fast bacilli". Ziehl-Neelsen stain and the fluorescent stain with auramine are two alternative techniques used for staining of mycobacteria. When replicative *M. tuberculosis* converts into non-replicating, dormant bacteria, it loses its acid- fastness. This is known as the Koch's paradox (Pai et al., 2016). The phenomenon has been linked to physiological metabolic changes and modifications of the cell wall composition in the dormant bacteria, which may be of importance for the diagnosis (Seiler et al., 2003). WHO has expressed the need of new tests for rapid TB diagnosis and multiple groups have taken the challenge. Bertozzi and colleagues have recently designed a 4-N,N-dimethylamino1,8-naphthalimide–conjugated trehalose (DMN-Tre) probe that fluoresces when transitioned to an hydrophobic environment, such as the incorporation into the mycomembrane of Actinobacteria. The probe provided rapid results and was suggested as a possible new method for TB diagnosis. Furthermore, the results suggested that the probe can distinguish between metabolically active and inactive organisms. The group particularly pointed out their interest in whether the probe can detect non-replicating "dormant" cells. However, further studies are needed to determine the hypothesis (Kamariza et al., 2018a).

1.4.2.2 Culturing

Culturing remains the most sensitive method for detection of mycobacteria, thus remains the "gold standard" for laboratory diagnostics of TB. However, culturing is a time-consuming process that can take several weeks. The process includes collection of

representative clinical specimens, transport to the laboratory, decontamination to remove the commensal flora, inoculation into appropriate media and incubation in under the right conditions. Further, growth must be monitored and detected before performing additional tests for identification of phenotypic drug susceptibility (Asmar and Drancourt, 2015).

1.4.2.3 Molecular methods

Molecular methods provide rapid detection of M. tuberculosis and drug resistance mutations. Also, molecular methods are more sensitive than microscopy, especially in samples with a low number of bacilli (Pai et al., 2016) The use of molecular tests is increasing globally. Among the rapid molecular tests available, WHO (2018) recommends Cepheid Xpert® MTB/RIF assay which can provide results within two hours. The assay is based on real- time PCR technique and detects both the presence of *M. tuberculosis* and the genotypic rifampicin resistance

1.5 Treatment

The drug regimens are variable and depend on several factors such as the characteristics of the patient, for instance age, weight, pregnancy, co- infections or other diseases. Moreover, the regimens depend on whether it is a new or previous TB case, antibiotic drug resistance and the available resources in the relevant country, including possibilities for directly observed therapy (DOT). Standard treatment for TB calls for six months and comprises four first-line anti-TB drugs, which are isoniazid, rifampicin, pyrazinamide and ethambutol (WHO, 2010). Isoniazid is a prodrug that after activation by the bacterial enzyme, catalase peroxidase (KatG), generates reactive species that are potent inhibitors of enzymes involved in the biosynthesis of cell wall lipids and nucleic acids. Antibiotic resistance towards the drug occur as a result of mutations in the *katG* gene (Timmins and Deretic, 2006). Rifampicin binds to the β- subunit of the bacterial RNA polymerase, thus inhibiting DNA replication and transcription. Drug resistance is due to mutations in the *rpoB* gene encoding the RNA polymerase β-subunit (Campbell et al., 2001). Pyrazinamide (PZA) is a prodrug that after activation by pyrazinamidase, encoded by the *pncA* gene in the bacteria, inhibits multiple targets including energy production and trans- translation (mechanism of quality control pathways in bacteria). Resistance is mostly caused by mutations in the *pncA* gene. Opposite from other TB drugs that primarily affect growing bacilli, PZA kill non-

replicating bacteria (Zhang et al., 2013). Ethambutol is a bacteriostatic drug that targets cell wall formation by interfering with the enzyme responsible for arabinogalactan biosynthesis, arabinosyl transferase. Resistance is caused by mutations in the genes *embCAB*, encoding the enzyme. Without going into detail, there are also available second-line anti-TB drugs such as fluoroquinolones, amikacin, kanamycin, capreomycin, macrolides, linezolid and new TB- drugs, including bedaquiline, and more (Palomino and Martin, 2014).

Treatment of LTBI is of importance for the elimination and control of TB worldwide. WHO (2018) recommends treatment with isoniazid or rifampicin or both for people with HIV, individuals who are in household with confirmed pulmonary cases and clinical risk groups (WHO, 2018)

2. The genus *Mycobacterium*

2.1 Taxonomy

The genus *Mycobacterium* is comprised of more than 170 closely related bacterial species in which most members can be referred to as non- tuberculous mycobacteria (NTM). NTM are mostly found in the environment, particularly in soil and water (Fedrizzi et al., 2017). The members of the MTBC, *M. leprae* and *M. lepromatosis* are separated from the NTM group and are inversely not found in the environment but have animals as their reservoir (Honap et al., 2018, Brites and Gagneux, 2017). The bacteria belongs to the phylum Actinobacteria, the order Corynebacteriales and the family Mycobacteriaceae (NCBI, 2018)

2.2 *Mycobacterium tuberculosis* complex (MTBC)

Mycobacterium tuberculosis complex (MTBC) is a group of closely related mycobacteria which have over 99 present nucleotide sequence identity (Brites and Gagneux, 2015) and identical 16srRNA sequences (Kirschner et al., 1993). Included in the complex are the pathogens *M. tuberculosis (K*och, 1982*)* and *M. africanum (Castets e*t al., 1968*)*, which have humans as their only known reservoir and are the main causative agents of human TB infection. Moreover, *M. bovis* (Smith, 1898), *M. caprae* (Aranaz et al., 1999), *M. microti* (Wells, 1937), *M. canettii* (van Soolingen et al., 1997), *M. pinnipedii* (Cousins et al., 2003), *M. mungi* (Alexander et al., 2010) , *M. orygis* (Lomme et al., 1976), *M. suricattae* (Parsons et al., 2013), the dassie bacillus (Wagner et al., 1958) and the chimpanzee bacillus (Mireia et al., 2013), are animal adapted members of the MTBC, often given their names according to the animal from which they were first isolated. It has been proposed that it is likely that the MTBC evolved from an organism similar to *M.canettii.* Further, it is plausible that the ancestor had an environmental reservoir before it adapted to animals and spread globally (Koeck et al., 2011). Smith et al. (2006) have suggested, due to their close relationship, that the members of the complex can be better considered as different ecotypes rather than different species, each having distinct host preferences and ecological niches. However, the host range of some of the species remain poorly defined. The animal adapted MTBC are only rarely isolated from humans. Being the most prominent, *M.bovis* and *M.caprae* cause only 1-3 % of human TB infections. In addition, causing infection outside of their ecological niche, rarely leads to transmission in between the "unpreferred" hosts (Borna et al., 2013).

2.2.1 MTBC lineages

The human adapted members of the MTBC can be divided into seven phylogenetic linages, L1 to L7. Lineage 5 and 6 belongs to *M.africanum*, the remaining to *M.tuberculosis*. Some of the lineages are distributed globally, whereas others exhibit strong geographical restrictions. Bioinformatical analysis of mycobacterial genomes by Comas et al. (2013), localizes the different lineages as follows: L1 in East Africa, the Philippines and Indian Ocean rim; L2 in East Asia; L3 in East Africa and Central Asian; L4 in Europe, America and Africa; L5 in West Africa; L6 in West Africa and L7 in Ethiopia.

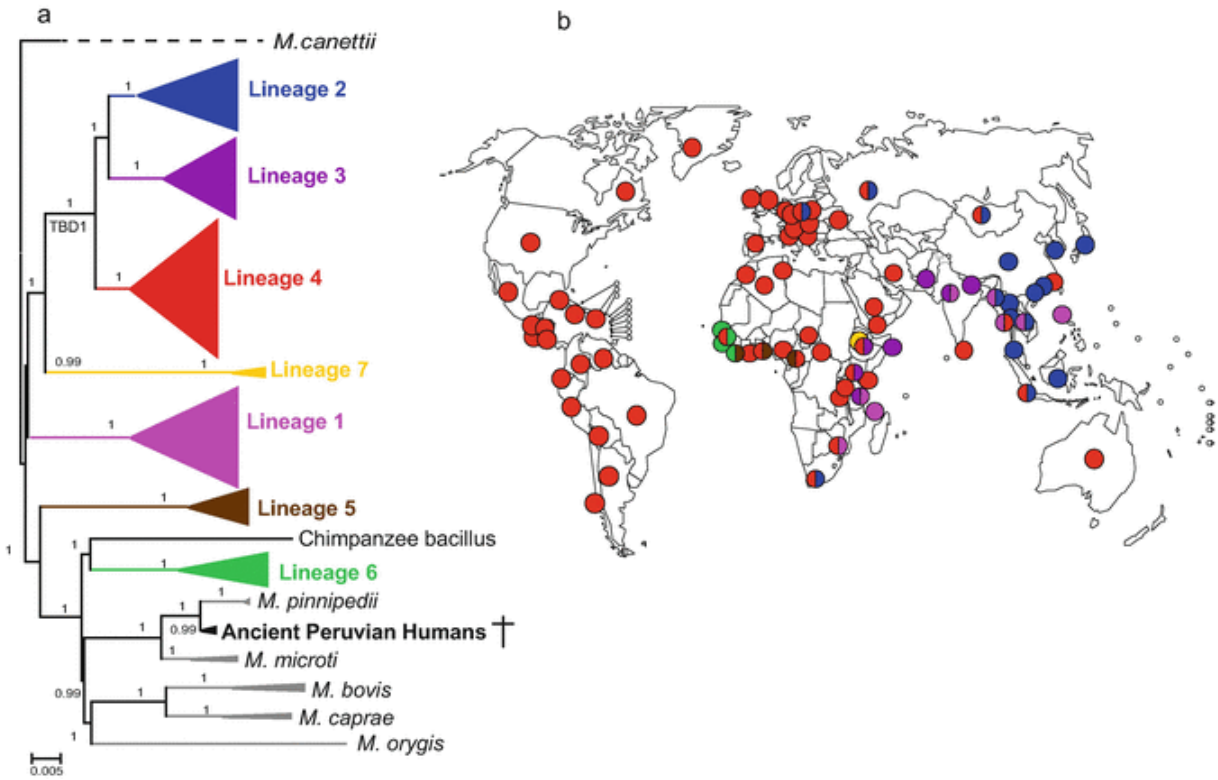

Figure 2. MTBC phylogenetic tree and geographical distribution

(Brites and Gagneux, 2017).

2.3 Physiology and characteristics

M. tuberculosis is an obligate aerobe and can only grow in presence of oxygen. Further, it is non- motile; lack the ability to move independently and non- sporulating; do not form endospores, a resilient structure containing the metabolically dormant bacterium. The bacterium is weakly gram positive, rod shaped (Willey et al., 2014) and 0.3–0.5 µm in diameter with variable length, ranging from 1.5 µm–4.0 µm. *M. tuberculosis* has a generation time of 16 hours or more at 37°C, are metabolically flexible and intracellular;

capable of growing inside host cells (Cook et al., 2009). *M. tuberculosis* has a large bacterial genome comprised of about 4.4 x 10^6 base pairs with a high content, 65 % of the genome, of guanine and cytosine (G+C). Further, it contains around 4000 genes (Cole et al., 1998).

2.3.1 Mycobacterial cell wall

Mycobacteria have a complex cell wall comprised of several layers, each with various chemical modifications and structures. The peptidoglycan layer is made up of polymers of the disaccharide N-acetyl glucosamine (NAG)- N-acetyl muramic acid (NAM). Compered to many other bacteria, the peptidoglycan in mycobacteria is heavily crosslinked. Further, is has modifications such as glycolation of NAM and amidation of residues of the peptide side chains. Surrounding the peptidoglycan is a layer of arabinogalactan, which is comprised of repeating units of the disaccharide, galactan, modified with long arabinan polymers. However, some galactan chains remain free of arabinan. Moreover, most of the arabinan is ligated with long chains of mycolic acids, which is characteristic for mycobacteria and an important virulence factor (Kieser and Rubin, 2014). As much as 60% of the cell wall is lipids. The composition and arrangement of the mycobacterial cell wall are still not fully known. An additional layer referred to as the mycomembrane, found in between the mycolic acids layer and the capsule have been demonstrated. This layer consists of glycolipids, phospholipids and lipoglycans together with proteins. Among the mentioned are the glycolipids, trehalose- 6-6-dimycolate (TDM), also known as cord factor, and lipoarabinomannan (LAM) (Chiaradia et al., 2017).

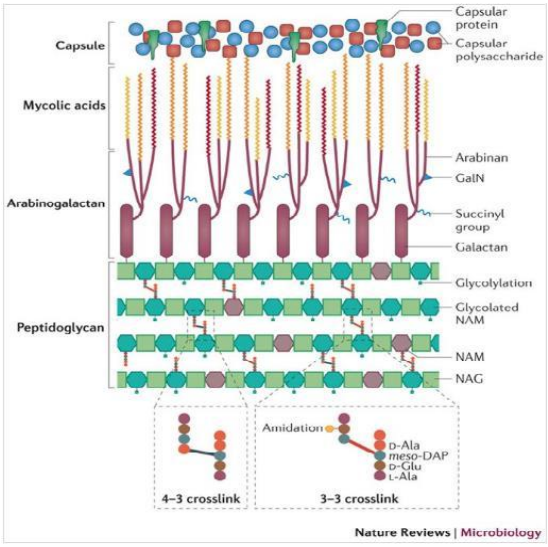

Figure 3. Mycobacterial cell wall. (Kieser and Rubin, 2014)

3. Metabolism of *M. tuberculosis*

Metabolism can be referred to as a set of pathways that cover the consumption and generating of biomass (anabolism), and degradation of organic macromolecules for the generating of new macromolecules and energy (catabolism). Some pathways are essential for the respond to changing environments and the survival of the bacteria in unfavourable conditions. *M. tuberculosis* enable a flexible metabolism which is central for its success as a pathogen (Warner, 2015).

3.1 Energy and carbon sources

M. tuberculosis is a heterotrophic bacterium and obtain carbon and energy from oxidation of organic compounds such as carbohydrates, lipids and proteins, which provide energy in the form of the high energy molecules ATP and reducing power in the form of NADH and $FADH_2$. NADH and $FADH_2$ further donate electrons to an electron transport chain with, in the case of aerobic respiring mycobacteria, oxygen as final electron acceptor. The processes also generate simple organic compounds needed for biosynthesis (Willey et al., 2014)

3.1.1 Central carbon metabolism

Carbohydrates is catalysed in the glycolytic Embden- Mayerhof pathway and the Pentose Phosphate pathway, which produces pyruvate, that can be further used in the Tricarboxylic Acid Cycle (TCA) pathway. Lipids are degraded into glycerol and fatty acids by lipases. Further, the glycerol can be converted to dihydroxyacetone phosphate, an intermediate metabolite of the Embden- Mayerhof Pathway. The fatty acids can be linked to CoA and oxidized in the β-oxidation pathway yielding acetyl-CoA and (Willey et al., 2014) propionyl-CoA, which is metabolized in the TCA, glyoxylate and methylcitrate cycles. The glyoxylate cycle is an alternative pathway of TCA used by *M. tuberculosis* when grown on fatty acids as the main carbon source. The two cycles have many enzymes in common apart from isocitrate lyases (ICL) and malate synthases (MLS), which is specific for the glyoxylate cycle. The Propionyl-CoA generated by β-oxidation is metabolised in the methylcitrate cycle. It has been suggested that ICL in the glyoxylate cycle might function as MCL in the methylcitrate cycle (Munoz-Elias et al., 2006). The pathways mentioned provide carbon precursor metabolites vital for the synthesis of macromolecules and is referred to as the central carbon metabolism (Warner, 2015). *M. tuberculosis* utilizes a wide range of

carbohydrates *in vivo.* However, it uses fatty acids as its main source of carbon and energy during infection. Compared to other many other bacteria, that metabolize carbon in a sequential dependent manner (carbon catabolite repression), *M. tuberculosis* has evolved the capacity to co-catabolize multiple carbon substrates simultaneously, thereby achieving faster growth on carbon source mixtures than on single ones (de Carvalho et al., 2010). In what manner and when *M. tuberculosis* segregates metabolism of single carbon sources from one another is still not fully understood (Ehrt et al., 2018). Further, the bacterium has been demonstrated to recyclyle lipids in, including cell wall glycolipid trehalose, in infected mice (Kalscheuer et al., 2010).

Protein and amino acids can also be utilized as a source of carbon and energy. Bacteria use enzymes called proteases to hydrolyse proteins into amino acids, which can be catabolized and converted into precursor metabolites. To do that, the amino acids have to be deaminated (removal of amino group) before they can be oxidized in the TCA cycle (Willey et al., 2014).

3.2 Energy and nitrogen sources

M. tuberculosis can use both organic, including amino acids, and inorganic compounds such as ammonium and nitrate as nitrogen sources. The organic sources, however, are preferred both *in vitro* and *in vivo*. The inorganic compounds are imported into the cell by transporters. Ammonium by transporters from the Amt family, nitrate by NarK2. Nitrate is further reduced by different enzymes to ammonium. To be used as a nitrogen source, the amino acids have to undergo deamination (removal of the amino, $-NH^{+}_{3}$ group) in order to release available ammonium (NH^{4+}). Deamination is performed by enzymes such as asparaginase, alanine dehydrogenase, serine deaminase, arginine deiminase and glutamate dehydrogenase (Gouzy et al., 2014b).

3.3 Amino acids

3.3.1 Physiology

Amino acids are the compounds of proteins. Each amino acid consists of a central carbon structure attached with an amino group ($-NH^{+}_{3}$) in one end and a carboxyl group ($-COO^{-}$) in the other. Also attached, a variable side group (-R) which is specific for each amino acid.

The carboxyl group of one amino acid can form a peptide bond with the amino group of another amino acid, creating polymers of amino acids which make up the different proteins. All proteins have a free amino and carboxyl terminus (Van Slyke, 1942). The natural standard repertoire consists of 20 amino acids, which can be divided into groups depending on the properties of the individual (-R) groups. There are several ways to classify amino acids, which includes grouping them in three; the non-polar amino acids; glycine, alanine, proline, valine, isoleucine, leucine, phenylalanine and methionine, the polar amino acids; serine, cysteine, threonine, asparagine, glutamine, tyrosine and tryptophan, the charged amino acids; aspartic acid, glutamic acid, lysine, arginine and histidine. Other amino acids, like hydroxyproline are produced due to post-translational modifications after the synthesis of the protein (Lesk, 2016). Cys**ti**ne is an oxidized form of the natural amino acid cyst**ei**ne (PubChem, 2004). All amino acids except glycine can occur in two isoforms because of their ability to form different orientations around the central carbon atom. The two isoforms are called L- and D- forms (Lam et al., 2009).

3.3.2 Amino acid biosynthesis

The carbon precursors produced by the central metabolism lack the functional amino- and, or sulfhydryl- groups, which have to be added later in the process. Some amino acids are made directly by the addition of an amino group to a precursor metabolite. For the synthesis of most amino acids, however, the process is more complex and acquire rearrangement of the metabolites. The generating of amino acids burdens the central metabolism for precursors. Many bacteria solve this by the use of alternative pathways for adequate supply (Willey et al., 2014). As mentioned above, the glyoxylate cycle can serve as such an alternative pathway in *M. tuberculosis* (Munoz-Elias et al., 2006).

3.3.3 *M. tuberculosis* utilization of amino acids *in vivo*

Which sources that are used by *M. tuberculosis* during infection is unknown (Gouzy et al., 2014b). *M. tuberculosis* has the potential to synthesize all the essential amino acids (Cole et al., 1998). However, it has also been suggested that amino acid uptake and degradation is required for multiplication and virulence in vivo. It has been indicated that asparagine and aspartate serve as nitrogen providers through the asparagine and aspartate transporters. Genetic deletion of the transporters, has been proven to result in decreased fitness and

virulence of the mycobacteria in mice (Gouzy et al., 2014a). Also, auxotrophic mutants (referring to an organisms inability to synthesize a particular compound required for growth) of arginine, lysine, leucine, methionine, proline and tryptophan, indicate that mycobacteria has a decreased ability to provide adequate sources of essential amino acids in vivo (Warner, 2015).

3.3.4 *M. tuberculosis* utilization of amino acids *in vitro*

Because deamination is performed by asparaginase, alanine dehydrogenase, serine deaminase, arginine deiminase and glutamate dehydrogenase, the amino acids aspartate, glutamate, asparagine and glutamine are assumed to be the most efficient nitrogen sources *in vitro* when provided as sole nitrogen sources (Gouzy et al., 2014b) (Lyon et al., 1970). However, it has been demonstrated that Mycobacterium species (*M. smegmatis* and avirulent *M. tuberculosis*) utilize the amino acids, asparagine, alanine and glutamine differently (Lyon et al., 1970).

4. Growth and reduced metabolic states

4.1 Mycobacterial growth and division

Unlike many bacteria, mycobacteria grow and divide asymmetrically, producing heterogeneous daughter cells of different sizes, growth rates and cell wall compositions, which may represent an advantage in variable environments encountered in the host, predicted to increase survival and potentially induce LTBI (Kieser and Rubin, 2014). Two hypotheses have been suggested explaining how mycobacteria divide. Aldridge et al. (2012) reported that both of the poles of the bacilli elongate, however, that the new pole grow slower than the old pole, inherited from the mother cell, giving rise to daughter cells of different sizes. Santi et al. (2013) suggests that growth of the poles is symmetric until cytokinesis, then growth predominantly occur at the old pole. Cytokinesis is the last phase of cell division when the cytoplasm is separated resulting in two daughter cells. Even though the hypotheses are different, there is agreement regarding the production of cells of different sizes, which elongate at different rates. Thereby, daughter cells are phenotypically different although genetically identical (Kieser and Rubin, 2014).

The synthesis and regulation of the cell wall are complicated in mycobacteria as they have to synthesize and hydrolyse peptidoglycan, arabinogalactan and mycolic acids simultaneously. The synthesis and hydrolysis of peptidoglycan is coordinated by penicillin-binding proteins and hydrolases. *M. tuberculosis* encode the peptidoglycan hydrolase, RipA which has been demonstrated essential for growth and separation of cells (Kieser and Rubin, 2014). The hydrolase can further interact with resuscitation promoting factor B (RpfB), another peptidoglycan hydrolase (Hett et al., 2008) that will be further mentioned in correlation to resuscitation of dormant cells. The synthesis of arabinogalactan is regulated through the *embCAB* operon, which encodes the transferases EmbA, EmbB and EmbC. The transcription factor EmbR activate the production of arabinogalactan through *embAB*, whereas phosphorylation of the same transcription factor activates the whole regulon, *embCA*, resulting in enhanced production, which may enable adaptation to the stresses encountered inside macrophages. The synthesis of mycolic acids is firmly regulated as it is energetically expensive. The synthesis is regulated through the transcription factors MabR and FasR which binds to the FAS (fatty acid synthase complex) operon. Binding by MabR

decrease the production, while FasR increase the production of mycolic acids (Kieser and Rubin, 2014).

4.1.1 Bacterial growth curve

When bacteria are inoculated into a fresh medium, the bacterial growth dynamics typically can be divided into four phases. In the initial lag phase, the bacteria adjust to the environment and do not replicate. In the log phase, the bacteria replicate exponentially before the growth halts in the stationary phase because of limited nutrients and accumulation of toxic products. In the death phase the bacteria lose their ability to divide and start to die exponentially. However, different cell populations become more heterogeneous in the stationary phase. Some populations die while others survive and remain viable (Wang et al., 2015) (Zhang, 2004). Mycobacteria display a remarkable ability to survive the stationary phase (Smeulders et al., 1999).

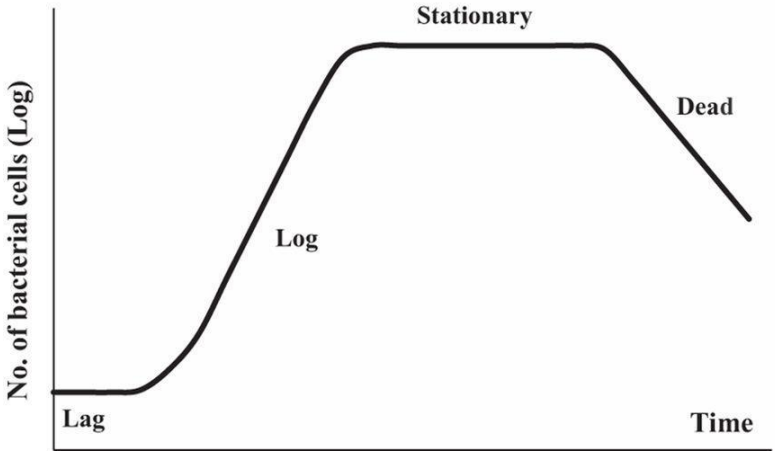

Figure 4. Bacterial growth curve (Wang et. Al 2015).

4.2 Definitions of reduced metabolic states

4.2.1 Dormancy

The term "dormancy" can be defined as a state of bacterial metabolic shutdown which is reversible. In such dormant state the metabolism of the bacteria is low. They are non-replicative and do not form colony forming units (CFU) immediately on conventional culture media. However, they remain viable and can be referred to as viable but not culturable (VBNC). Dormant bacteria further exhibit the ability to resist various stresses and antimicrobial agents. Dormancy is a debated term as it has been used to address multiple conditions regarding reduced metabolic activity in bacteria. The confusion is linked to the

definition of dormant bacteria requiring resuscitation by addition of exogenous factors to reverse from the dormant state and again form CFU. It has been pointed out that various models of dormancy do not generate truly dormant cells as they are still CFU forming. However, they may represent a pre-state towards dormancy (Chao and Rubin, 2010, Zhang, 2004, Dutta and Karakousis, 2014).

4.2.2 Persistence

The term "persistence" can be defined as the ability of bacteria to tolerate or persist various stress conditions. Persistence is a consequence of dormancy, a phenotypically trait probably caused by the transformation of bacteria into a reduced metabolic and non- replicative state. The term is often used in context with antibiotic pressure; the bacterial capacity to survive indefinitely despite continued exposure to appropriate drugs. The organisms are phenotypically resistant to drugs even though genetically susceptible (Gengenbacher and Kaufmann, 2012, Dutta and Karakousis, 2014).

4.3 Population heterogeneity *in vivo* and *in vitro*

McCune and Tompsett (1956), McCune et al. (1966) presented one of the most convincing evidence regarding *M. tuberculosis* enduring in a dormant state *in vivo*. After infecting and treating mice with TB drugs, no growth of the bacilli could be demonstrated in the mice tissue. However, when treated with immunosuppressive drugs, growth was demonstrated in tissues from almost all the mice. Traditionally, LTBI has been considered comprised of bacteria in a dormant state. However, it has been suggested that a portion of the bacteria are, at a minimal rate, probably replicating. The theory is based on the observation that isoniazid, a TB drug that only targets replicating bacteria, can prevent reactivation of disease (Houk et al., 1968). Medlar et al. (1952) found that in lung tissue from patients who had received treatment for TB, all lesions smear-positive, growth was only obtained from lesions of open cavities. In solid necrotic lesions, growth was only detected in 7% of the cases, leading to the hypothesis that not all the bacterial populations could be resuscitated using conventional culturing techniques. Later, Hobby et al. (1954) managed to obtain growth from 78% of closed, necrotic lesions from patients treated for TB. However, for a portion of the lesion samples, growth was first detected after a prolonged time of incubation, leading to the suggestion that the bacterial cells in such lesions is in a reversible

suppressed metabolic state. At present time, the physiological status of bacteria residing inside granulomas is unknown, however, conditions within granulomas suggests that they contain heterogeneous populations of replicating and non-replicating cells (Gideon and Flynn, 2011).

Zhang (2004) conveyed the unpublished observation that in aged mycobacterial cultures, dormant cells are rarely found as a homogenous population. Instead they are found in a mixture of cells with different metabolically states, including dead cells. Garton et al. (2008) revealed nonreplicating persistence in *M. tuberculosis* smear- positive sputum samples. Also, Mukamolova et al. (2010) found that the cell population of smear- positive sputum samples was dominated by cells that could only be grown in the presence of resuscitation-promoting factors (RpF), proteins that promote growth, indicating the presence of metabolically heterogenous populations, including dormant and metabolically active cells.

Altogether, it is likely that *M. tuberculosis* coexist in different metabolically stages in infected individuals. Although speculative, Gengenbacher and Kaufmann (2012) suggest an equilibrium between the dormant and replicative bacteria. During active TB, metabolically active bacteria outnumber cells with low metabolism, while under LTBI, the dormant outnumber some active replicating bacteria. Further, this equilibrium may distinguish the course of the infection.

4.4 Models of dormancy and persistence

It is obvious that *in vitro* models are unable to copy the complex host-pathogen interactions underlying LTBI. However, models that mimic selected environmental features of the condition would still be useful in the study of dormancy. Nutrient deprivation, iron restriction, acidification, ROS, RNS, antimicrobial agents and hypoxia are all features that *M. tuberculosis* are assumed to be exposed to when residing inside host macrophages and in granulomas. Several *in vitro* models have been developed based on these stress factors to induce states of reduced metabolism and dormancy in *M. tuberculosis* (Dutta and Karakousis, 2014).

In comparison to *in vitro* models, *in vivo* models represent the entire repertoire of host immune defences and could induce dormancy in a more authentic way. However, no animal

model has yet been able to copy tuberculosis in humans. Murine, mouse model is the most extensively used for the study of *M. tuberculosis* pathogenesis. Unlike human tuberculosis, mice can control a heavy bacterial burden for months before it causes early death of the animals. Further, the physiological state of the bacteria during this chronic phase in murine infection, remain unclear (Chao and Rubin, 2010, Dutta and Karakousis, 2014).

4.4.1 Wayne model

The Wayne model (Wayne and Hayes, 1996), named after its developer Lawrence Wayne, is established as an *in vitro* model for inducing dormancy in *M. tuberculosis* by progressive hypoxia. However, it has been pointed out that the model does not represent a true dormant state according to the definition of dormancy, which state that bacteria in such a state would need to be resuscitated by exogenous factors to reverse from the dormant state and form CFU (Zhang, 2004). When oxygen is reintroduced to the Wayne culture, the bacteria will start replicating again (Hu et al., 1998). However, it is not yet determined which states that are most relevant in human disease (Chao and Rubin, 2010). The model has been able to identify factors related to persistence *in vivo* and appear to produce a significant number of dormant cells when inoculated for an extended time (Shleeva et al., 2002). Moreover, the model is effective, simple and has been studied extensively, thus provided knowledge on the physiology of hypoxic, non- replicating *M. tuberculosis*, which is useful as the model probably represent a pre- state on the way towards dormancy (Chao and Rubin, 2010).

In the Wayne model, *M. tuberculosis* is cultivated with gradual depletion of oxygen. As *M. tuberculosis* is an obligate aerobic microbe, the depletion of oxygen will result in a non-replicating persistent culture capable of surviving in microaerobic and anaerobic surroundings. It has been suggested that a non- replicative persistent state, which is induced by the Wayne model, can correlate to the physiological state of the bacteria when residing in granulomas during LTBI (Wayne and Sohaskey, 2001) (Via et al., 2008). In the model, a culture of *M. tuberculosis* is slowly stirred in sealed tubes with a defined head space ratio of air to culture medium. As the bacteria replicate the oxygen content decrease until the culture becomes hypoxic. Meanwhile, the bacterial cells adapt to the decreased oxygen tension entering two stages of non- replicating persistence (NRP). At the first stage, NRP1,

the oxygen content decreases below 1%, the cell replication and transcription are terminated, and the cell wall of the bacteria is thickened. In the second stage, NRP2, the oxygen saturation is below 0.06% and the optical density stabilizes (Wayne and Hayes, 1996). Moreover, the culture is insensitive to isoniazid, as mentioned is only active against dividing cells. Inversely, the culture is susceptible to the antibiotic, metronidazole (Wayne and Sramek, 1994), which require activation by reduction to be effective. The respiratory chain reduces the drug, which only cause DNA damage under hypoxic conditions. Under aerobic conditions the reduced species instead reacts with molecular oxygen (Lin et al., 2012).

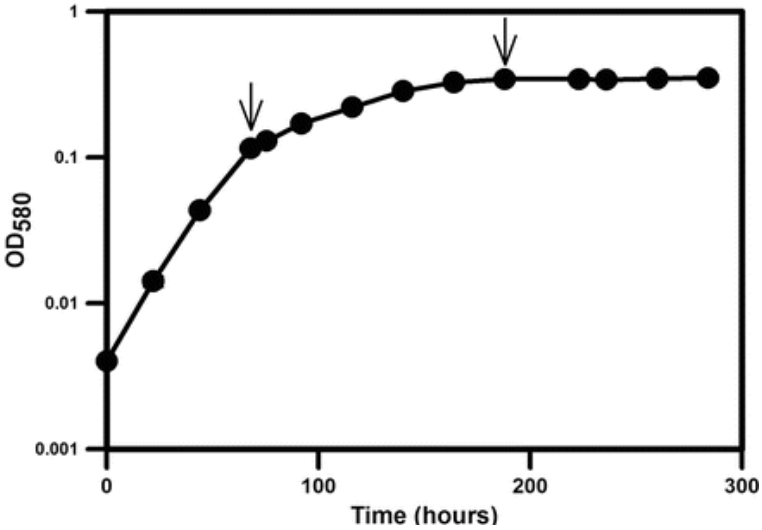

Figure 5. Bacterial growth curve in the Wayne model. (Sohaskey and Voskuil, 2015)

4.5 Regulation of persistent and dormant *M. tuberculosis*

Persistent and dormant states in *M. tuberculosis* is generally characterized by downregulation of the core metabolism and upregulation of proteins involved in stress and alternative metabolic pathways (Chao and Rubin, 2010).

4.5.1 The *dos* regulon

The *dos* regulon consists of a family of about 50 genes and the two-component regulatory system DosS/DosR which control the entry of mycobacteria into a dormant state. DosS is a heme- containing sensor, also called DevS, that activate DosR (DOrmancy Survival Regulator), a protein that can bind to DNA, thus induce gene expression. DosT is another sensor related to DosS, which also contribute to the response (Sivaramakrishnan and de Montellano, 2013). Voskuil et al. (2004) demonstrated upregulated expression of the *dos*

regulon in *M. tuberculosis* when exposed to hypoxia in the Wayne model. Other stresses linked to growth- limiting conditions, including macrophage infection and phases of infection in mice support the role of the dos regulon (Dutta and Karakousis, 2014).

4.5.2 Energy and carbon

The persistence of *M. tuberculosis* inside a host has been related to lipid metabolism and the glyoxylate pathways (Ghazaei, 2018). Munoz-Elias and McKinney (2005) demonstrated that the ICL enzymes in the glyoxylate cycle are essential for growth and persistence of M. tuberculosis in macrophages and mice. The deletion of both the enzymes ICL1 and ICL2 resulted in impairment of replication and rapid elimination from the lungs. As mentioned in chapter 3.1.1; Central carbon metabolism, *M. tuberculosis* has been demonstrated to recyclyle lipids in, including the cell wall glycolipid trehalose, in infected mice (Kalscheuer et al., 2010). Also, exposure to hypoxia have shown increased trehalose catabolism, suggested as preparation for later re-initiation of peptidoglycan biosynthesis and growth (Eoh et al., 2017).

4.5.3 Toxin- antitoxin systems

Toxin- antitoxin systems (TA) consist of two proteins, the "toxin", which is neutralized by the other protein, the "antitoxin" under normal conditions. However, under altered conditions, the expression of the antitoxin protein can be repressed by the organism, leading to accumulation of toxin. M. tuberculosis has multiple TA- systems which may contribute to the establishment and maintenance of persistence and LTBI. Under such conditions, the toxin may act as a ribonuclease (RNase) that cleaves mRNA, resulting in inhibition of protein synthesis and bacterial growth (Peddireddy et al., 2017)

5. Culturomics and resuscitation from reduced metabolic states

5.1 Culturomics

As mentioned initially (Introduction, chap. 1.3) culturing is the "gold standard" for laboratory diagnostics of tuberculosis. Both solid and liquid media are available for culturing of mycobacteria. Liquid culture methods supplementary to automated detection (for instance the BD Bactec 960 system), are 10 % more sensitive in comparison to the solid media, which on the other hand is less expensive. Another limitation, liquid methods are more prone to contamination and overgrowth by other microorganisms and must, depending on the origin of the specimen, be decontaminated prior to cultivation. Unfortunately, decontamination is also harmful to mycobacteria (WHO, 2015). There is no consensus on which medium is best for routine isolation(GlobalLaboratoryInitiativ, 2014).

One of the first medias for cultivation of *M. tuberculosis*, the Lowenstein–Jensen (LJ) medium, containing egg-yolk, glycerol, sodium pyruvate, gelatine, sodium glutamate, sodium pyruvate, activated carbon, oligonucleotides, and malachite green to inhibit contaminants, still remains the most commonly used medium worldwide. The medium is inexpensive, has high sensitivity and reveal the characteristic morphology of colonies. Disadvantages includes sensitivity towards the quality of the added eggs, long preparation time and short shelf life. The solid agars, Middlebrook 7H10 and 7H11 contains mineral salts, glucose, albumin bovine, amino acids, oleic acids and sodium pyruvate. The 7H11 medium additionally contains enzymatic digest of casein providing nitrogen, vitamins and amino acids. Inversely from the Lowenstein–Jensen (LJ) medium, they are expensive, thus rarely used routinely in countries with limited resource (Asmar and Drancourt, 2015).

Many mycobacteria, *M. tuberculosis* in particular, have the ability to aggregate and form clumps in liquid media. The morphotype remains attached during replication, thus forming compact colonies containing structures referred to as cords (Brambilla et al., 2016) (Julián et al., 2010). Koch (1982), who discovered *M. tuberculosis,* already back then, referred to the structures as "*densely bunched and frequently small braided groups*". Presence of cords are considered to be the reason why mycobacteria *in vitro* cultures show fluctuations and poor

correlation between CFU and OD (Caceres et al., 2013). Furthermore, the presence of tween in the growth medium reduce clumps (Leisching et al., 2016) (Caceres et al., 2013).

Among liquid media, Middlebrook 7H9 broth contains inorganic salts and sodium citrate and can be supplemented with growth promoting compounds such as glycerol, polysorbate 80 and OADC solution (oleic acid, bovine albumin, dextrose and detoxifying catalase). Modified versions of the media are incorporated into different automated systems, such as the BD Bactec MGIT 960 system, which provide continuously monitoring of the growth (Asmar and Drancourt, 2015). Attention has been devoted to liquid media which are more attractive as they offer significantly shorter time for the detection of mycobacteria (Hillemann et al., 2006).

Although the combination of liquid media and automated growth detecting systems have provided decreased detection time, the slow growth of mycobacterial cultures remain a major problem for the diagnostics of Tb. Currently, the average detection time for primary cultures are two to four weeks, and phenotypically susceptibility testing can take an additional two to four weeks (Ghodbane et al., 2014).

5.2 Growth (resuscitation)-promoting factors

Resuscitation prompting factors (Ppf) is a term used for enzymes which presumably are involved in the resuscitation and regrowth of dormant bacteria. The effect of Rpf proteins have been demonstrated both *in vitro* (Mukamolova et al., 2002, Kana et al., 2008) and *in vivo (Downing et al., 2005)*, but the exact mechanism and regulation of the Rpfs are still unknown. However, suggested to involve alterations of the bacterial cell wall (Kana and Mizrahi, 2010). Rpf- dependent bacteria are those who cannot be cultured on conventional media without the help of Rpfs to reinitiate growth. *M. tuberculosis* possesses five Rpf-encoding genes, *rpfA* to *rpfE*. The action of the Rpf proteins is predicted to cleave glycosidic bonds in peptidoglycan, which is essential for cell wall integrity, thereby inducing a signal cascade which may initiate upregulation of metabolism (Chao and Rubin, 2010).

AIM OF THE STUDY

The main aim of this thesis was to identify amino acids exhibiting a growth promoting effect on *Mycobacterium tuberculosis* cultures with reduced metabolism.

Sub-goals

With the assumption that sputum samples from TB patients host heterogeneous mycobacterial populations with single cells in different states of metabolic activity, ranging from dormant to exponentially replicating, a sub goal was to induce reduced metabolism in *Mycobacterium tuberculosis* H37Rv reference strain, in order to generate a model for obtaining clues to whether the addition of different amino acids, in various concentrations to cultivation media, could improve the effectiveness and sensitivity of cultivation diagnostics

A second goal was to study the effect of added amino acids to cultivation media at a transcriptional level

The third goal was to use the staining probe 4-N,N-dimethylamino1,8-naphthalimide–conjugated trehalose (DMN-Tre), kindly provided by Bertozzi and colleagues, to obtain clues whether the probe can distinguish between non-replicating "dormant" cells and replicating ones.

MATERIALS AND INSTRUMENTS

1. General consumables

Powder free examination gloves (Latex)	6402	OneMed
Powder free examination gloves (Nitrile)	6202	OneMed
Pipette tips 1000 long	613-4705	VWR
Pipette tips 1000 short	613-4708	VWR
Pipette tips 200	70.760.211	Sarstedt
Pipette tips 10	70.1130.210	Sarstedt
Eppendorf tube 1.5 mL	72.690.001	Sarstedt

2. Culturing

2.1 Medium and supplements

Chemical	Catalogue No.	Supplier
Middlebrook 7H9 liquid medium		
BD Difco™ Middlebrook 7H9 Broth	DF0713-17-9	BD
TWEEN® 80	421501J	VWR
ADC growth supplement		
Albumin bovine serum	421501J	VWR
D-(+)-Glucose	G7021	Sigma-Aldrich
Sodium chloride	7647-14-5	Merck
Catalase		
BACTEC™ MGIT™		
BACTEC™ MGIT™ growth indicator tubes	245122	BD
BACTEC™ MGIT™ 960 supplement kit	245124	BD
Amino acids		
L-Alanine, BioUltra ≥ 99.5%	05129	Sigma-Aldrich
L-Arginine monohydrochloride, BioUltra ≥99.0%	11040	Sigma-Aldrich
L-Glutamine, BioUltra ≥99.5%	49419	Sigma-Aldrich
L-Histidine monohydrochloride monohydrate, BioUltra ≥ 99.0%	53370	Sigma-Aldrich
Trans-4-Hydroxy-L-Proline, BioUltra ≥99.0%	56250	Sigma-Aldrich
L-Lysine monohydrochloride, BioUltra ≥99.5%	62929	Sigma-Aldrich
L-Proline, BioUltra ≥ 99.5%	81709	Sigma-Aldrich
L-Serine, BioUltra ≥99.0%	84960	Sigma-Aldrich
L-Threonine, BioUltra ≥99.0%	89180	Sigma-Aldrich
L-Valine, BioUltra ≥99.0%	94620	Sigma-Aldrich
L-Tryptophan, BioUltra ≥98%	T0254	Sigma-Aldrich
L-Asparagine, BioUltra ≥99.0%	11150	Sigma-Aldrich
L-Aspartic acid, BioUltra ≥99.5%	11189	Sigma-Aldrich
L-Cysteine, BioUltra ≥98.5%	30089	Sigma-Aldrich
L-Cystine, BioUltra ≥99.7%	30200	Sigma-Aldrich
L-Glutamic acid, BioUltra ≥99.0%	49450	Sigma-Aldrich
Glycine, BioUltra ≥99.0%	50050	Sigma-Aldrich

L-Lucine, BioUltra ≥98%	L8000	Sigma-Aldrich
L-Methionine, BioUltra ≥99.0%	64320	Sigma-Aldrich
L-Tyrosine, BioUltra ≥98%	T3754	Sigma-Aldrich
L-Phenylalanine, BioUltra ≥95%	P2126	Sigma-Aldrich
L-Isoleucine, BioUltra ≥99.0%	58880	Sigma-Aldrich
Plating for determination of cell density		
Middlebrook 7H10 solid medium		

2.2 Plating for calculation of cell concentration (CFU/mL)

Equipment	Catalogue No.	Supplier
Plastic spreaders	12342048	Fisher Scientific
Eppendorf tube 1.5 mL	For details: general consumables	
Chemical		
PBS	444057Y	VWR

2.3 Inducing non-replicating persistence in *M. tuberculosis* using The Wayne Model

Instrument	Catalogue No.	Supplier
Multi-position magnetic stirrer	4-42-1094	VWR
GENESYS™ 20 Visible spectrophotometer	4001-00	Thermo Scientific
GENESYS™ 20 Spectrophotometer Test Tube Holder for 1 in. Tubes	4015-000	Thermo Scientific
Equipment		
DURAN® Test tubes with SVL® screwcaps: 20 mm diameter by 150 mm tall	212-0379	VWR
PTFE covered magnetic stirring bars: 8x1.5 mm	442-0364	VWR
Parafilm	291-1214	VWR
Chemical		
Methylene blue	M6900-50G	Sigma-Aldrich
Middlebrook 7H9 medium	For details: medium and supplements	
ADC growth supplement	For details: medium and supplements	

3. RNA isolation

Instrument	Catalogue No.	Supplier
MagnaLyser (2.0)		Roche
Microcentrifuge		ThermoFischer
NanoDrop (One)		ThermoFischer
Equipment		
RNase-free Microfuge Tubes (1.5 mL)	AM12400	Ambion
FastPrep Lysing Beads – Matrix B	116911050	MP
Rør 50 mL, sterile, RNase-free	62.547.264	Sarstedt AG
Chemical		
Agarose Ultrapure	.	
Chloroform:Isoamyl (24:1)	C0549-1PT	Sigma-Aldrich
DMPC-water	8187580100	Merck Millipore
Ethanol	100983	Merck Millipore
Isopropanol	109634	Merck Millipore
RNaseZap	AM9780	Ambion
TURBO DNA-free Kit	AM1907	Ambion
UltraPure Glycogen	10814010	ThermoFisher
10x TBE	B52	ThermoFischer
2x Gel Loading Buffer II (Denaturing PAGE)	AM8546G	Ambion

4. Staining

5. Instrument	Catalogue No.	Supplier
Elvira PS. 1		Zeiss
Equipment		
Aluminium foil	293-4321	VWR

Coverslips	22-050-231	FischerScientific
Microscope slides	22-050-233	FischerScientific
Poly-L-Lysine	P4707	Sigma-Aldrich

Chemical		
Nile Red	19123-10MG	Sigma-Aldrich
Vectashield antifade mounting medium	H-1000	Vector labs
Nail polish	100491-940	VWR
DMN-Tre probe	Provided by Bertozzi and collegues	

6. Bacterial strain

Mycobacterium tuberculosis, linage 4, H37Rv reference strain was used in all the bacterial cultures futured in the thesis. Seed cultures were stored at ~ 20ºC.

METHODS

1. General methods

1.1 Working in a biosafety level 3 laboratory

All work handling M. tuberculosis cultures were conducted in a biosafety level 3 laboratory, also referred to as P3 laboratory (pathogen or protection level 3). Procedures involving the infectious agent were done within a class II safety cabinet. All equipment, chemicals and containers (MGIT growth indicator tubes, Eppendorf tubes etc.) containing the bacterium were decontaminated with PeraSafe™ for a minimum of 10 minutes before removed from the cabinet. Waste from the laboratory was put into sealed containers which were decontaminated by autoclaving before shipped for burning. Used glassware that had contained the bacterium were decontaminated with PeraSafe™, UV radiated and autoclaved before cleaned outside of the P3 facility. Samples that were to be analysed further outside of the P3 laboratory, were inactivated by 4% formaldehyde (SRM staining using DMN-Tre/Nile Red) or by Trizol/MagnaLyser (RNA isolation). Protective laboratory equipment and clothing were worn at all times when inside the P3 laboratory, including a long-sleeved lab gown outside of regular lab clothing, respiratory mask with filter and gloves. A second set of gloves were used when working inside the safety cabinet, which, when moving out of the cabinet, were thrown in the garbage and replaced with new ones when work again was to be conducted inside.

1.2 Aseptic working technique

Aseptic working technique is a prerequisite when working with culturing to avoid contamination of unwanted microbes. Media were autoclaved, supplements sterile-filtered, tools and equipment were either sterile disposables or autoclaved prior to use.

1.3 RNase control

When working with RNA, precautions were taken to avoid contamination of RNA degrading enzymes, RNases. Pipette tips, Eppendorf and falcon tubes were RNase-free. Further, gloves were used to avoid contamination from the skin. The gloves and equipment were frequently treated with RNase Zap® during the protocol.

2. Preparation of growth media and supplements

2.1 Middlebrook 7H9 liquid medium

Difco™ Middlebrook 7H9 Broth was suspended in Milli-Q® Ultrapure Water, and Tween® 80 was added before autoclaved at 121ºC for 10 minutes. The medium was stored at 2-8 ºC protected from light.

Middlebrook 7H9 medium (conventional)	
Contents	**Concentration**
Difco Middlebrook 7H9 Broth base	5.9 mg/mL
Ammonium sulfate	50 mg/mL
Disodium phosphate	2.5 mg/mL
Monopotassium phosphate	1.0 mg/mL
Sodium citrate	0.1 mg/mL
Magnesium sulfate	0.05 mg/mL
Calcium chloride	0.5 µg/mL
Zinc sulfate	1 µg/mL
Copper sulfate	1 µg/mL
Ferric ammonium citrate	40 µg/mL
*L-Glutamic acid**	0.5 mg/mL
Pyridoxine	1 µg/mL
Biotin	0.5 µg/mL
Tween® 80*	0.5 µL/mL

*Components which are not included in BBL® MGIT 7H9 medium
** Alternatively, glycerol can be used

2.2 Modified Middlebrook 7H9 (in BBL® MGIT growth indicator tube)

BBL® MGIT growth indicator tubes arrives from the supplier containing modified Middlebrook 7H9 liquid medium. The tubes were stored as recommended, in room temperature (2ºC-25ºC).

Modefied Middlebrook 7H9 (BACTEC™ MGIT™)	
Components	**Concentration**
Broth base	
Ammonium sulfate	0.5 mg/mL
Disodium phosphate	2.5 mg/mL
Monopotassium phosphate	1.0 mg/mL
Sodium citrate	0.1 mg/mL
Magnesium sulfate	50 mg/mL
Calcium chloride	0,5 µg/mL
Zinc sulfate	1 µg/mL
Copper sulfate	1 µg/mL
Ferric ammonium citrate	40 µg/mL

*L-Asparagine**	1.25 mg/mL
Pyridoxine	1 µg/mL
Biotin	0.5 µg/mL
*Casein peptone**	1,25 mg/mL
*Sodium glutamate**	0.5 mg/mL
Glycerol	3.1 µL/mL

*** Components which are not included in conventional 7H9 medium**

(BectonDickinson, 1996)

2.3 Middlebrook 7H10 solid agar medium

These agars were produced by the Unit of Control and Production at the Department of Microbiology, Oslo University Hospital, following protocols from the manufacturer. The agar was used for determination of cell concentration and was not used for the purpose of identifying growth prompting substances. Further description of the content is therefore not discussed in detail.

2.4 OADC/ADC/DC enrichment supplements

2.4.1 OADC growth supplement (conventional)

OADC is a growth enrichment supplement consisting of oleic acid, bovine serum albumin, dextrose and catalase which normally are added to the 7H9 medium. However, because of the nature of this thesis, modifications of the supplement were used. Removal of oleic acid for ADC supplement and removal of both oleic acid and bovine serum albumin for DC supplement. The different components were suspended in Milli-Q® Ultrapure Water. Because the supplement should not be autoclaved, it was filter sterilized (0.2µM) and stored dark at 2-8°C.

(A)DC enrichment supplement (conventional)	
Components	**Concentration**
Bovine serum albumin	50 mg/mL
D-glucose	20 mg/mL
Catalase	40 µg/mL
Natrium Chloride	8.5 mg/mL

2.4.2 BACTEC™ MGIT™ 960 OACD Growth Supplement

BACTEC™ MGIT™ 960 OACD Growth Supplement and BBL™ MGIT™ PANTA™ Antimicrobic mixture, consisting of the antibiotics Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin (BectonDickinson, 1999) was made as described by the

supplier, by mixing the solved growth supplement (liquid) with the antibiotics (lyophilized). The finished product was stored in the dark at 2 to 8°C. The supplement was added to Bactec MGIT 960 growth indicator tubes for a complete medium.

MGIT OADC/PANTA Supplement	
BACTEC™ MGIT™ 960 OADC Growth Supplement	
Compound	**Concentration**
Oleic acid	0.1 mg/mL
Bovine serum albumin	50 mg/mL
Catalase	30 µg/mL
Dextrose	20 mg/mL
Polyoxyethylene stearate	1.1 mg/mL
BBL™ MGIT™ PANTA™ Antibiotic Mixture	
Polymyxin B	400 units/mL
Amphotericin B	40 µg/mL
Nalidixic acid	160 µg/mL
Trimethoprim	40 µg/mL
Azlocillin	40 µg/mL

2.5 Amino acids

Solvents were added to the different amino acids (lyophilized) accordingly to the solubility specifications on the containers. For the amino acids, for which solvent and solubility concentration was not stated on the containers, the information was found on the product specification sheets collected at the supplier's website. The amino acid solutions were sterile filtrated (0.2µM) and used fresh in growth media within 12 hours.

Solubility of amino acids	
Amino acid	**Solubility**
Solved in Milli-Q® Ultrapure Water (H_2O)	
L-Alanine	H_2O: 1M
L-Arginine	H_2O: freely
L-Glutamine	H_2O, 0.1 M
L-Histidine monohydrochloride monohydrate	H_2O, 1g/10mL
Trans-4-Hydroxy-L-Proline	H_2O, 50mg/mL
L-Lysine monohydrochloride	H_2O, 50mg/mL
L-Proline	H_2O, 1M
L-Serine	H_2O, 359.7g/L
L-Threonine	H_2O, 50 mg/mL

L-Valine	H_2O, 50 mg/mL
Solved in Hydrochloric acid (HCl)	
L-Tryptophan	0,5 M HCl, 50 mg/mL
L-Asparagine	1M HCl, 100 mg/mL
L-Aspartic acid	1M HCl, 0.5 M
L-Cysteine	1M HCl, 1M
L-Cystine	1M HCl, 0.5g/10 mL
L-Glutamic acid	1M HCl, 100 mg/mL
Glycine	1M HCl, 1M
L-Lucine	1M HCl, 50 mg/mL
L-Methionine	1M HCl, 50 mg/mL
L-Tyrosine	1M HCl, 50 mg/mL
Solved in Ammonium hydroxide (NH_4OH)	
L-Phenylalanine	1M NH_4OH, 50 mg/mL
L-Isoleucine	1M NH_4OH, 50 mg/mL

3. Determination of cell density

3.1 Measurement of optical density (OD)

Optical density refers to a medium's ability to delay transmission of light. The slower the light, the higher optical density of the medium. In contrast, absorbance refers to the ability to absorb light and is a measure of the loss of light through the medium(Burtis et al., 2008). In the thesis two different instruments were used to measure the absorbance, using the wavelength 600 nM (OD_{600}).

3.1.1 GENESYS™ 20 Visible spectrophotometer

The instrument was used to measure the OD of the DURAN® Test tubes used in the Wayne model (Chap. 4.2, "Methods").

3.1.2 Thermo Electron Corporation, Spectronic 20 D+

The instrument was used to measure the OD of the Nephelo flasks (Chap. 4.1, "Methods").

3.2 Plating for colony-forming units (CFU)

To determine the density of bacterial cells used in different culture set-ups, known volumes from dilutions of the original culture were plated on 7H10 agar, and the colonies were counted for calculations of CFU and CFU/mL.

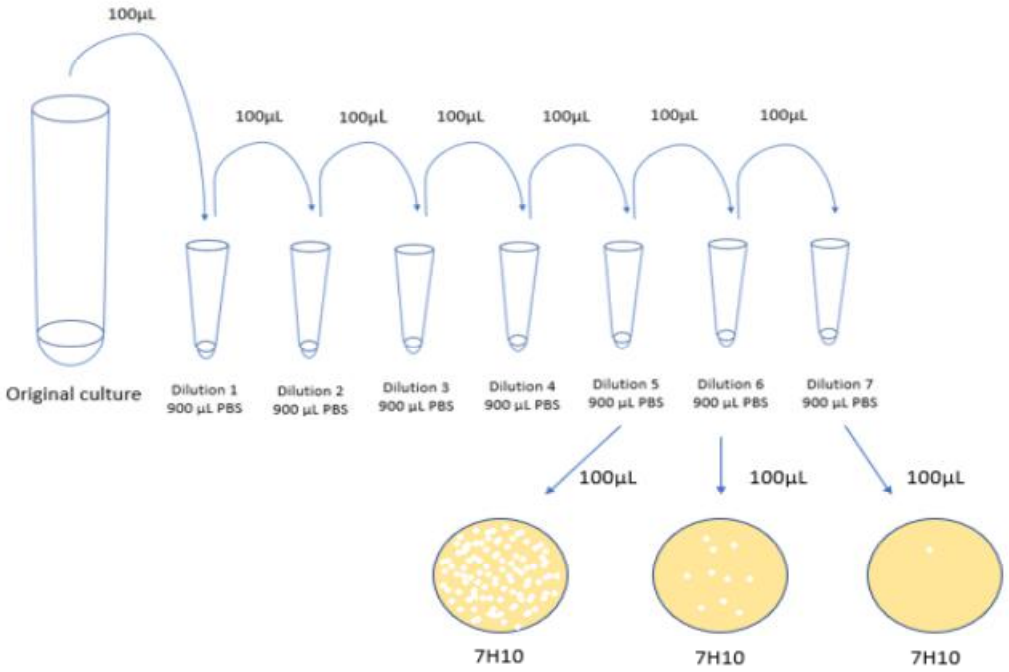

Figure 6. Ten-fold serial dilution and plating

1. Eppendorf tubes was prepared with 900µL of PBS
2. From the original culture, 100 µL was transferred to the first Eppendorf tube, creating a 1/9 dilution. Further, 100 µL from the dilution was transferred to the next Eppendorf tube, continuing until the seventh dilution
3. From dilutions 5-7, 100µL sample was transferred to 7H10 agars and spread with plastic spreaders
4. The agars were put into small plastic bags and incubated at 37°C
5. Cell concentration was calculated:

   CFU/mL = counted cells x dilution factor (10^x) /volume transferred to plates

   Inoculation size (number of cells) was calculated:

   CFU = (CFU/mL) / (volume used) / (dilution used)

4. Culturing

4.1 Culturing using Nephelo flasks

Nephelo culture flasks, glass flasks with a sidearm which can be inserted into a spectrophotometer, were used to grow *M. tuberculosis* cultures until a mid- log, exponential phase. Because of the side arm, the growth of the cultures could be monitored without having to transfer the material. Mid-log, exponential phase cultures were acquired in several of the set-ups in the thesis.

1. A 300 mL flask was added with 9 mL Middlebrook 7H9 medium, 1 mL ADC supplement and 1 mL of *M. tuberculosis* culture
2. The flask caps were properly sealed with Parafilm and incubated at 37°C /120 rpm.
3. The culture was grow until $OD_{600} \approx 0.5 - 0.6$
4. The absorbance was measured with Thermo Electron Corporation, Spectronic 20 D+

4.2 Inducing non- replicating persistence in *M. tuberculosis* using The Wayne Model

In the model, *M. tuberculosis* is cultivated with gradual depletion of oxygen. The depletion of oxygen results in a non-replicating persistent culture capable of surviving in microaerobic and anaerobic surroundings. More detailed description of the principle is found in chapter 5.4.1 under introduction.

The model uses a head space ratio of 0.5 between the medium and head space air. The test tubes used in this thesis could hold a total volume of 30 mL:

$$X + 0.5X = \text{total volume} \rightarrow X = 30/1.5 \rightarrow x = 20$$

20 mL 7H9/ADC/bacterial culture + 10 mL air = 30 mL total volume.

The tubes were inoculated with a mid-log (exponential) phase culture of *M. tuberculosis* to an initial cell density of 1.1×10^6 CFU/mL. When a culture reaches the NRP state (non-replicative persistent state), it should contain approximately 6.6×10^7 CFU/mL.

The protocol used was modified from Sohaskey and Voskuil (2015).

1. Middlebrook 7H9 medium was made as described in chapter 2.1. Each DURAN® Test tube containing a magnetic stirring bar was added with 17 mL of the medium before autoclaved
2. Further added to the DURAN® Test tubes: ADC growth supplement (2 mL) and 1mL of Mid-log phased ($OD_{600} \approx 0.6$) *M. tuberculosis* culture.
   (Assuming that a mid-log phase culture is approximately 10^8 CFU/mL, 1 mL culture was added to achieve an initial concentration of $\approx 10^6$ CFU/mL).
3. One of the tubes was added with 200 µL 1mg/mL Methylene blue (10µL/mL), which functioned as a control for monitoring of oxygen levels. As a redox indicator, methylene blue is deep blue in the presence of oxygen, while colourless under anaerobiosis
4. The tubes were thoroughly sealed with screwcaps and Parafilm and positioned on the magnetic stirrer which was placed inside an incubator
5. Incubated at 37°C / ≈ 120 rpm (position 1 on the magnetic stirrer)

6. Optical density ≈ OD_{600} was measured in GENESYS™ 20 Visible spectrophotometer. The frequency of the measurements varied between the different set- ups. In the first set- ups the OD was measured daily in the beginning to ensure that the system worked as expected. Later set- ups were measured less frequently, generally late in the process just to confirm that the cells were non- replicating.

4.3 BD Bactec MGIT 960 System

4.3.1 Principle

The BACTEC™ MGIT™ 960 System is developed for detection of mycobacteria. The system is composed of BBL™ MGIT™ Mycobacteria Growth Indicator Tubes, when inoculated with specimen and placed in the BACTEC MGIT™ 960 instrument, is monitored for emitting increased fluorescence, indicating presence of viable microorganisms. The tubes have a fluorescent compound embedded in silicone rubber on the bottom, sensitive to the presence of oxygen. Oxygen present initially of the analysis quenches the emissions. However, when respiring microorganisms in the added specimen metabolize the oxygen, the relative amount of fluorescence will be detected when irradiated with UV light, thus reporting the tube as instrument positive. If the tubes are not reported as positive within the protocol time of 42 days, they are stated as negative (BectonDickinson, 1999).

4.3.2 Bactec MGIT 960 instrument

The Bactec MGIT 960 instrument is made up of three incubator drawers, each capable of holding 320 Bactec MGIT growth indicator tubes. The incubator drawers hold a temperature of 37°C. The tube stations are divided into 16 rows in which each has a detector underneath, monitoring the emitted fluorescens continuously, registering readings every hour. The fluorescent compound is activated by illumination from LEDs (365 nm), also placed underneath the tubes. The fluorescent signal is converted by the instrument to an algorithmic measure referred to as growth units. When the growth unit exceeds the cut off value of 75, the tube will be reported as "positive" by the instrument, usually containing a biomass around 10^5-10^6 CFU/mL. At this timepoint, clinical samples must be tested further to confirm the presence of mycobacteria and be checked for contamination. Occasionally, the machine report tubes as "threshold positive", represented graphically, by the growth curve being steeper than expected for a gradual curve generated by the growth of

mycobacteria. The phenomena usually occur due to contamination by bacteria with more explosive growth (GlobalLaboratoryInitiativ, 2014).

4.3.3 Bactec MGIT 960 growth indicator tubes

In addition to the fluorescent compound, each of the tubes contain 7 mL of modified Middlebrook 7H9 medium. For a complete medium, the BACTEC™ MGIT™ 960 OACD Growth Supplement and BBL™ MGIT™ PANTA™ Antimicrobic mixture are added (BectonDickinson, 1999). However, as mentioned above, also alternative supplements, other than the original MGIT OADC/PANTA supplement; ADC or DC mixed with single amino acids were used.

4.3.4 Protocols

The Bactec MGIT system was used in several set-ups. However, as mentioned above, with modifications from the original procedure for the purpose of the aim. Protocol 1.3.2.1 describes a general procedure, whereas chapters 4.3.2.2 – 6 elaborates the modifications used in the different set-ups. Set-ups 4.3.2.2- 5 were used for screening of amino acids with growth promoting effect on **non-replicative** *M. tuberculosis*, while in set- up 4.3.2.6, the effect was tested on an **exponential replicative culture** in order to check whether the effect of amino acid addition differed between exponential replicative and non-replicative cultures.

4.3.4.1 General protocol

1. Bactec MGIT growth indicator tubes containing modified 7H9 medium were added with 0.8 mL growth supplement (ADC or DC)
2. Tubes were added with 0.5 mL amino acid-PBS solution. Amino acids were first solved as described in chapter 2.5 under "preparation of growth media and supplements", then mixed with PBS to achieve wanted concentrations (total for the tube). The calculation sheet is found as "Appendix 1". Control tubes were added with 0.5 mL of PBS.
3. *M. tuberculosis* cultures were serial diluted (as illustrated in chapter 3.2 "Plating for CFU", only in a larger scale) with PBS.
4. Each tube was inoculated with 100µL of culture from the desired dilution

5. Inserted in the Bactec MGIT 960 instrument. After the tubes were given a status (positive, including threshold positive or negative) an instrument report with the information, was printed

4.3.4.2 Screening comparing ADC and DC as supplement

All amino acids, except from L- Cysteine were tested in duplicates with two different concentrations, 4 and 8 mM, and with the growth supplement ADC as one condition and DC as the other. Dilution 4 of a non- replicative culture induced in the Wayne model was used as inoculum.

4.3.4.3 Screening comparing different inoculum sizes and amino acid concentrations

Selected amino acids, L-Lysine monohydrochloride, L-Proline, Trans-4-Hydroxy-L-Proline, L-Histidine monohydrochloride monohydrate, L- Glutamine, L-Asparagine, L-Serine and L-Threonine were used. Further, two sets of concentrations were used for the different amino acids; 2, 4 and 8 mM, or 4, 8 and 10 mM. DC was used as growth supplement and all conditions were set up in triplicates. Two different non- replicative biological replicates (Wayne tubes) were used in four set-ups (A-D). Biological replicate 1; A) inoculate from dilution 3, B) inoculate from dilution 4. Biological replicate 2; C) inoculate from dilution 3, D) inoculate from dilution 4.

4.3.4.4 Screening comparing conventional and modified (MGIT) 7H9 medium

Selected amino acids giving selected concentrations, L-Lysine monohydrochloride, 10 mM, L-Proline, 8 mM, L-Histidine monohydrochloride monohydrate, 4 mM, L- Glutamine, 8 mM and L-Asparagine, 4 mM were used in a set- up to compare the performance of the amino acids when the modified (MGIT) 7H9 medium was replaced with conventional 7H9 medium. The modified MGIT 7H9 medium was removed from the Bactec MGIT tubes, which were rinsed with PBS twice, before 7 mL conventional 7H9 medium was added as a replacement. DC was used as growth supplement. Triplicates were used for all conditions. Dilution 4 of a non- replicative culture induced in the Wayne model was used as inoculum.

Further, two additional controls were added in the set-up. One control replacing DC with BACTEC™ MGIT™ 960 OACD / BBL™ MGIT™ PANTA™ supplement and one control using the original MGIT modified 7H9 medium instead of the conventional one.

4.3.4.5 Cultivation with L-Lysine monohydrochloride for RNA isolation

Tubes were added with DC and L-Lysine monohydrochloride giving 10 mM concentration or PBS (control). Each condition was repeated in 50 tubes, times two biological replicates (non- replicative, induced in the Wayne model). Tubes were inoculated with dilution 4.

4.3.4.6 Screening replacing the non- replicative culture with a replicating one

Two selected amino acids, L-Lysine monohydrochloride and L-Proline, with the concentrations 4, 8 and 10 mM were, together with DC, used as supplement in a set- ups using a mid-log, exponential phased culture (dilution 4) instead of a non- replicative one. The conditions were set up in triplicates.

5. RNA isolation

5.1 Protocol

The protocol used was developed by Marta Gomes Munoz, Ph.D. student in the Genome Dynamics group, which had modified the protocol from the TRIzol™ Reagent manual (Invitrogen, 2016).

5.1.1 Collection and preservation of RNA

RNA was collected in two rounds from two different set-ups. In one of the set- ups (Chapter 4.4.4.2, "Screening comparing different inoculum sizes and amino acid concentrations) the tubes inoculated with biological 1 was collected continuously as they were reported as positive. In the second set- up (Chapter 4.3.4.5, "Cultivation with L-Lysine monohydrochloride for RNA isolation") all tubes were collected from the instrument at the same time, which was when most of the tubes added with the amino acid were reported with growth units (not necessarily reported as positive).

The medium was transferred to 50 mL centrifugation tubes and spinned down for 15 minutes. Pasteur pipettes were used to collect and transfer bacterial cells from the bottom of the tubes into 1.5 mL microfuge tubes, which were centrifuged at 14 rpm. for 10 minutes. The supernatant was removed and RNAlater® was added to the pellets, before stored at ≈20 °C.

5.1.2 Cell lysis

1. The tubes with RNAlater® was thawed on ice (10-15 min.) before the RNAlater® was removed after centrifugation (13,000 xg / 4°C / 10 min.)
2. The pellets were washed with DMPC water (13,000 xg / 4°C / 5 min.)
3. Pellets were resuspended with Trizol (900 µL) and transferred to lysing matrix tubes, which were run in the MagnaLyser (Speed 6 / 30 sec.) three times. Between the runs the tubes were incubated on a -20°C block (1 min)

5.1.3 Organic extraction

1. Chloroform:Isoamyl (180 µL) was added and mixed (hand mixed 15 seconds) with the samples before incubated in room temperature (10 min)

2. After centrifugation (13,000 xg / 4°C / 15 min.) three phases were visible. The top; a colorless aqueous phase containing RNA, the middle; a white interphase containing DNA, and the bottom; a red phenol-chloroform phase containing proteins and lipids. The upper part containing RNA was transferred to fresh tubes

5.1.4 Isopropanol precipitation (preformed outside of the P3-laboratory)

1. Glycogen (1 µL 20µg/µl) and isopropanol (450µL) were added to each tube before mixed (vortex) and incubated in room temperature (10 min.)
2. Without disturbing the pellets, the supernatant was removed, and the pellets washed with ethanol (1 mL 75%) (7500xg / 4°C / 5 min.). The step was repeated twice
3. After removal of the ethanol, the pellets were air dried with lids open in room temperature (10 min.)
4. DMPC water (25µL) was added to each tube and incubated to resuspend the pellets (65°C / 5 min. / shaking (800-1000rpm))

5.2 RNA measurements

5.2.1 Nanodrop

NanoDrop™ was used to obtain optical measurements of RNA-concentrations and for determination of the product's purity. DMPC water was employed for blanking the instrument, before 2 µl of the RNA samples was applied to the pedestal. The absorbance was measured at 260 nm, which is absorbance maximum of nucleic acids. The concentration was returned in ng/ml while the purity was emitted by the instrument as the 260/280 and 260/230 absorbance ratios. The ratios should be approximately 2.0 to be accepted as "pure". Ratios different from the target may indicate presence of protein, phenol or other contaminants (ThermoFisherScientific, 2010).

5.2.2 Agilent 2100 Bioanalyzer

The Agilent 2100 Bioanalyzer was used in combination with Bioanalyzer RNA 6000 Nano assay to determine the RNA quality. The Bioanalyzer software generates an electropherogram, a gel-like image which presents a visual estimation of the quality by determining he RNA integrity number (RIN). The RIN is an objective measure of total RNA quality ranging from 10, highly intact RNA to 1, completely degraded RNA. Indications for RNA degradation are presented as decreasing ratio of ribosomal bands, additional peaks

below the ribosomal bands, decrease in the overall RNA signal and shifts towards shorter fragments (AgilentTechnologies, 2018).

5.2.3 Protocol

5.2.3.1 Preparing the Gel and Gel-Dye Mix

1. 550 µl of RNA 6000 Nano gel matrix was pipetted into the spin filter provided in the kit and centrifuged at 1500 g ± 20 % for 10 minutes.
2. Filtered gel were aliquoted into 0,5 ml RNase-free microfuge tubes, 65 µL per tube.
3. RNA 6000 Nano dye concentrate were held at room temperature to equilibrate for 30 min, then vortexed for 10 seconds.
4. 1 µl of RNA 6000 Nano dye were pipetted into filtered gel.
5. The solution was vortexed and spinned down at 13000 g for 10 min before use.

5.2.3.2 Loading the Gel-Dye Mix

1. A new RNA 6000 Nano chip were placed on the chip priming station and 9.0 µl of gel-dye mix were pipetted in the wells marked" **G**".
2. The plunger was positioned at 1 ml before closing the chip priming station.
3. Then the plunger was pressed until it is held by the clip.
4. The chip priming station was opened before pipetting 9.0 µl of gel-dye mix in the wells marked" G".
5. The remaining gel-dye mix was discarded.

5.2.3.3 Loading the Agilent RNA 6000 Nano Marker, Ladder and Samples

1. 5 µl of RNA 6000 Nano marker were pipetted in all 12 sample wells and in the well marked with a ladder-sign.
2. 1 µl of the ladder and sample were pipetted in sample and ladder wells.
3. The chip was placed horizontally in the IKA vortexer and vortexed for 1 min at 2400 rpm before running the chip on the Agilent 2100 bioanalyzer.

6. Staining using DMN-Tre and Nile Red

6.1 DMN-Tre probe

DMN-Tre (4-N,N-dimethylamino-1,8-naphthalimide–conjugated trehalose) is a dye which is linked to trehalose, a sugar that makes up the mycomembrane of Actinobacteria. The principle is based on the incorporation of trehalose in metabolically active *M. tuberculosis*. The dye emits fluorescence that undergoes >700-fold increase in intensity when transitioned from aqueous to hydrophobic environments, such as when incorporated into the hydrophobic mycobacterial membrane. This enhancement occurs upon metabolic conversion of DMN-Tre to trehalose monomycolate (Kamariza et al., 2018b). The probe was visualized with Elyra PS.1

6.2 Nile Red

Nile Red is a lipophilic dye which stains intracellular lipids. The dye emits florescence when transitioned to a lipid rich, non-polar environment, while in water and other polar solvents it is almost non-fluorescent (Daniel et al., 2011). The stain was visualized with Elyra PS.1.

6.3 Protocol

The protocol used was developed by Marta Gomes Munoz, Ph.D. student in the Genome Dynamics group, which had modified the protocol from Kamariza et al. (2018b), Rodriguez-Rivera et al. (2018), Kamariza et al. (2018b) and Deb et al. (2009).

6.3.1 Staining

1. *M. tuberculosis* culture (500 µL), both non-replicative and exponential replicative was mixed in different Eppendorf tubes with DMN-Tre (5µl 10mM), to reach a final concentration of 100µM
2. Tubes were further incubated for A) 10 minutes and B) 30 minutes (37°C/120rpm)
3. After incubation, tubes were centrifuged (3000xg / 10min.) before the supernatant were removed and pellets were resuspended with PBS (500 µL), Nile Red (2.5µl Nile Red (2mg/ml)), and incubated at 37°C (10 min.)
4. Samples were centrifuged (3000xg / 5 min.), the supernatant discarded, and PBS (500 µL) was added. The step was repeated twice

5. The samples were incubated with 4%formaldehyde (1 hour) in room temperature with rotation 250 rpm, before the supernatant was discarded after centrifugation (3000xg / 10 min) and PBS (500 µL) was added to the tubes. Further work was continued outside of the P3 laboratory
6. Cell suspensions (100 µL) were added to coverslips coated with Poly-L-Lysine (protocol 6.3.2) and incubated in room temperature (1 hour) before the liquid was removed
7. Vectashield® (6µL), a mounting medium which inhibits photobleaching of fluorescent dyes, was added in-between the coverslips and the microscope slide which was sealed with nail polish
8. The stains were visualized using high resolution microscopy (below, chapter 6.4)

6.3.2 Preparation of coverslips coated with Poly-L-Lysine

1. Poly-L-Lysine (100 mg) was solved in water (100 mL) before filter sterilized (0.2 µM)
2. Solution (100 µL) was further added to cover glasses, with thickness no.1 1/2; high performance; D=0,170, that had been cleaned with ethanol (96%)
3. The slides were incubated in 37°C (1 hour) before the solution was removed by pipetting and dried (minimum 2 hours)

6.3.3 Super Resolution Microscopy (SRM)

Elyra PS.1 Super Resolution Microscopy was used to image Nile Red and DMN-Tre probe with excitation and emission settings as described above. SRM is light microscopy which uses several techniques to obtain high resolution on images. Techniques involved includes SIM Structure Illumination Microscopy (SIM), Stochastic Optical Reconstruction Microscopy (STORM), Photo Activated Localization Microscopy (PALM) and Total Internal Reflection Fluorescence (TIRF). The DMN- Tre probe was imaged; excitation: 405nm Diode Laser 50mV, emission: MBS-405 BP420-480/LP750 and MBS-488 BP495-575/LP750. Nile red was imagined; excitation: 561nm OPSL Laser 200mV and emission: MBS-642 LP655.

7. Statistical analysis

Statistical analysis was performed on results presented in Figure 16 (Chapter 1.2.3, "Results"). A paired t-test was used because it takes into consideration the average differences within the same experiment. The Shapiro- Wilk normality test was used to check it the data was normally distributed. R- was used to perform the statistical analysis.

RESULTS

Presented below are results regarding culturing, RNA isolation and super resolution microscopy. The chapter concerning culturing contains results from inducing of non-replicative *M. tuberculosis* using the Wayne model. The cultures produced from the model were further used in a series of set-ups for screening of amino acids with growth promoting effect on non- replicative *M. tuberculosis*. In the first set-ups, the screening of amino acid was done using two different additional supplements, DC or ADC. In the next set- ups using DC as additional supplement, different inoculum amounts, and amino acid concentrations were used. In one set- up, the modified 7H9 (MGIT medium) was replaced (except form a control) with a conventional 7H9 medium. DC and selected amino acids were then added. In hope of achieving enough material for RNA isolation. The last set- up using a non-replicating culture, contained a number of replicates added with the amino acid, L-Lysine. In order to reveal if the effect seen in the earlier set- ups was unique for the non- replicative cultures, a last set-up was done using an exponential culture instead of a non- replicative one.The two other chapters contain results regarding RNA- isolation and quality, and results from staining of cells using different dyes, visualised with super resolution microscopy.

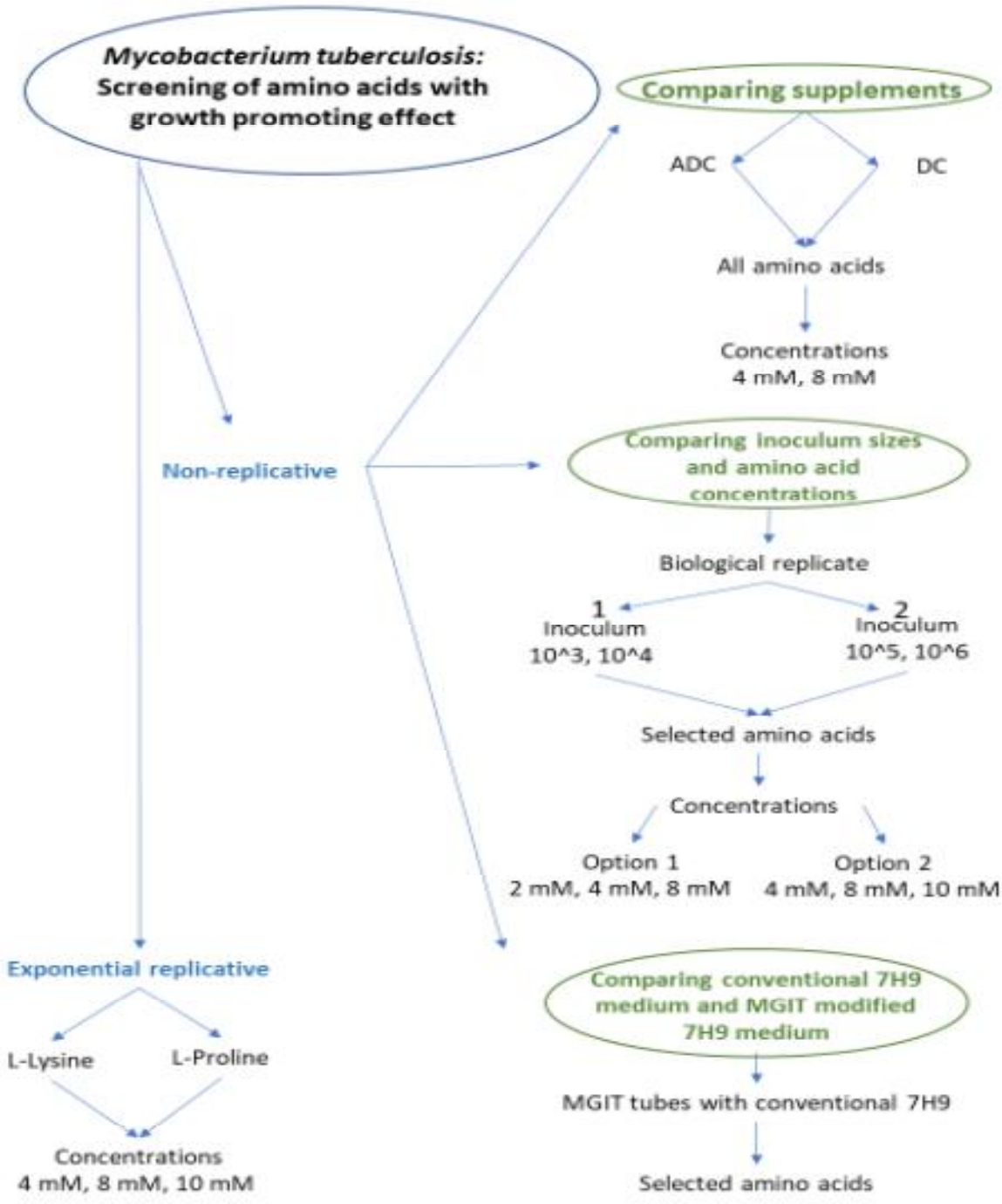

Figure 7. Overview of the experimental set- ups concerning culturing

1. Culturing

1.1 Wayne model

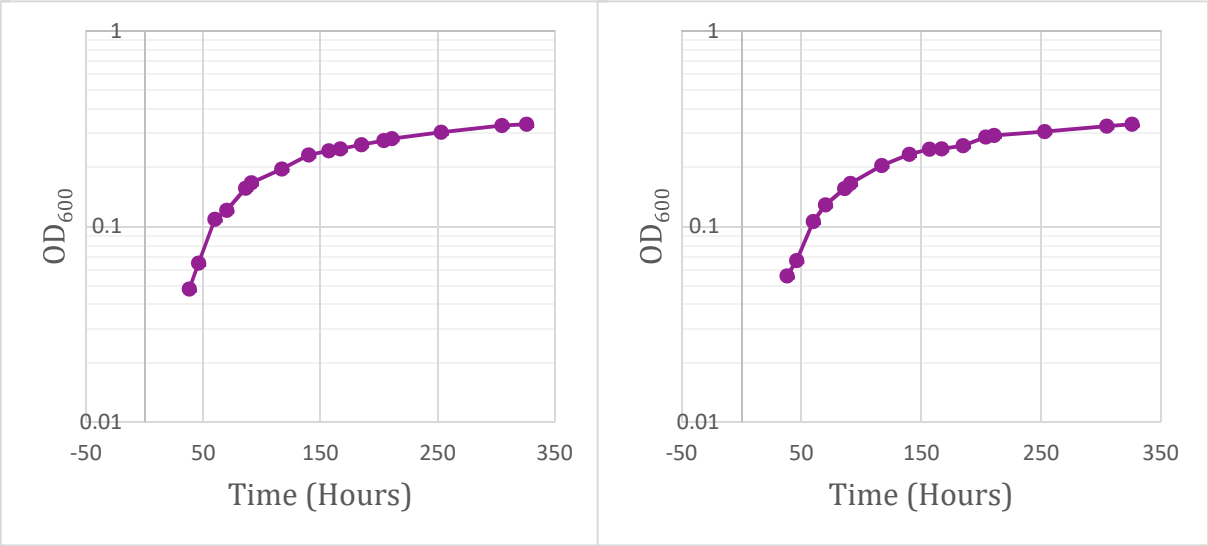

Figure 8. Experimental growth curves during the Wayne model

Based on the measured absorbance, the cultures induced in the thesis behaved as expected, showing the same tendency as described by the model's developer (Introduction; figure 5). Only the results from the two first set- ups are presented. The controls added with methylene blue turned transparent and the OD stopped increasing in all of the set- ups used.

1.2 Screening of amino acids with growth promoting effect on non-replicative *M. tuberculosis*

In total, 22 different amino acids or isoforms of amino acids were screened for growth promoting effect on non-replicative *M. tuberculosis*, reference strain H37Rv. The MGITs were inoculated with bacterial cultures previously induced into a non-replicating state using the Wayne model. PBS was used as a control in all the set-ups. Because of the difficulty in standardizing the inoculum amounts of *M. tuberculosis* due to cord factor (aggregation of the cells), the results are presented relative to the control to provide comparable results between different experiments. Also, two or three technical replicates were used for each condition in all the set-ups. Outliers were removed from the results.

MGITs with the different conditions were incubated and monitored in the BD BACTEC MGIT instrument, which reports the time that takes for a tube to flag as positive after being inserted into the instrument. It also reports the negative and the threshold positive tubes.

In the presented results, some amino acids are addressed with shortened names, this includes L-Histidine monohydrochloride monohydrate (L-Histidine), Trans-4-Hydroxy-L-Proline (Hydroxy-Proline) and L-Lysine monohydrochloride (L-Lysine).

1.2.1 Screening compering ADC and DC as supplement

In the first set-ups, two different concentrations of amino acids were added to MGITs supplemented with either 1) DC (dextrose and catalase) or 2) ADC (bovine serum albumin, dextrose and catalase). The amino acid concentrations used, 4 mM and 8 mM, were determined based on literature (Vilchèze et al., 2017, FisherScientific, 2018).

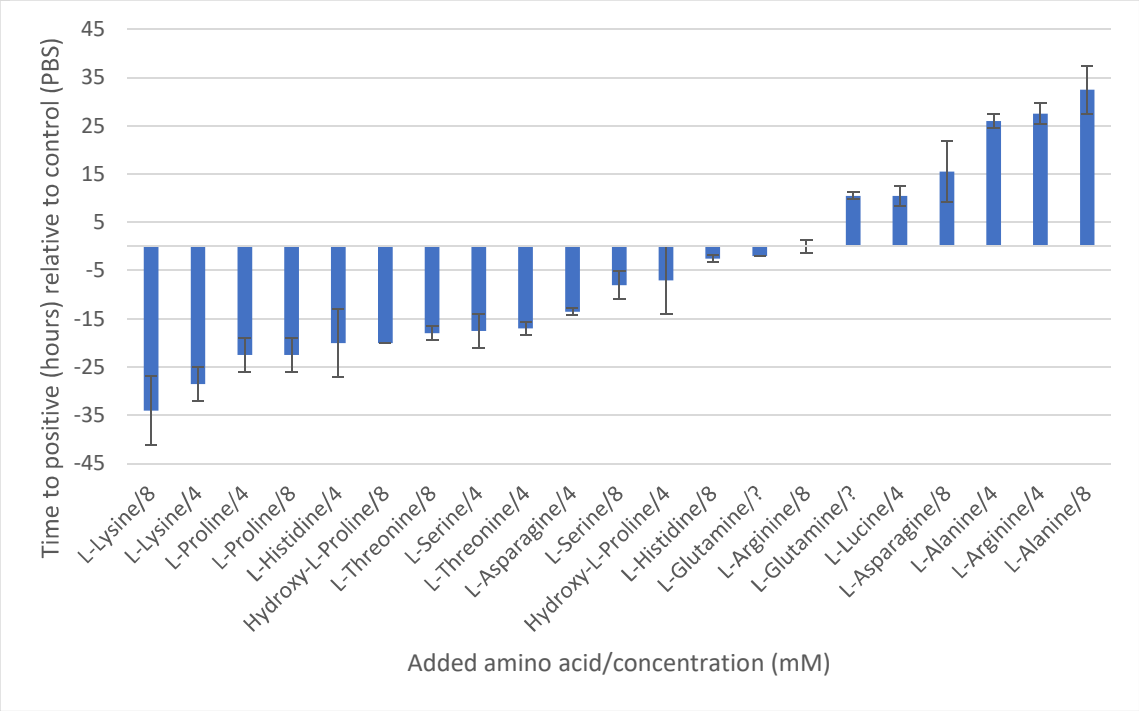

Figure 9. ***Screening of amino acids with growth promoting effect on non- replicative M. tuberculosis using MGITs supplemented with DC.*** *Each bar represents the mean time (n=2) since a MGIT was placed in the BD Bactec MGIT instrument until it reported as positive. Results are presented relative to the control (PBS), which was reported positive after 413 hours (mean, n=2). The tubes contained different concentrations of amino acids and* ***DC*** *(dextrose and catalase) and were inoculated with ≈2.2 x 10^3 cells of non-replicating (Wayne model) M. tuberculosis H37Rv*

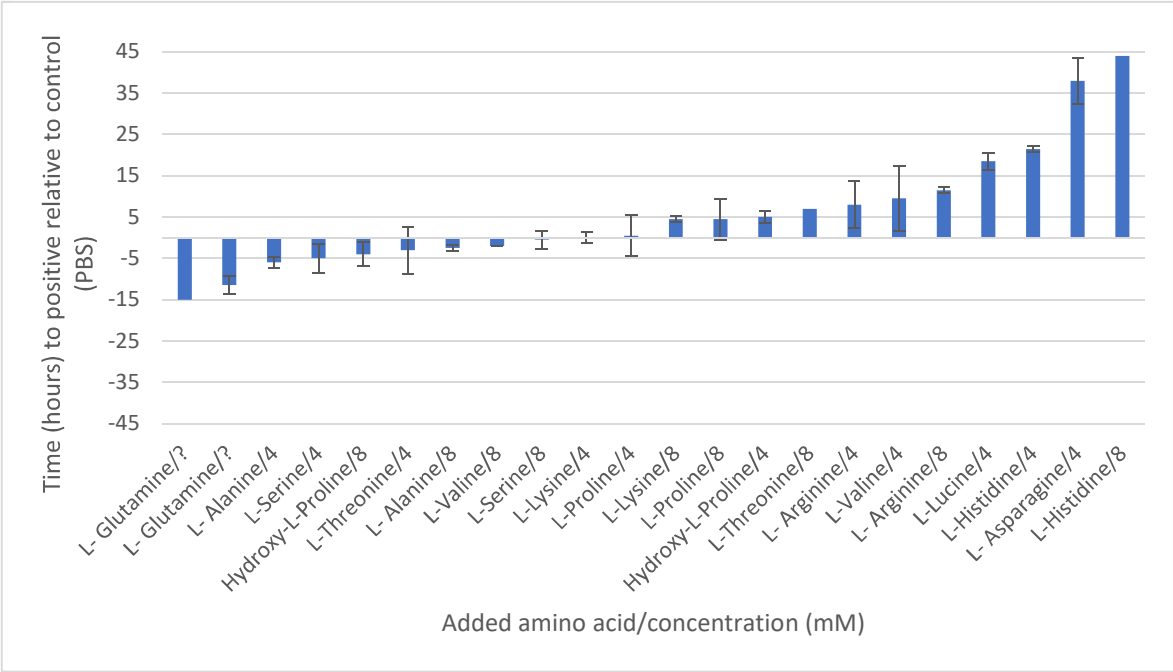

Figure 10. ***Screening of amino acids with growth promoting effect on non- replicative M. tuberculosis using MGITs supplemented with ADC.*** *Each bar represents the mean time (bars with error bar n=2, bars without n=1) since a MGIT was placed in the BD Bactec MGIT instrument until it reported as positive. Results are presented relative to the control (PBS), which was reported positive after 298 hours (mean, n=2). The tubes contained different concentrations of amino acids and* ***ADC*** *(bovine serum albumin, dextrose, catalase) and were inoculated with ≈2.2 x 10^3 cells of non-replicating (Wayne model) M. tuberculosis H37Rv*

Several amino acids including L-Aspartic acid, L-Cys**ti**ne, L-Glutamic acid, L- Isoleucine, L-Methionine, Glycine, L-phenylalanine, L-Tyrosine, L-Tryptophan (Fig. 8 and 9) and L-Valine (Fig. 8) were excluded from the presented results for various reasons: no growth detection within the protocol time (42 days), considerable growth delay compared to the control (over 45 hours after control) or reported as threshold positive. L-Cyst**ei**ne was used in a previous test set-up and was repeatedly reported as threshold positive for all tested concentrations. The amino acid was therefore not included in further experiments.

The remaining twelve amino acids, which are included in the presented results, supported growth before or within 45 hours after the control, both in tubes supplemented with ADC and DC. The amino acids included, correspond to amino acids with positively charged side chains (e.g. L-Lysine, L-Histidine and Arginine) and negatively charged (glutamic acid), with polar uncharged side chains (e.g. L-Threonine, Glutamine, Asparagine and L-Serine) and the special amino acid L-Proline and its isoform, Hydoxy-L-Proline. Of the eight amino acids with hydrophobic side chain, only L-Lucine, Alanine and (for the ADC set-up) L-Valine

supported growth within 45 hours after the control. The concentration of L-Glutamine used is unknown due to a calculation error that was detected later. It is likely that the amino acid was not properly dissolved before sterile filtration due to insufficient amount of solvent, thus resulting in an unknown concentration. Still, the results were of interest and is therefore included.

In general, the samples used decreased time before reported as positive in the tubes supplemented with ADC than in those with DC. The controls added with PBS instead of amino acids used an average time of 298 hours for tubes supplemented with ADC and 413 hours for the tubes supplemented with DC. However, several of the amino acids managed to grow without the albumin and showed decreased detection time compared to the control. Those were L- Lysine, L-Proline, Hydroxy-L-Proline, L-Histidine, L-Serine, L-Threonine, L-Glutamine and L-Asparagine.

1.2.2 Screening with different inoculum sizes and amino acid concentrations

Results from four experimental set- ups are presented in, graph A-D. The four graphs present results from three (exceptions) technical replicates of tubes added with selected amino acids. Each amino acid is set up with three different concentrations. Option 1; 2, 4 and 8 mM or option 2; 4, 8 and 10 mM. The set- ups represent four different inoculum sizes and two biological replicates. All tubes were added with DC in addition to the amino acids.

A. Biological replicate 1, inoculum ≈ 1.4 x 10^4 cells (3-fold dilution)

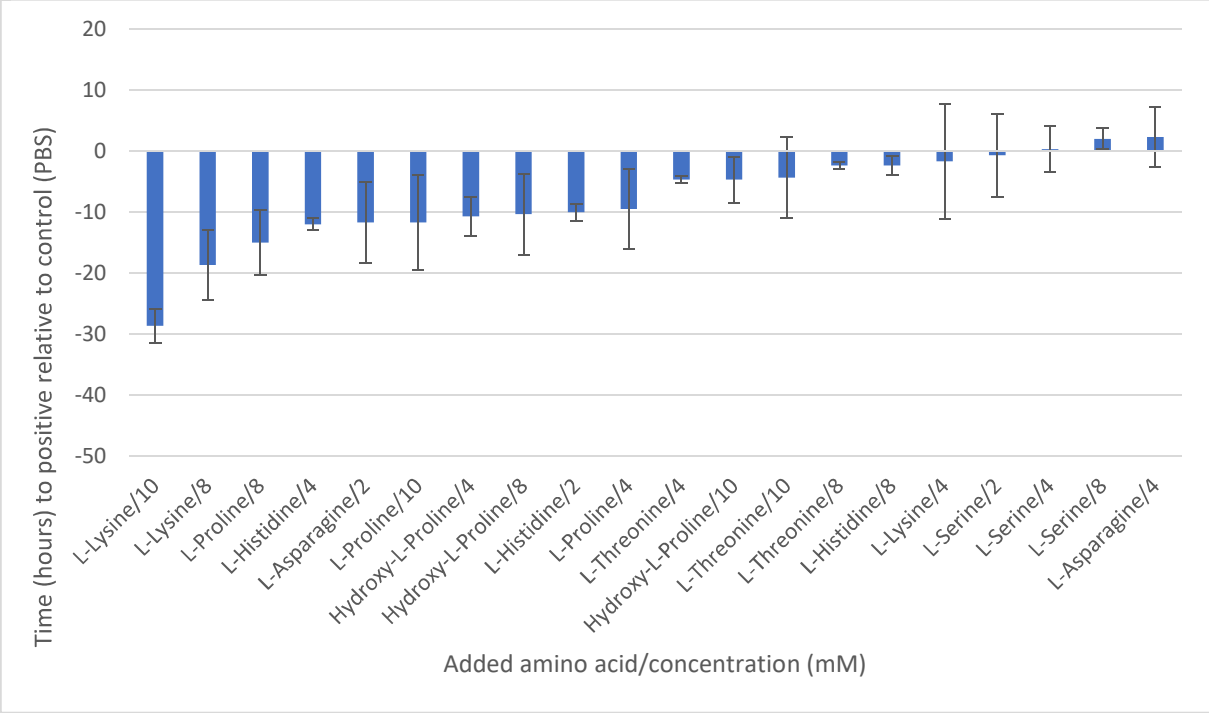

Figure 11. ***Screening of amino acids with growth promoting effect on non- replicative M. tuberculosis. Comparing the effect of amino acid concentrations on cultures containing ≈ 1.4 x 10^4 cells (3-fold dilution). Biological replicate 1****. Each bar represents the mean time (L- Histidine/2 and L-Proline/4, n=2, other conditions, n=3) since a MGIT was placed in the BD Bactec MGIT instrument until it reported as positive. Results are presented relative to control (PBS), which was reported positive after 368 hours (mean, n=3). The tubes contained different concentrations of amino acids, DC (dextrose and catalase) and were inoculated with non-replicating (Wayne model) M. tuberculosis H37Rv.*

L-Lysine exhibited the most prominent effect and had the shortest detection time of all the amino acids tested. Further, the highest concentrations of L- Lysine tested (8 and 10 mM) had the best effect, while 4 mM L-Lysine exhibited a high variation within the replicates and did not show an apparent effect on the growth rate. L-Proline, Hydroxy-L-Proline and L-Histidine also showed a decreased time to detection but did not seem to depend on the concentration as much as L-Lysine. L-Threonine and L- Serine did not have a convincing

effect on the growth rate. L-Asparagine showed a variable effect; tubes containing 2 mM reduced the detection time with approximately twelve hours, while the tubes containing 4 mM had no apparent effect. Tubes containing 8 mM had an increased time to detection compared to the control and are removed from the graph. Unfortunately, due to an error during the set-up, tubes with L-Glutamine was not analysed and are therefore not represented in the graph.

B. Biological replicate 1, inoculum ≈ 1.4 x 10^3 cells (4-fold dilution)

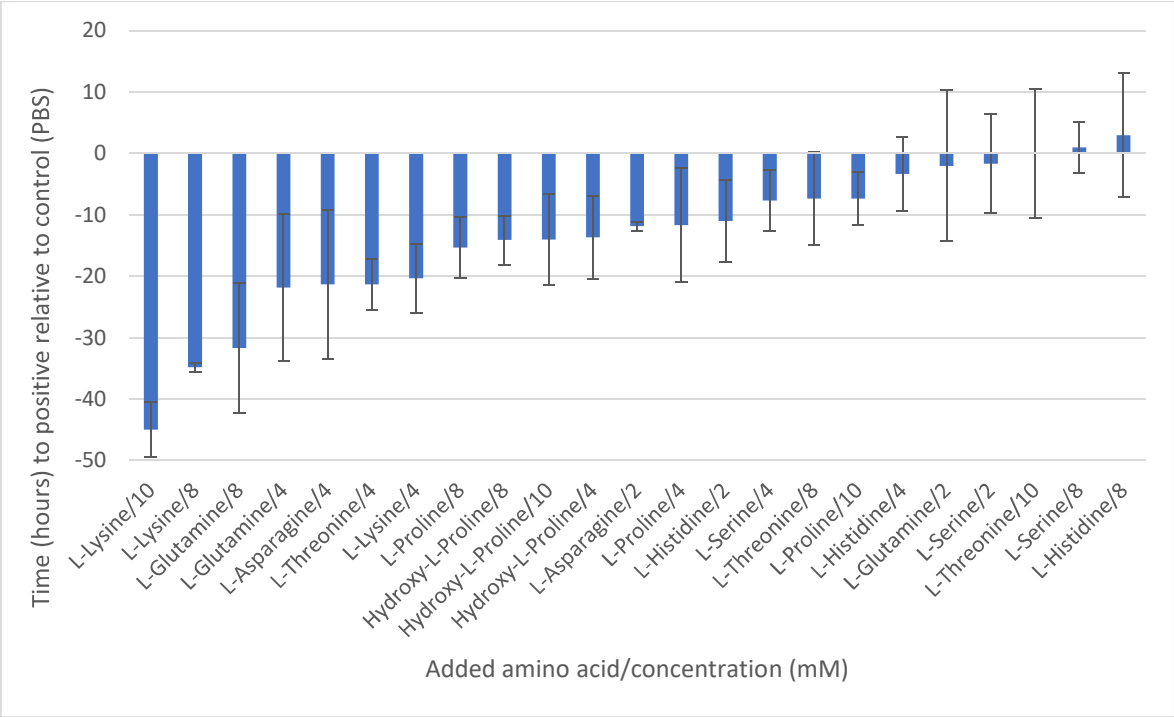

Figure 12. ***Screening of amino acids with growth promoting effect on non- replicative M. tuberculosis. Comparing the effect of amino acid concentrations on cultures containing ≈ 1.4 x 10^3 cells (4-fold dilution). Biological replicate 1****. Each bar represents the mean time (L- Lysine/4/8, L-Glutamine/4, L-Asparagine/2 and Proline/10, n=2, other conditions, n=3) since a MGIT was placed in the BD Bactec MGIT instrument until it reported as positive. Results are presented relative to control (PBS), which was reported positive after 445 hours (mean, n=3). The growth tubes contain different concentrations of amino acids, DC (dextrose and catalase) and were inoculated with non-replicating (Wayne model) M. tuberculosis H37Rv.*

Similar to the set-up with the same biological replicate (Fig.10), L-Lysine stand out as the amino acid with the shortest detection time. Also, similar to the previous set- up, the two highest concentrations show the best effect. Although the standard deviation is high for L-Glutamine, the two highest concentrations (4 and 8 mM) show a decreased time to detection. L-Proline and Hydroxy-L-Proline show some reduced and stable detection time for all tested concentrations. The results for 8 mM L-Asparagine are as for the set-up with the same biological replicate, excluded due to increased detection time. L- Histidine and L-

Serine have slight, or no increased effect, while L-Threonine only have convincing reduced detection time for tubes containing 4 mM.

C. Biological replicate 2, inoculum ≈ 1.5 x 10^6 cells (3-fold dilution)

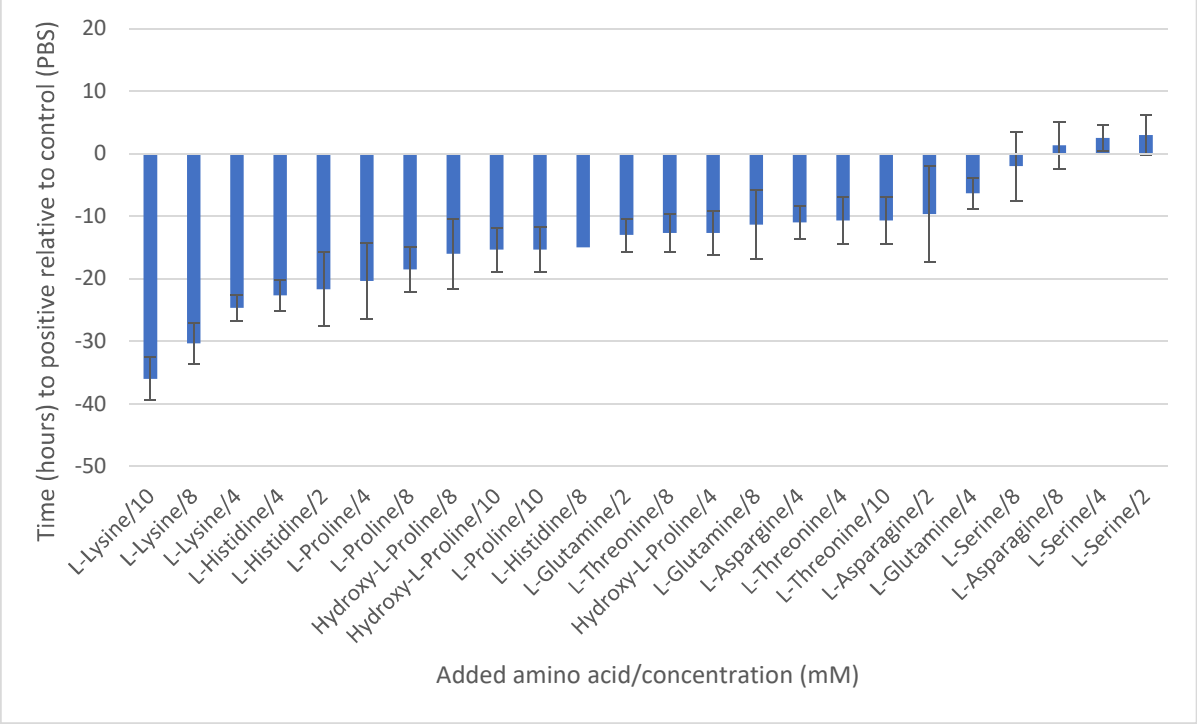

Figure 13. ***Screening of amino acids with growth promoting effect on non- replicative M. tuberculosis. Comparing the effect of amino acid concentrations on cultures containing ≈ 1.5 x 10^6 cells (3-fold dilution). Biological replicate 2*** *Each bar represents the mean time (L- Histidine/8, n=1, Hydroxy-L-Proline/4, L-Serine/4 and L-Proline/8, n=2, other conditions, n=3). since a MGIT was placed in the BD Bactec MGIT instrument until it reported as positive. Results are presented relative to control (PBS), which was reported positive after 334 hours (mean, n=3). The growth tubes contain different concentrations of amino acids, DC (dextrose and catalase) and were inoculated with non-replicating (Wayne model) M. tuberculosis H37Rv.*

This second biological replicate presents the same trend in results as seen for the two set-ups from biological replicate 1. Once again, tubes added with L- Lysine are reported as positive before all of the other tubes, including the control. However, the results suggest a more rapid detection time for L-Histidine, the opposite for L-Glutamine in comparison to biological replicate 1. L-Proline, Hydroxy-L-Proline, Asparagine (2 and 4 mM) and Threonine show some reduced detection time, while L-Serine and Asparagine (8 mM) continues to show no or increased effect on the growth rate.

D. Biological replicate 2, inoculum ≈ 1.5 x 10^5 cells (4-fold dilution)

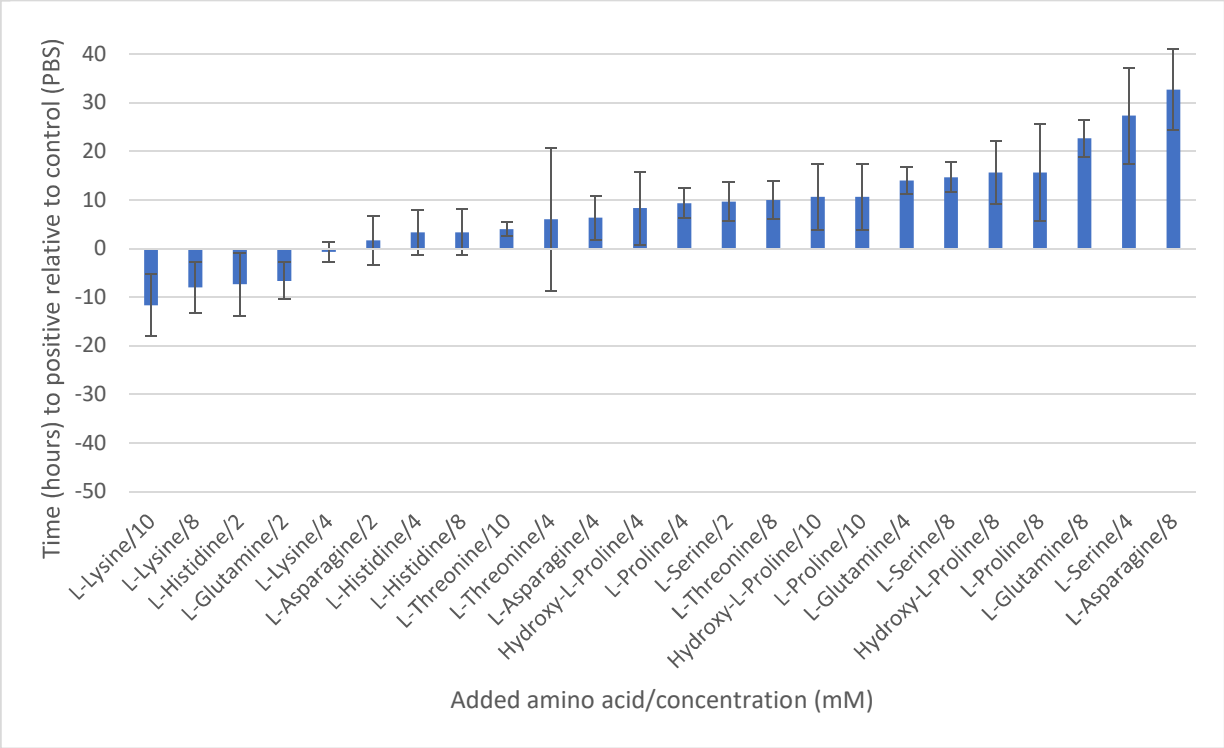

Figure 14. ***Screening of amino acids with growth promoting effect on non- replicative M. tuberculosis. Comparing the effect of amino acid concentrations on cultures containing ≈ 1.5 x 10^5 cells (4-fold dilution). Biological replicate 2****. Each bar represents the mean time (L- Threonine/10 and L-Glutamine/4, n=2, other conditions, n=3) since a MGIT was placed in the BD Bactec MGIT instrument until it reported as positive. Results are presented relative to control (PBS), which was reported positive after 386 hours (mean, n=3). The growth tubes contain different concentrations of amino acids, DC (dextrose and catalase) and were inoculated with non-replicating (Wayne model) M. tuberculosis H37Rv.*

All the amino acids tested, except L-Lysine with concentration 8 and 10 mM, L-Histidine and L-Glutamine with 2 mM, show an increased detection time compared to the control. Further, the detection time for L-Lysine is increased compared to the previous set-ups. It is worth noticing that the inoculum of, 1.5 x 10^5 cells is in between the calculated inoculums 1.5 x 10^6 cells (biological replicate 2), 1.4 x 10^3 cells and 1.4 x 10^3 cells (biological replicate 1).

1.2.3 Summary of L-Lysine performance

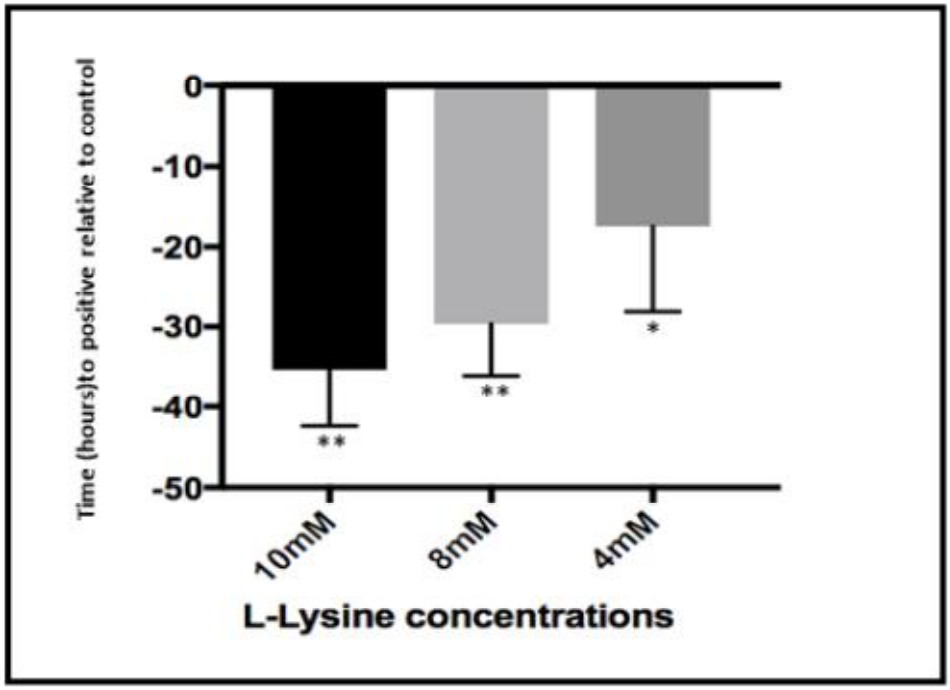

Figure 15. ***Summary of L- Lysine performance.*** *Each bar represents the mean time of the performance from four different set- ups. Since a MGIT was placed in the BD Bactec MGIT instrument until it reported as positive. Results are presented relative to the mean time of controls (PBS) from the same set -ups. The growth tubes contain concentrations of amino acids, DC (dextrose and catalase) and were inoculated with non-replicating (Wayne model) M. tuberculosis H37Rv. * = < 0.05 **= < 0.005 significance*

The graph sums up the results for L-Lysine compared with control (PBS) from the previous set- ups (figure 9, 11-13). The average of all detection times for the same L- Lysine concentration was calculated. The same was done for the controls belonging to the different set- ups. Statistical analysis using pared T- test showed that the differences in detection time between the control and addition of L- Lysine in the different concentrations were statistical significant.

1.2.4 Screening comparing conventional and MGIT modified 7H9 medium

Selected amino acids with the concentrations providing the best effects on growth rates in previous set-ups were used. In addition, two additional controls were added in the set-up. One control replacing DC with OADC (oleic acid, bovine serum albumin, dextrose and catalase) and one control using the original MGIT modified 7H9 medium instead of the conventional one.

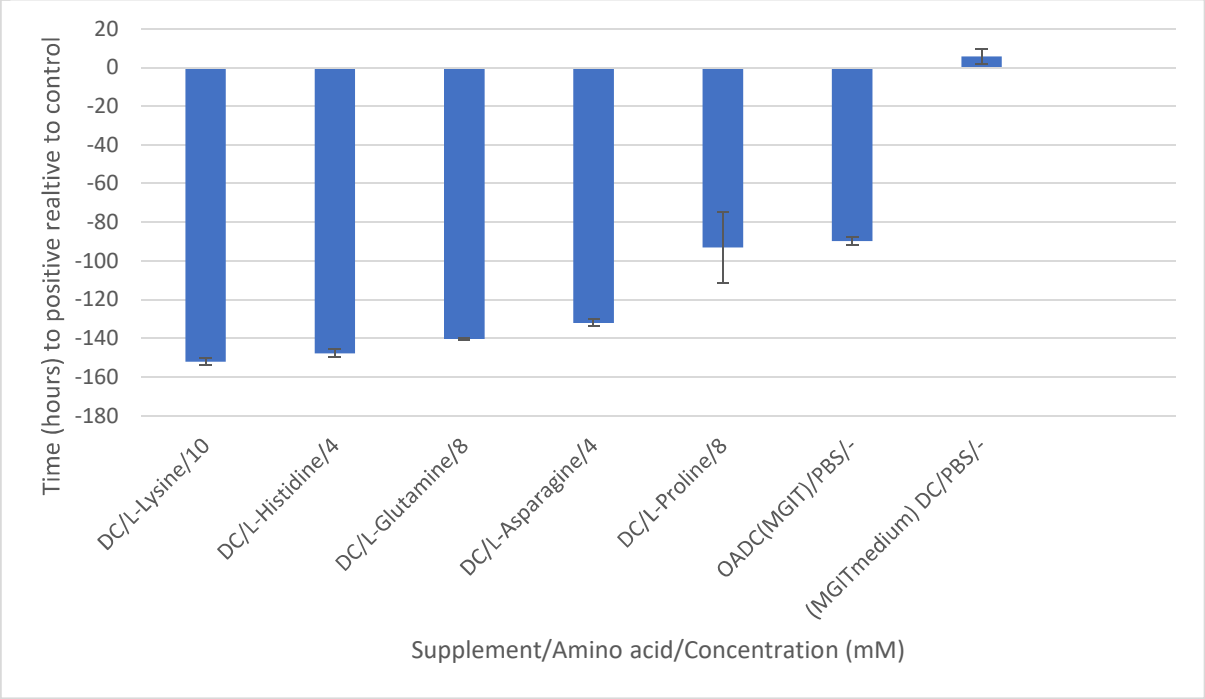

Figure 16. ***Screening of amino acids with growth promoting effect on non- replicative M. tuberculosis. Compering the effect of conventional 7H9 medium without added casein peptone and modified 7H9 medium added with casein peptone (MGIT medium).*** *since a MGIT was placed in the BD Bactec MGIT instrument until it reported as positive. Results are presented relative to control (PBS), which was reported positive after 279 hours (mean, n=3). The growth tubes contain different concentrations of amino acids, DC (dextrose and catalase) and were inoculated with ≈ 10^5cells of non-replicating (Wayne model) M. tuberculosis H37Rv. Additionally, a control replacing DC with MGIT OADC (oleic acid, bovine serum albumin, dextrose and catalase), and a control replacing the conventional 7H9 medium with the original MGIT modified 7H9 medium.*

Notably, the results suggest that the conventional 7H9 medium in combination with the tested amino acids drastically reduced the detection time compared to the control. The general detection time was also reduced compared to other set- ups using DC as supplement (fig. 9, 11-13). The control containing the original MGIT modified 7H9 did not differ in detection time compared to the conventional 7H9 medium. The control replacing DC with OADC was reported with a reduced detection time compared to the control, however, the tubes added with amino acids were reported as positive earlier, with the exception of L-Proline, which had a similar detection time.

1.3 Screening of amino acids with growth promoting effect replacing the non- replicative culture with an exponential replicative

To check if the effect of the amino acid additive differed between exponential and non-replicative cultures, an exponential culture of *M. tuberculosis* was used in another set-up. Only L-Lysine and L-Proline of the amino acids were used together with the control.

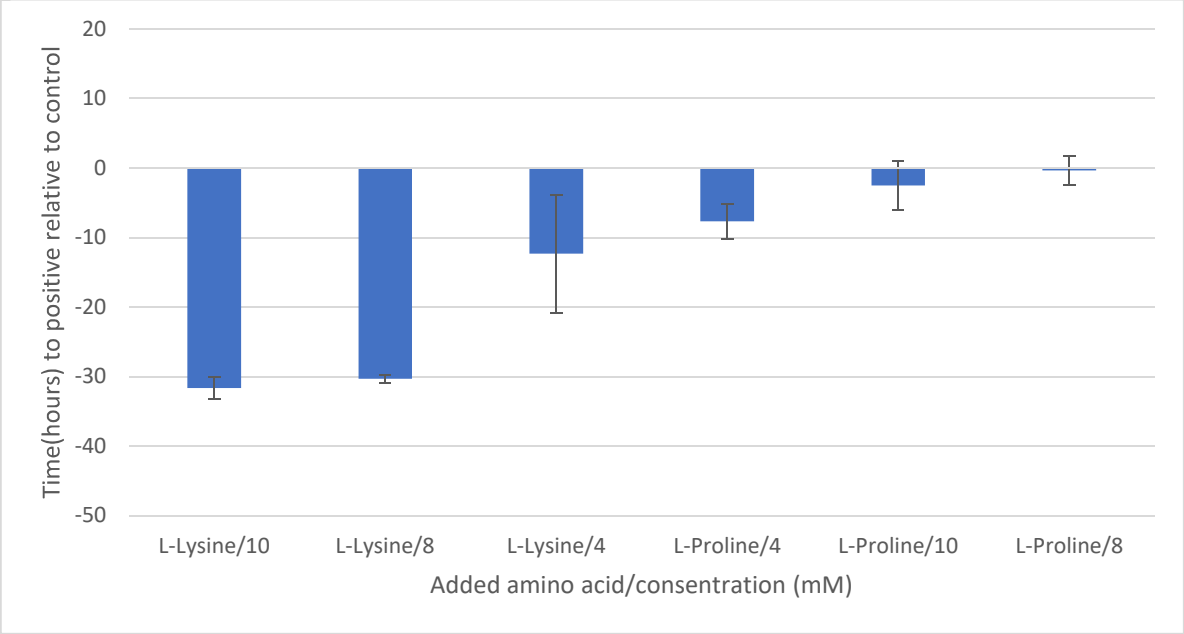

Figure 17. ***Screening of amino acids with growth promoting effect on replicative M. tuberculosis****. The bars represent the mean time (n=3) from a MGIT growth indicator tube was placed in the BD Bactec MGIT instrument until reported as positive. Results are presented relative to control (PBS), which was reported positive after 289 hours (mean, n=3). The growth tubes were added with different concentrations of amino acids, supplemented with DC (dextrose and catalase) and inoculated with an exponential replicative culture of M. tuberculosis H37Rv.*

The exponential replicative culture used shorter detection time compared to the non-replicate cultures in the other set-ups (Fig.9, 11-13) using DC as supplement. However, the results suggest that for the amino acids tested in this set-up, reveal a similar effect on growth rate compared to the control, both in exponential replicative and non-replicative cultures.

2. RNA isolation

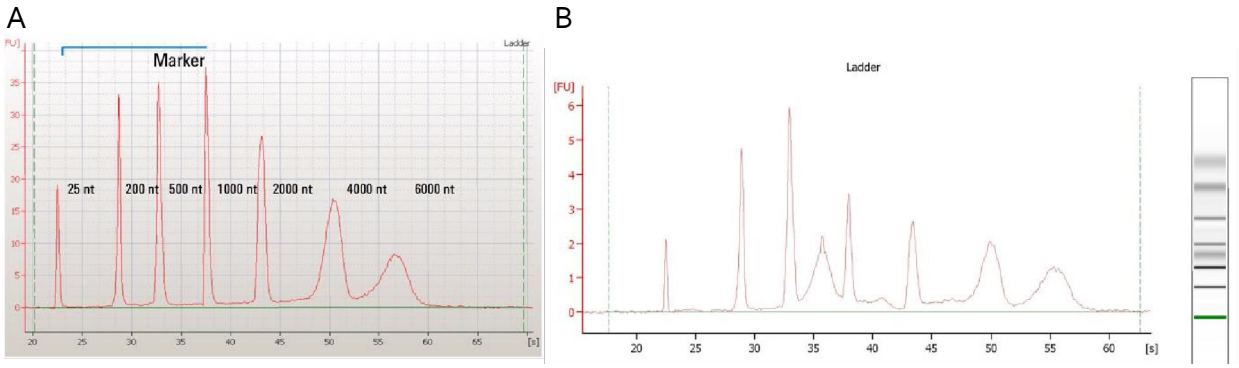

Figure 18. Agilent RNA 6000 Nano ladder well results. *The Figure 18A shows how the electropherogram of the ladder should look like according to the manufacturer's protocol (picture taken from the Agilent RNA 6000 Nano Kit Guide). The figure 18B shows the electropherogram of the ladder obtained after running our samples.*

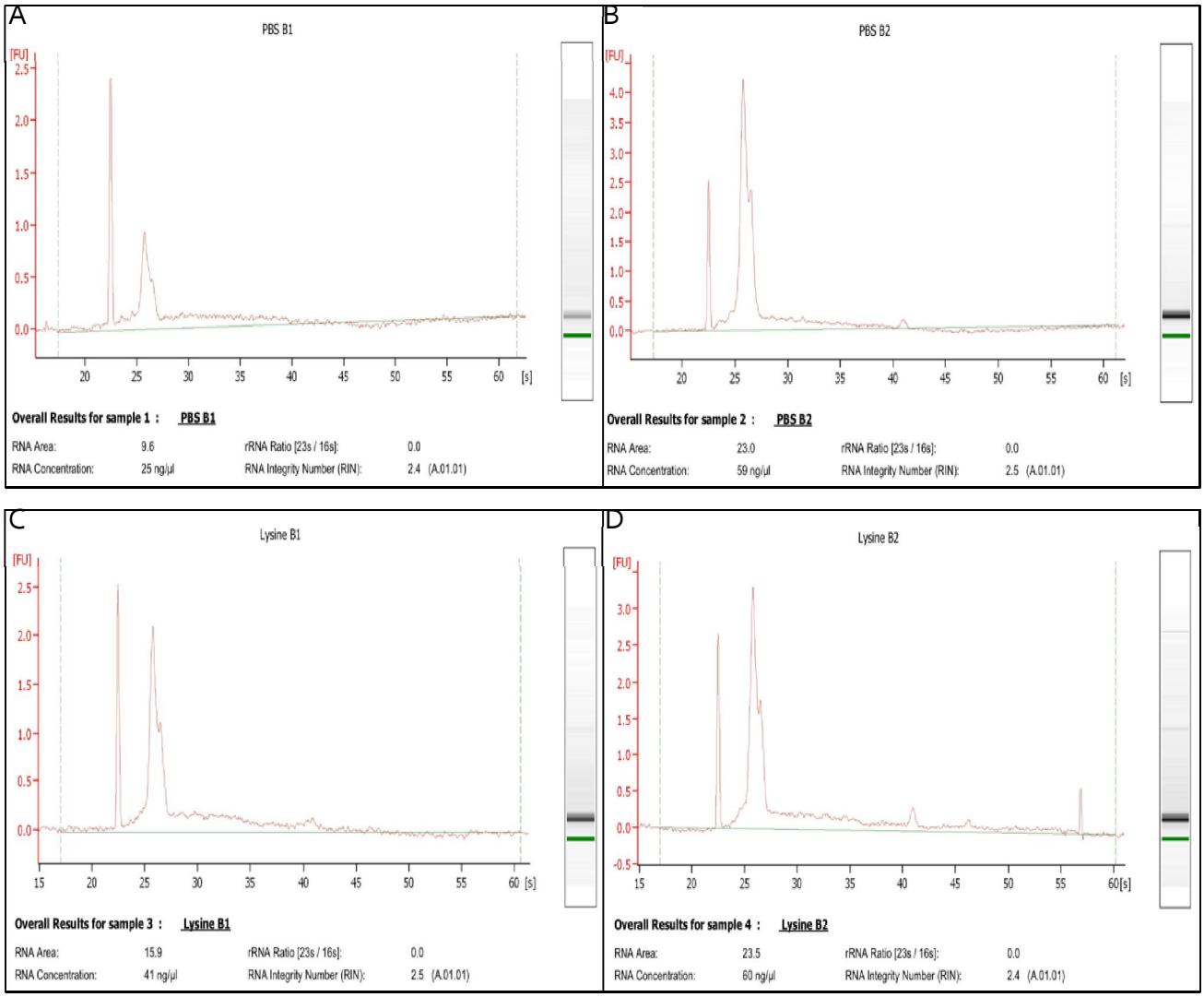

Figure 19. Agilent RNA 6000 Nano sample well results. *The Figures 19A and 19B show the electropherograms of the PBS controls 1 and 2 together with their gel images. The figures 19C and 19D show the electropherogram of the Lysine samples 1 and 2 besides their gel images*

Results A- D in figure 19 show a RIN number around 2, indicating that the RNA isolated were highly degraded RNA. Figure 18A shows how the electropherogram should have looked like according to the manufacturer's protocol. The RNA concentrations ranged between 25 and 60 ng/µL.

3. Staining using DMN-Tre and Nile Red

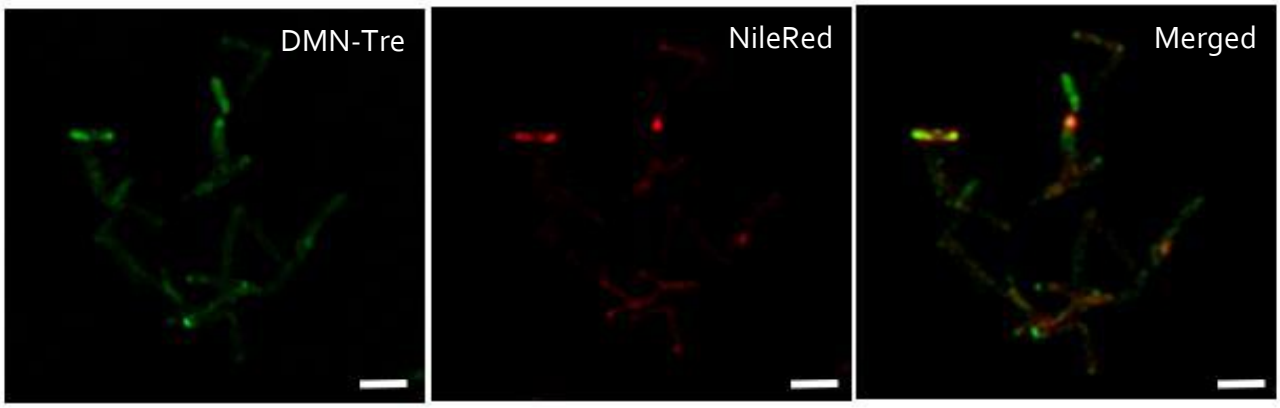

Figure 20. Superresolution structured illumination images of M. tuberculosis cells in a non- replicative state*. The bacteria were stained with DMN-Tre (green) for 30 minutes and Nile-Red (red). Bar: 2 µm. DMN-Tre was kindly provided by Bertozzi's Group from Standford University. The picture was taking with the ELYRA PS.1 from Zeiss (Images by Marie T. Noer and Marta Gómez Muñoz).*

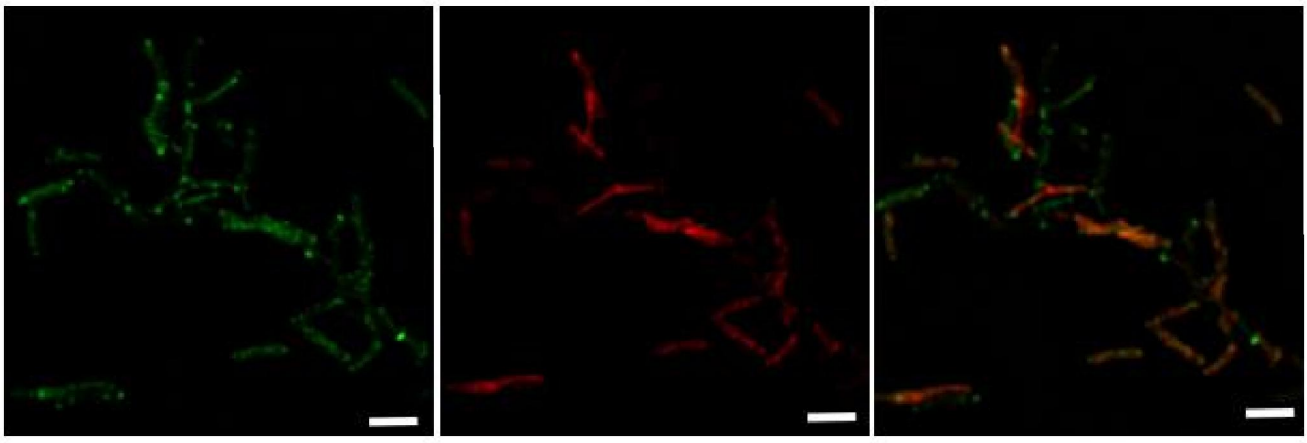

Figure 21. Superresolution structured illumination images of M. tuberculosis cells in exponential phase*. The bacteria were stained with DMN-Tre (green) for 30 minutes and Nile-Red (red). Bar: 2 µm. DMN-Tre was kindly provided by Bertozzi's Group from Standford University. The picture was taking with the ELYRA PS.1 from Zeiss (Images by Marie T. Noer and Marta Gómez Muñoz).*

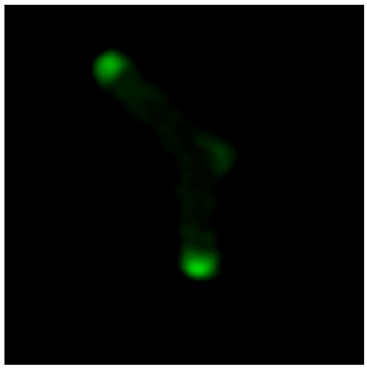

***Figure 22. Superresolution structured illumination image of Mtb cells in exponential phase**. The bacteria were stained with DMN-Tre (green) for 30 minutes. Bar: 1 µm. DMN-Tre was kindly provided by Bertozzi's Group from Standford University. The picture was taking with the ELYRA PS.1 from Zeiss (Images by Marie T. Noer and Marta Gómez Muñoz).*

The results show the DMN-Tre probe was incorporated in both replicating an non-replicating.

DISCUSSION

Tuberculosis is a major public health problem caused by the infectious agents of the *M. tuberculosis complex* (MTBC) *(WHO, 2018).* The pathogenesis of the bacterium is strongly linked to its ability to survive within its host. Further, the adaptive and flexible metabolism plays a central role for its success (Pai et al., 2016). *M.tuberculosis* can utilize a wide range of carbon, energy and nitrogen sources, has the capacity to co-metabolize multiple carbon sources simultaneously, (de Carvalho et al., 2010) recycle lipids (Kalscheuer et al., 2010) and encodes enzymes for alternative metabolic pathways to ensure adequate supply(Munoz-Elias et al., 2006). Also, there are strong indications that the bacterium has the ability to reduce its metabolism, including entering a state of dormancy. In such a state, the bacteria are non-replicative and cannot form colony forming units (CFU) immediately on conventional culture media. However, they remain viable, capable of resuscitation and can return to a metabolically active state(Chao and Rubin, 2010). Additionally, unlike many bacteria, mycobacteria grow and divide asymmetrically, producing heterogeneous daughter cells of different sizes, growth rates and cell wall compositions, which may represent an advantage in variable environments (Kieser and Rubin, 2014).

It is likely that *M. tuberculosis* coexist in different metabolically stages in infected individuals. Moreover, it has been demonstrated that the cell population of smear- positive sputum samples are dominated by cells that only can be grown in the presence of resuscitation- promoting factors (RpF), proteins that promote growth, indicating the presence of cells with reduced metabolism. Mukamolova et al. (2010) have pointed out that the existence of such standard culture- invisible populations should be recognized as important for the development of enhanced diagnostic methods.

Culturing remains the "gold standard" for laboratory diagnostics of TB due to high sensitivity (Asmar and Drancourt, 2015) However, culturing is a time- consuming process that can take several weeks. Currently, the average detection time for primary cultures are two to four weeks. Phenotypical susceptibility testing can take further days or weeks in addition. Although the combination of liquid media and automated growth detecting

systems have provided decreased detection time compered to solid medium, the slow growth of mycobacterial cultures remain a major problem for the diagnostics of TB Although *M. tuberculosis* has the potential to synthesize all the essential amino acids, *in vivo* experiments suggest that amino acid uptake and degradation is required for multiplication and virulence. Most media contain nitrogen sources in the form of complex protein, such as albumin and enzymatic digest of casein. The 7H9 media used in this thesis are also added with L-Glutamine or L-Asparagine. The reason may be that those amino acids together with aspartate are considered to be the most efficient nitrogen sources *in vitro* when provided as sole nitrogen sources (Ghodbane et al., 2014).

Detecting mycobacteria in sputum samples from TB patients requires time, especially if the bacteria is in a non-replicative and dormant state. Implementing the culture media to recover cells faster is therefore very important. Most media contain a complex amino acid mix. Based on this, we wanted to investigate if there was any amino acid that exhibited a growth promoting effect on M. tuberculosis cultures with reduced metabolism. Learning about the independent contribution of each amino acid during culturing, could help to design an amino acid cocktail that the bacteria could use more efficiently.

1. Culturing

1.1 Wayne model

There are several ways to induce non- replicative *M. tuberculosis* cultures. However, compared to others, the Wayne hypoxia model is relatively time efficient and easy to use. Another advantage is that it has been extensively used and described earlier. Also considered used in the thesis was the Loebel nutrient starvation model. In this model, bacteria is transmitted from a nutrient-rich medium into phosphate-buffered saline, resulting in a gradual downshift as the nutrients are consumed (Alnimr, 2015). An obvious advantage with the latter model is that it acquires a minimum of equipment. Also, like the Wayne model, it is easy to use. In the Wayne model the bacteria will start replicate again once reintroduced to oxygen. This is a major disadvantage as it is difficult to control whether the growth is a result of the added components or the oxygen. In the Loebel model, this variable would have been avoided. However, due to the long starvation time of

at least six weeks (Betts et al., 2002) compared to the ~14 in the Wayne model, the latter one was chosen.

In general, *in vitro* models are unable to copy the complex host-pathogen interactions encountering *in vivo*. Further, as already addressed in the introduction, it has been pointed out that the model does not represent true dormancy in accordance to the definition. However, it still represents cells with reduced metabolism (non-replicating), which probably are more comparable to the states found in patient sputum than exponential cultures.

Based on the measured absorbance, the cultures induced in the thesis behaved as expected, showing the same tendency as described by the model's developer (figure 4 "introduction").

1.2 Screening comparing ADC and DC as additional supplements

In the original protocol for cultivation using the BD BACTEC MIGIT system, tubes are added with OADC/PANTA solution. Because the MGIT instrument only measures the consumption of oxygen, it cannot distinguish the mycobacteria from other microbes, which is why, prior to inoculation the samples are decontaminated. NALC (N-acetyl-L-cysteine), a mucolytic agent, mixed with NaOH (sodium hydroxide) is one of the most widely used methods to reduce the number of contaminating microbes. An unfortunate disadvantage however, the decontamination can result in killing of many of the mycobacteria as well (Peres et al., 2009). The balance between recovery of mycobacteria and elimination of contaminants are therefore delicate, which is why the antibiotic mixture (PANTA) is added as an extra insurance. Given that we wanted to modify the original supplement in the futured set- ups, the complete one belonging to the MGIT system was replaced by a "conventional" supplement that could be mixed as desired. Pure cultures with low probability of being contaminated was used, which is why the decontamination and the antimicrobic mixture, except in a control, were left out. Albumin is a rich source of amino acids, so to be able to measure the effect of the added amino acids, this protein was removed from the supplement in almost all of the set-ups. However, because of the uncertainty initially, whether the bacterium would be able to grow at all on a single amino acid source, ADC was included as a second condition in the first screening. Nevertheless, it must be said that the MGIT 7H9 medium are added with both L- Asparagine and casein peptone, which also are a

source of amino acids. Based on this knowledge, our initial believe, that the added amino acids would serve as the sole amino acid source, was incorrect. The theme will be addressed further in 1.5, "Screening comparing conventional and MGIT modified 7H9 medium".

Oleic acid was also excluded from the supplements as it has been demonstrated that, even though supportive of rapid growth (Dubos, 1945), free fatty acids are toxic to mycobacteria (Dubos, 1947). To avoid the latter problem, albumin is usually added to media to neutralize the toxicity by binding to the fatty acids (HiMediaLaboratories, 2018). Thus, because albumin was excluded, the oleic acid was not added to the growth supplement to avoid a toxic effect. In addition, albumin protects the cells from substances such as heavy metals, chlorine, phenolic compounds and detergents (Dubos, 1947). If "growth promoting" is defined as reduction in detection time, a protective effect would be considered growth promoting. Having this in mind, the removal of oleic acid may represent a disadvantage, as the potential protective effect of the amino acids would not have been revealed.

The results from the screening compering the supplements showed that time to detection on the average were faster in the tubes added with ADC and single amino acids, than in the ones added with DC and amino acids. This suggests that the complex source is more growth promoting than single amino acids. Still, the results, not only from this set- up (fig. 8-16), reveal that the amino acids are utilized at different rates, indicating a potential for improvement of the amino acid cocktail added to growth media.

Tubes added with the non-polar (except L-Alanine) and negatively charged amino acids were not reported as positive at all or exhibited prolonged time to detection compared to the controls. This was the case for tubes added with both ADC or DC, suggesting that the particular amino acids on the contrary impeded the growth. It this is related to the properties of the amino acids or are concentration related (as mentioned, the medium contains casein peptone with unknown content of amino acids). Selected members of the polar and the positively charged amino acids displayed a growth promoting effect when added to tubes without albumin.

1.3 Screening with different inoculum and amino acid concentrations

Based on the results from "screening comparing ADC and DC as supplement", we wanted to do additional set- ups using DC and the amino acids that have had decreased detection time compared to the control. The purpose was to check if the results were reproducible, but also to investigate if different inoculum sizes and amino acid concentrations would affect the outcome. The previous results (figure 9, results) suggested that, at least for some, the amino acid concentration was relevant for their growth promoting performance. The though behind the use of different inoculum sizes was linked to the assumption that the number of cells compared to the concentration of amino acid is relevant. However, subsequently the this represent two sides of the same story. The selected concentrations were based on the previous results. If the highest concentration (8mM) showed decreased detection time compared to the lower one (4mM), an additional concentration of 10 mM was added to the future set-ups. In the case of the opposite outcome 2 mM was included. The four set- ups taken together (figure 11-14, results) represented three technical replicates for each concentration, four different inoculum sizes and two biological replicates. The MGIT instrument usually reports a tube as "positive" when containing a biomass around 10^5-10^6 CFU/mL. The aim was therefore to use inoculates that provided lower concentration of cells than the detection concentration. In the set- ups "screening comparing ADC and DC as supplement" dilution 3 provided approximately 10^2 cells/mL. Based on this result; dilution 3 and dilution 4 of both biological replicates were used in the set -ups "Screening with different inoculum and amino acid concentrations". Nevertheless, this provided different concentrations; about 10^2 and 10^3 cells/mL for biological replicate 1, and about 10^4 and 10^5 cells/mL for biological replicate 2. These results highlight one of the biggest challenges faced when working with mycobacterial culturing; their ability to aggregate, which makes standardization difficult. Replicates in general are important for the production of more reliable results and statistics and can be used to evaluate and reveal sources of false variations. Biological replicates can capture random biological variation while the technique replicates can reveal variation associated with protocols and equipment (Blainey et al., 2014). Considering the aggregation and standardization difficulties, replicates is of extra importance when working with mycobacteria.

Aside from figure. 14, the results suggest that L- Proline and Hydroxy-L-Proline in general exhibited reduced detection time compared to the control, while L-Glutamine, L-Histidine, L-Asparagine (2 and 4 mM) and L-Threonine show more varying results between the different set-ups and amino acid concentrations. However, the results Also, these results suggest that L-Serine have no effect on the detection time. L- Lysine is the one amino that sticks out from the rest displaying a significant, stable and reduced detection time compared to the controls in all the described set-ups were albumin was not added. Also, it can be suggested that, of the tested concentrations, especially 8 and 10 mM decreased the detection time.

The results presented in figure 14 is unexpected, completely different from the other four set-ups (figure 9, 11-13, results) using DC as additional supplement, suggesting that almost all the tested amino acids impeded the detection time. Tubes added with L-Lysine (8 and 10 mM) were, as in the other set- ups reported as positive before the control. Anyhow, the time difference was not as convincing as in the other set- ups. It is difficult to explain why in particular this set- up behaved differently from the others. The inoculum came from the same biological replicate/tube that was used in the parallel set- up, in which the results were as expected (figure 13, results). Also, the exact same amino acid and supplement solutions were used. The results may simply be due to an unrevealed human error during the preparation of the analysis, or perhaps it can be explained by the already mentioned problem of standardizing the inoculums due to aggregation. The ten-fold dilution used to prepare the plates for counting of CFU was not the exact same dilution as used to inoculate the tubes. Although speculative, it is tempting to suggest that the inoculum calculated may not representative for the actual inoculum size used, and that maybe a biomass over the instruments detection concentration (10^5-10^6 CFU/mL) could have affected the results. Nevertheless, the question will remain unanswered. Because we do not trust the results presented in this particular parallel set- up, they are excluded from the general validation of the results. They are also excluded from the statistical test results presented for L-Lysine.

Our result may relate to earlier findings. It has been demonstrated that mutant *lysA M. tuberculosis* (*ΔlysA*) could not replicate in mice. Diaminopimelate decarboxylase (LysA) is an enzyme involved in the synthesis of the amino acid L-lysine. Suggesting an important role

of L-Lysine for growth (Pavelka et al., 2003). Also, it has been suggested that mycobacterial lysine ε-aminotransferase, Rv3290c (LAT), an enzyme which catalyse the transfer of the amino group of L-lysine to alpha-ketoglutarate (Mycobrowser, 2018), is significantly upregulated during hypoxia persistent model, proposing a role of LAT in persistence. Further suggested, that LAT controls the intracellular amino acid balance in dormancy formation (Duan et al., 2016).

1.5 Screening comparing conventional and MGIT modified 7H9 medium

As explained earlier, albumin was removed from the supplement in order to measure the effect of the added amino acids without the interference of amino acids from other sources. However, we discovered after all of the screening set-ups, that the 7H9 medium used in the MGIT growth indicator tubes is modified with the addition of casein peptone, an amino acid source. The medium further contain L- Asparagine. Given the background, it was of interest to compare the MGIT medium to a conventional 7H9 medium, which does not contain casein peptone, to check the possible interference of casein peptone with the previous results. However, the conventional medium is also added with an amino acid, but with L-Glutamine instead of L-Asparagine. In comparison to the OADC supplements, what could be manipulated by mixing the compounds as desired, the MGIT 7H9 medium and the conventional 7H9 broth already contained the mentioned amino acids/ amino acid mix (casein peptone). The additional amino acid sources found in the media are clearly a disadvantage to the method, which is why our results only can be interpreted using the media as baseline. Anyway, the background for us wanting to do the set- up comparing the two media, was to hopefully get a clue about the extent of interference the casein peptone represented.

Surprisingly, the results (figure. 16, results) suggested that the casein peptone probably did not interfere much with the previous results, given that the control containing MGIT medium (casein peptone) did not decrease the detection time compared to the control (conventional 7H9, without casein peptone). The results also suggest that albumin (and oleic acid) is a more potent growth promoting factor in comparison to casein peptone in the given concentrations, revealed by the difference between the control containing

conventional medium supplemented with OADC and the control containing MGIT medium supplemented with casein peptone (no oleic acid or albumin). Another interesting observation, tubes supplemented with MGIT OADC have a longer detection time compared with tubes supplemented with DC and one of the amino acids; L-Lysine, L- Histidine, L-Glutamine or L- Asparagine. L- Proline had approximately the same detection time. This differs with the result that was proposed in one of the earlier set- ups (fig. 9), were MGIT tubes (original medium) supplemented with "conventional" ADC did not as convincingly decrease the detection time relative to the control. However, differences between the two set- ups; the one using MGIT medium and conventional ADC did not contain oleic acid or antibiotic mixture, while in this set- up it did (chapter 2.1, 2.2 and 2.4.1, 2.4.2 "Methods). Actually, the latter set- up contained oleic acid from two sources, both from the OADC supplement and the Tween80, which is a component of the conventional 7H9 medium. A bold suggestion that may explain the observation, is that the albumin perhaps was bound to the oleic acids in the one set- up, which possibly made the protein less available for metabolism. It could also be an effect due to the antibiotic mixture or simply unknown variables. Nevertheless, more set- ups and investigations must be conducted in order to check the reproducibility.

1.6 Screening of amino acids with growth promoting effect replacing *the* non- replicative culture with an exponential replicative

The results suggest that the amino acids not necessarily "resuscitate" the bacteria from a reduced metabolically state.

2. RNA isolation

The purpose of RNA isolation was to obtain material for RNA sequencing, which could have provided information about changes in gene expression due to the addition of amino acids. However, the attempts turned out to be challenging, as the isolations did not provide enough RNA or RNA with good enough quality. The tube materials were put into RNA later at an early time point, before (based on the growth units) or just after the MGIT instrument had reported the tubes as positive, thus providing small numbers of RNA due to low cell concentrations. In one of the set- ups to provide RNA, each condition was set up with 50

technical replicates. I therefore find it strange that the extractions did not provide enough RNA (minimum 1 mg). Degradation of the RNA by RNases is one explanation. However, precautions were taken to avoid contamination of RNases by using RNase-free equipment and gloves at all time to avoid contamination from the skin. The gloves and equipment were also frequently treated with RNase Zap® during the protocol. Still, heavy degradation was identified using the Bioanalyzer, which may be due to contamination from an unidentified source. It was also observed that it was difficult to obtain and keep the cell pellets throughout the protocol. Aggregated cells in the form of clumps was observed sticking to the tube walls and inside the pipettes. By experience, more than usual. The phenomenon could be due to the lack of added detergent (e.g. polyoxyethylene stearate or Tween80®), which in the MGIT system usually are provided through the OADC/PANTA supplement (conventional was used). A more speculative suggestion for why the RNA was degraded, is that the lack of detergent may make it easier to collect degraded and dormant cells than alive, replicating ones, due to their differences in cell wall properties (Pai et al., 2016).

3. DMN- Tre to distinguish non- and replicative cells

It has been suggested that the DMN-Tre probe can distinguish between metabolically active and inactive organisms, however, that further studies are needed to determine the hypothesis (Kamariza et al., 2018b). The probe was used to stain both a non- replicative culture induced in the Wayne model and a mid- log exponential one to determine if any differences could be recognized. The cells were also dyed with Nile Red, which would stain all the cells regardless of metabolic state, in order to identify cells that had not incorporated the DMN-Tre probe. In our results it looks like that the DMN- Tre probe was incorporated in the cells from both the Wayne model and the mid- log exponential cultures. Yet, differences can be acknowledged. Seemingly, there are accumulations of the DMN- Tre probe in the poles of many of the bacteria from the exponential culture. In the cells from the non- replicative culture the colour is more even. Inversely, accumulations of Nile Red may be indicated in the non- replicative culture, whereas in the exponential culture, the dye is more evenly distributed. Our results suggest that the DMN- probe is incorporated at different rates in cells with different metabolic activity, however, that it cannot be used to distinguish a non- replicative from an exponential replicating culture.

4. Future perspectives

Further validation of wildtype and mutant M. tuberculosis strains in well- defined media and growth conditions are required to discover the mechanism of action of the Lysine- induced growth enhancement. These findings are highly relevant for elucidating the state of dormancy in latent TB and for designing and optimized culture medium for isolation of mycobacterial isolates from clinical specimens.

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Appendix – 1: Amino acid supplement used in MGIT

Growing amino acids in MGIT																				
Each tube containing: 7 mL of 7H9 + 800 µL of ADC or DC + 100 µL dormant culture + 500 µL amino acid/ PBS solution																				
Amino Acid	Lot#	Solubility	Molar weight (g/mol)	Mol	Dry material (g)	Volume added (µL)	C1 Molar (mmol/mL)	C2 Molar (mmol/mL)	C2 Molar (mmol/mL)	C2 Molar (mmol/mL)	C2 Molar (mmol/mL)	V2 Volume (mL)	V1 Volume (µL) (0,002)	PBS (0,002)	V1 Volume (uL) (0,004)	PBS (0,004)	V1 Volume (µL) (0,008)	PBS (0,008)	V1 Volume (µL) (0,01)	PBS (0,01)
L-Alanine	BCBV0750	H_20: 1M	89,09	0,00224492	0,2	2245	1		0,004	0,008		8,4			34	466	67	433		
L-Arginine	0001371738	H_20: freely	210,66	0,0009494	0,2	949	1		0,004	0,008		8,4			34	466	67	433		
L-Asparagine	0001371738	1M HCl, 100 mg/mL	132,12	0,00151378	0,2	1992	0,76	0,002	0,004	0,008		8,4	22	478	44	456	88	412		
L-Aspartic acid	BCBV6094	1M HCl, 0.5 M	133,1	0,00150263	0,2	3005	0,5		0,004	0,008		8,4			67	433	134	366		
L-Cysteine	BCBV4905	1M HCl, 1M	121,16	0,00165071	0,2	1651	1		0,004	0,008		8,4			34	466	67	433		
L-Cystine	BCBT3618	1M HCl, 0.5g/10 mL	240,3	0,00083229	0,2	3963	0,21		0,004	0,008		8,4			160	340	320	180		
L-Glutamine	BCBR7569V	H_20, 0.1 M	146,14	0,00136855	0,2	13686	0,1	0,002	0,004	0,008		8,4	168	332	336	164	672	-172		
L-Glutamic acid	0001423805	1M HCl, 100 mg/mL	147,13	0,00135934	0,2	1999	0,68		0,004	0,008		8,4			49	451	99	401		
Glycine	0001119375	1M HCl, 1M	75,07	0,00266418	0,2	2664	1		0,004	0,008		8,4			34	466	67	433		
L-Histidine monohydrochloride monohydrate	BCBT9895	H20, 1g/10mL	209,63	0,00095406	0,2	1988	0,48	0,002	0,004	0,008		8,4	35	465	70	430	140	360		
Trans-4_Hydroxy-L-Proline	BCBL2666V	H20, 50mg/mL	131,13	0,0015252	0,2	4014	0,38		0,004	0,008	0,01	8,4			88	412	177	323	221	279
L-Isoleucine	0001423806	1M NH_4OH, 50mg/mL	131,17	0,00152474	0,2	4012	0,38		0,004	0,008		8,4			88	412	177	323		
L-Lucine	BCBV3850	1M HCl, 50mg/mL	131,17	0,00152474	0,2	2006	0,76		0,004	0,008		8,4			44	456	88	412		
L-Lysine monohydrochloride	BCBN9886V	H_20, 50mg/mL	182,65	0,00109499	0,2	3911	0,28		0,004	0,008	0,01	8,4			120	380	240	260	300	200
L-Methionine	0001423807	1M HCl, 50mg/mL	149,21	0,00134039	0,2	3942	0,34		0,004	0,008		8,4			99	401	198	302		
L-Phenylalanine	BCBV1428	1M NH_4OH, 50mg/mL	165,19	0,00121073	0,2	3906	0,31		0,004	0,008		8,4			108	392	217	283		
L-Proline	BCBP4505V	H_20, 1M	115,13	0,00173717	0,2	1737	1		0,004	0,008	0,01	8,4			34	466	67	433	84	416
L-Serine	0001336081	H_20, 359.7g/L	105,09	0,00190313	0,2	2003	0,95	0,002	0,004	0,008		8,4	18	482	35	465	71	429		
L-Threonine	BCBD4901	H_20, 50mg/mL	119,12	0,00167898	0,2	3998	0,42		0,004	0,008	0,01	8,4			80	420	160	340	200	300
L-Tryptophan	BCBT3974	0,5 M HCl, 50mg/mL	204,23	0,00097929	0,2	4080	0,24		0,004	0,008		8,4			140	360	280	220		
L-Tyrosine	BCBV6144	1M HCl, 50mg/mL	181,19	0,00110381	0,2	3942	0,28		0,004	0,008		8,4			120	380	240	260		
L-Valine	BCBM0163V	H_20, 50mg/mL	117,15	0,00170721	0,2	3970	0,43		0,004	0,008		8,4			78	422	156	344		

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