THE EFFECT OF SELF-GENERATED HYPOXIA ON THE EXPRESSION OF TARGET GENES CODING FOR ELECTRON TRANSPORT RELATED PRODUCTS IN *MYCOBABCTERIUM TUBERCULOSIS*

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A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy, Medical Science

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University of KwaZulu-Natal

2010

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ABSTRACT

The effect of self-generated hypoxia on the expression of target genes coding for electron transport related products in *mycobabcterium tuberculosis*

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The work presented here aims at identifying whether the genes identified in the genome of *Mycobacterium tuberculosis* that code for products involved in anaerobic metabolism are active or inactivated genes. The study consists of three distinct parts.

In part one, serial dilutions of sputum of patients with pulmonary tuberculosis (PTB) were grown on agar surface and in high columns of un-agitated broth. The highest dilution from which mycobacteria was grown was for all patients significantly higher in the broth cultures than on the plates suggesting the presence of anaerobically metabolizing mycobacteria in the lungs of patients with PTB.

Part two of the study identified gene expression by measuring the concentration of transcripts for 5 genes involved in aerobic or anaerobic pathways. This was done over a period of 15 weeks using un-agitated broth cultures (the Wayne method). Undulating patterns of gene expression were found

with the genes coding for anaerobic metabolic pathway components expressed at higher levels than those coding for aerobic pathway components while the cultures grew older.

Part three aimed at measuring transcription products of the same set of genes directly in sputum specimens. Although quantitation at bacterial cell level in the sputum could not be achieved, expression of all genes was established with on average larger quantities of transcripts of genes coding for the anaerobic pathway components.

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DECLARATION

This manuscript was prepared by the author based on independent experimental work and analysis. All figures were created or captured by the author unless stated otherwise.

ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to Professor AW Sturm for his revered assistance in the preparation of this manuscript, and to the staff and students of the Department of Medical Microbiology, University of KwaZulu-Natal, School of Medicine. In addition the author acknowledges the staff of the Medical Research Council who assisted with clinical specimen acquisition for this project.

SUMMARY OF KEY CONCEPTS

Summary of Key Concepts and Definitions

H37Rv Genome

Cole and colleagues (1998) published the complete genome of *M. tuberculosis* H37Rv. This exercise revealed many unknown features that the bacterium could potentially use.

Dormancy Regulon

Voskuil and colleagues reported a set of approximately 48 genes that allow *M. tuberculosis* to adjust to environmental changes.

Wayne Model

This is a culture model for growth of *M. tuberculosis* in liquid culture medium that allows for the formation of a self-generated oxygen gradient. The tubes are left undisturbed during incubation and remain sealed. There is no oxygen circulating in these tubes and the cells undergo what is described as an orderly shut-down.

Non-Replicating Persistence

When grown under gradual oxygen depletion in broth, *M. tuberculosis* survives in the culture media without increasing in number (i.e. cell division ceases). This happens in 2 phases known as NRP-1(micro-aerophilic) and NRP-2 (anaerobic).

Anaerobiosis

The occurrence of metabolic processes in the abscence of molecular oxygen is referred to as a state of anaerobiosis.

Hypoxia

This refers to a diminished availability of oxygen.

PCR definitions Threshold: Usually 10X the standard deviation of Rn for the early PCR cycles (background activity). The threshold should be set in the region associated with an exponential growth of PCR product (which may be easier in the log-view of the amplification plot) and not as high as the linear or plateau sections of the curve. It should be above the highest baseline signal level. It is assigned for each run to calculate the C_t.

C_t (threshold cycle): Threshold cycle reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. It is inversely correlated to the logarithm of the initial copy number. The C_t value assigned to a particular well thus reflects the point during the reaction at which a sufficient number of amplicons have accumulated. This is also called the crossing point (C_p) in LightCycler terminology.

Rn (normalized reporter signal): The fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. Rn+ is the Rn value of a reaction containing all components, including the template and Rn– is the Rn value of the negative

control. The Rn– value can be obtained from the early cycles of a real-time PCR run (those cycles prior to a significant increase in fluorescence), or a reaction that does not contain any template.

ROX: (**6-carboxy-X-rhodamine**) Used as passive reference dye for normalization of reporter signal. The emission recorded from ROX during the baseline cycles (usually 3 to 15) is used to normalize the emission recorded from the reporter due to amplification in later cycles. The use of ROX improves the results by compensating for small fluorescent fluctuations such as bubbles and well-to-well variations that may occur in the plate.

FAM: (**6-carboxy fluorescein)** A reporter dye at the 5' end of a TaqMan® probe.

INTRODUCTION

Over the last few decades, *Mycobacterium tuberculosis* has developed mechanisms of resistance against most of the available anti-tuberculosis drugs. For many years these drug resistant strains were thought to have limited ability to spread within communities. Currently, a limited number of successful strains have acquired the ability to be transmitted as resistant phenotype. That includes multi-drug resistant (MDR) as well as extensively drug resistant (XDR) strains (Pillay and Sturm, 2007; Filliol, et al 2003). There is therefore an urgent need for the development of new drugs for the treatment of patients infected with MDR or XDR *M. tuberculosis*. The study of metabolic pathways in *M. tuberculosis* can consequently lead to the identification of new drug targets.

M. tuberculosis has been historically characterized as a strictly aerobic organism. This has hampered the understanding of the persistence and latency of tuberculosis. Reports disputing the aerobic nature of the organism arose as early as 1933. In the 1970s the concept of *M. tuberculosis* as a facultative species was reintroduced by Wayne et al (1976). When *M. tuberculosis* is grown in liquid medium exposed to air, and the culture is left undisturbed, equilibrium is established between the rate of replication, oxygen diffusion and the rate of bacilli settling at the bottom of the tube. In addition, actively replicating bacilli die rapidly when they are abruptly derived of oxygen (Wayne and Lin, 1982). They have however been shown to shift into a stage of dormancy when they are allowed to adapt to gradually decreasing oxygen (Wayne, 1976). We therefore chose to use this liquid culture model, to investigate the role of selected genes in adaptation of *M. tuberculosis* under stress conditions.

This study provides novel insights into the manner of gene expression patterns in *M. tuberculosis*, leading to a better understanding of its persistence mechanisms. We provide insight into the simultaneous occurrence of the forward and reverse reactions of the conversion of fumarate providing new information regarding the operation of the forward and reverse cycles of the TCA. We also report on the expression of a cytochrome b reductase component, under anaerobic conditions, despite reports of this being an aerobic component. In addition we provide preliminary evidence based on gene transcript detection, that cells present *in vivo* are predominantly anaerobic in nature. In addition we provide the first report of the expression of the *men*B gene *in vivo* and in vitro. Since this represents a critical pathway that occurs in bacteria only, it proves to be an attractive drug target, having already been theoretically marked as such. We further demonstrate the up regulation of the ferredoxin gene *fdx*A in oxygen depleted cultures and the detection of its transcripts in sputum specimens of patients with pulmonary tuberculosis. This justifies further study into the potential application of metronidazole for the treatment of tuberculosis infection.

Section 1

Literature Review

Section 1: Literature Review

An Introduction to tuberculosis

1.1. Preamble

"*The struggle against tuberculosis is not dictated from above, and has not always developed in harmony with the rules of science, but it has originated in the people itself, which have finally correctly recognized its mortal enemy. It surges forward with elemental power, sometimes in a rather wild and disorganized fashion, but gradually more and more finding the right paths.*"

(Robert Koch, 1905)

Robert Koch, known as the father of bacteriology, identified and described the infectious agent causing tuberculosis, *Mycobacterium tuberculosis*, as early as 1882 (Kaufmann, 2003). His presentation on 24 March of that year facilitated experiments to elucidate the nature of the ancient disease and to develop remedies against the infection. In 1890 he reported on a glycerine extract of the tubercle bacilli as such a "remedy" (a vaccine) for tuberculosis. He named the extract 'tuberculin', a term still widely used for Purified Protein Derivative (PPD) which is derived from Koch's extract (Parekh, 2009). Although tuberculin was not effective as a therapeutic or prophylactic vaccine, it was later adapted by von Pirquet in a test to diagnose pre-symptomatic tuberculosis. Mantoux modified von Pirquet's test in 1907 and the Mantoux

test, in which tuberculin is injected in the skin, became the most frequently used diagnostic test for sub-clinical tuberculosis for decades to come. This test is still used for this purpose.
Koch opened his Nobel Lecture in 1905 by indicating the difficulties and hurdles that were still faced by tuberculosis researchers in trying to neutralize the disease and indeed, progress has been very slow for at least a century

This is corroborated by the fact that *M. tuberculosis* is currently the leading bacterial cause of human mortality in the world (Rao and Vijaya, 1995). It claims millions of lives per year and is responsible for more deaths annually than any other single bacterial pathogen (Bloom and Murray 1992; Kochi, 1991). It is a facultative intracellular organism and its entry of, its subsequent replication in, and its ability to avoid destruction by the macrophage, are critical to its virulence. Since *M. tuberculosis* is capable of residing within host cells for a long period of time, it must have mechanisms to overcome nutrient limitations.

Hippocrates described a disease that he named "phthisis" which means "consumption", since it was characterized by wasting. He reported this disease to be the most widespread and fatal disease of his time. Later observations make it likely that "phthisis" was indeed tuberculosis. It is often stated that tuberculosis is as old as mankind. The presence of abnormalities characteristic of tuberculosis in the skulls and spines of Egyptian mummies, provides evidence that tuberculosis has been plaguing humans for at least 4000 years. Salo and colleagues (1994) reported the detection of insertion element DNA, unique to *M. tuberculosis* in mummified human remains found in Peru. Fletcher and colleagues reported in 2003 similar findings in

naturally mummified remains of a mother and two daughters which were located in an 18th century Hungarian crypt.

More than a century after Koch discovered *M. tuberculosis*, the World Health)Organization still reports alarming statistics: The World Health Organization (WHO) estimates that the largest number of new TB cases in 2008 occurred in the South-East Asia Region, which accounted for 35% of incident cases globally. However, the estimated incidence rate in sub-Saharan Africa is nearly twice that of the South-East Asia Region, at nearly 350 cases per 100 000 population'' (World Health Organisation, 2010).

1.2. Mycobacterial Physiology

Mycobacterium tuberculosis is a non-motile rod-shaped bacterium related to the *Actinomycetes*. Its generation time is 15-20 hours, making it one of the slowest replicators amongst pathogenic bacteria. This characteristic may have a role in its pathogenesis. Many other chronic diseases are caused by bacteria with long generation times, for example, *M. leprae* causes leprosy, *Treponema pallidum* causes syphilis, and *Borrelia burgdorferi* causes Lyme disease (Orme 1988 (b)). The cell wall structure of *M. tuberculosis* is distinctive among prokaryotes and it is a major determinant of virulence for the bacterium.

The insoluble cell wall core (formed after the removal of soluble proteins, lipids and carbohydrates), is chemically composed of three covalently linked macromolecules: highly cross-linked peptidoglycan, arabinogalactan (AG), mycolic acids (Cole, et al, 1998). Mycolic acids are high-molecular-weight fatty acids consisting of a short β-hydroxy chain with a longer α-alkyl side chain. They are found in the genera *Mycobacterium, Nocardia, Corynebacterium* and *Rhodococcus* (Brennan and Nikaido, 1995). The outer leaflet, consisting of covalently linked arabinogalactan, mycolic acids and other lipids, constitutes a very hydrophobic barrier. These components make up a significant fraction of the dry weight of the mycobacterial cell envelope.

Mycolic acids are thought to play a significant role in virulence in *M. tuberculosis*. They protect extracellular mycobacteria from complement deposition and phagocytosis. They also confer intrinsic resistance to certain antimicrobial drugs (Nikaido, 2001). Hoffmann and colleagues

reported recently (2008) the structural nature of the mycobacterial cell wall. They advise that the structure of the mycobacterial outer layer requires revision. They used cryo-electron tomography and vitreous sections to reveal the lipid bilayer structure. They report that proof of the structure of the mycobacterial outer layer will have an impact on the design and interpretation of experiments aimed at elucidating the translocation pathways for nutrients, lipids, proteins, and anti-mycobacterial drugs across the cell envelope.

Serpentine cording is a phenomenon that was found to be distinctive in virulent mycobacteria and was thought to be the result of the production of cord factor (Bloch, 1950). This "cord factor" was later found to be a group of mycolic acid-containing glycolipids (Indrigo, 2003). Trehalose-6, 6' -dimycolate (TDM) is the most prominent and best-studied representative of this class of molecules (i.e. cord factor molecules). Indrigo and colleagues (2003) further reported TDM as having a range of biological activities including immune-modulation. They reported that in murine models, immune responses to purified TDM mimic aspects of natural *M. tuberculosis* infection, including production of pro-inflammatory cytokines (IL-1b, IL-6 and TNF-α), development of granulomas, pro-coagulant activity and decreased cortisol in the blood (Actor et al., 2000; Behling et al., 1993; Perez et al., 2000). Additionally, TDM is reported to prevent the fusion of phospholipid vesicles (Crowe et al, 1994; Indrigo et al, 2003; Spargo et al, 1991). This implies that TDM may contribute to intracellular survival of *M. tuberculosis* by inhibiting phagosome–lysosome fusion after invasion of macrophages.

The high concentration of lipids in the cell wall of *M. tuberculosis* has been associated with protective properties of the bacterium like impermeability to stains and dyes, resistance to many

antibiotics, resistance to killing by acidic and alkaline compounds, resistance to lysis via activation of the alternative complement pathway, resistance to lethal oxidants and resistance to elimination from macrophages (Indrigo, 2003).

Macrophages are fundamental for the initiation of immune responses. Through a variety of cell surface receptors, macrophages internalize microbes into phagosomes that undergo maturational events allowing phagosome-lysosome fusion that expose the microbes to acid, lytic enzymes, oxygenated lipids and fatty acids (Nathan and Shiloh, 2000). The ability to persist in macrophages is not unique to *M. tuberculosis*. This ability to conceal itself within macrophages confers a degree of protection from interactions with other immune components as well as antimicrobial agents.

Lipoarabinomannan (LAM) is a phosphatidylinositol anchored lipoglycan composed of a mannan core with oligoarabinosyl-containing side-chains with diverse biological activities (Hunter and Brennan, 1990). The tubercle bacillus can bind directly to mannose receptors on macrophages via the cell wall- associated mannosylated glycolipid, LAM, or indirectly via complement receptors or Fc receptors (Chatterjee and Khoo, 1998). *M. tuberculosis* is known to impede the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis by two mechanisms. Firstly, compounds including glycolipids, sulfatides and LAM down regulate the oxidative cytotoxic mechanism. Secondly, macrophage uptake via complement receptors may bypass the activation of a respiratory burst (Hunter and Brennan, 1990; Chatterjee and Khoo, 1998).

The antigen 85 complex is composed of a group of proteins secreted by *M. tuberculosis* that bind fibronectin. It is coded for by the *fbp*ABC genes. The observation that live *M. tuberculosis* elicits protective immunity more effectively than dead bacilli prompted extensive investigation of proteins that tubercle bacilli secrete as potential inducers of protective immune responses (Bloch and Segal, 1955; Orme, 1988(a)). The fibronectin binding proteins may aid in walling off the bacteria from the immune system and are involved in the first steps in pathogenesis as any ligand that binds to fibronectin assists in adhesion, and ultimately invasion of the host by the pathogen. The antigen 85 complex is one of the best characterized groups of secreted proteins of *M. tuberculosis*.

M. tuberculosis is perceived to be an obligate aerobe**,** even in recent years (Cox, 2003, Weinstein et al, 2005), despite the fact that there are several publications to the contrary. This is supported by several observations in animal models and in vitro. The Wayne Model discussed in detail later clearly indicates that *M. tuberculosis* can grow under conditions of a self-generated oxygen gradient. This capability could serve the organism in its interactions with macrophages. Inhalation of *M. tuberculosis* results in phagocytosis by alveolar macrophages. Before macrophages are immunologically activated, *M. tuberculosis* blocks maturation of phagosomes preventing the formation of phagolysosomes (Russell, 2001). Surviving bacteria are believed to enter a period of non-replicating persistence in the phagosome until waning host immunity leads to reactivation from the latent state and the onset of disease (Wayne and Sohaskey, 2001). This non-replicating state may reflect anaerobic metabolism.

1.3. Extrapulmonary tuberculosis

Mycobacterium tuberculosis proliferates in the lung which is oxygen rich relative to deeper tissue within the host, thus fueling the concept that *M. tuberculosis* is a strict aerobe.

M. tuberculosis can colonize and cause disease in any organ system of the body. Even though pulmonary tuberculosis remains the most common form of the disease, extra pulmonary tuberculosis (EPTB) remains a significant clinical problem (Sharma and Mohan, 2004). The lungs are indeed a site rich in oxygen. However, in order for oxygen to be used as a final electron acceptor, the redox conditions need to be electropositive. Even the surface of the skin has an electronegative redox potential, and the level of electro-negativity increases in deeper tissues of the body. This therefore impedes the utilization of oxygen as final electron acceptor (Mclane et al, 1999).

1.4. Anti-mycobacterial drugs and drug resistance

1.4.1. Treatment and Drug resistant tuberculosis

In the last decade, the prevalence of tuberculosis (TB) has increased 20% worldwide with the highest burden in the most impoverished communities. If these trends continue, the TB incidence is expected to increase by 41% in the next twenty years (Outsourcing-Pharma.com, 2005). Fifty years since the introduction of an effective chemotherapy, TB remains a leading public health problem.

Even in industrialized nations with some of the best healthcare systems in the world, TB outbreaks transpire. In the early 1990s New York City witnessed an outbreak of drug-resistant TB and spent $1 billion fighting 4,000 cases (Fogarty International, 2007). In 2007 London was becoming the TB capital of the world, with double the level of New York's case load during that outbreak (All-Party Parliamentry Group, 2007)

Drug-resistant tuberculosis has been reported since the early days of the introduction of chemotherapy. Most of this was reported from developed countries. In 1992, the Third World Congress on Tuberculosis concluded that there was little recent information on the global magnitude of multidrug-resistant tuberculosis (MDR-TB), defined as resistance to at least isoniazid and rifampicin. MDR-TB is now at critical levels in specific regions of the world. Areas where MDR-TB occurs with high frequency include Estonia, Latvia, the Oblasts of Ivanovo and Tomsk in Russia, and the Henan and Zhejiang Provinces in China (Toungoussova, et al, 2006). The emergence of multi-drug resistant strains has hastened the search for more

effective treatments.

The importance of rapid detection of drug resistant tuberculosis is constantly in the spotlight. The answer is sought in the application of molecular assays. Tests of this nature may offer rapid alternatives to current diagnostic procedures allowing for higher throughput of samples. The most widespread application of molecular techniques in the study of tuberculosis focuses on the study of the evolution of resistance mechanisms and through that the development of tests to detect these.

Isoniazid (INH) is a pro-drug that requires activation by susceptible tubercle bacilli. It was introduced in the 1950s and soon after, it was observed that clinical isolates manifesting resistance to this drug, lost its catalase and peroxidase activity. In addition, it is noted that the activity of INH is restricted to actively "growing bacilli" and is not active against "non-growing" cells (Siddiqi et al, 2007; Zhang 2004). The association with the *kat*G gene and resistance to isoniazid was confirmed in the early 1990's when this gene was cloned and sequenced (Herrera et al, 2004). A mutation was detected that allowed the bacteria to retain up to 50% of its catalase activity without activating the drug – and thus a highly effective resistance mechanism was born. It has also been proposed that isoniazid blocks the production of mycolic acids, a critical cell wall component in mycobacteria.

One of the foremost causes of treatment failure and fatal clinical outcome in tuberculosis patients is the resistance to rifampicin (Mitchison and Nunn, 1986). This drug serves to inhibit transcription by inhibiting the DNA-dependant, RNA polymerase (coded for by the *rpo*B gene).

Pyrazinamide (PYZ) is reported to target an enzyme involved in fatty acid synthesis. (Zimhony et al, 2000) It is also a pro-drug that is converted to its active form, pyrazanoic acid, by the enzyme pyrazinamidase (Somoskovi, et al, 2001).

1.4.2. The Evolution of *Mycobacterium tuberculosis*: eukaryotic traits and recent developments

Mycobacteria belong to the order of gram positive rods known as *Actinomycetales*. There have been several studies in recent years using genomic technologies that have revealed some unexpected characteristics of *Mycobacterium tuberculosis*. Av-Gay and Everett (2002) revealed in their review the presence of genes coding for eukaryotic-like kinases that played a role in cellular signaling pathways. Protein phosphorylation is a mechanism by which extracellular signals are translated into cellular responses. This process is controlled by highly specific protein kinases and is directly linked to de-phosphorylation reactions that are controlled by protein phosphatases.

In prokaryotic cells this system consists of histidine kinase sensors and their associated receptors (Parkinson, 1993). In eukaryotic cells signaling transduction pathways are maintained by phosphorylation of serine, threonine or tyrosine. Previously, serine, threonine and tyrosine phosphate and their respective phosphatases were thought to be exclusive to eukaryotic cells (Verma and Maurelli, 2003). However, in 1994 Chow and colleagues showed that eukaryotic-like signal transduction occurs in *M. tuberculosis* as well as in some other bacteria including *Streptomyces* (a related genus), *Anabaena* and *Myxococcus*. Av-Gay and Davis (1997) further

demonstrated that at least 6 proteins were phosphorylated *in vitro* confirming that the genes were not just present but functional as well. Full genome sequencing of strain H37Rv by Cole and colleagues (1998) showed that there were in fact eleven genes coding for putative eukaryotic-like protein kinases present. Av-Gay and Everett (2002) proposed an interesting hypothesis that implied these protein kinases to be involved in various mechanisms of pathogenesis. These propositions are in the process of being tested.

A further example of the presence of eukaryotic genes is that of genes coding for the cytochrome P450 enzymes. Azole antifungals are reported to be potent inhibitors of cytochrome P450 mono-oxygenases and bacterial growth in mycobacteria and streptomycetes (McLean et al, 2002). Seven of these CYP450 enzymes were genetically coded for in *Bacillus subtilis*. This was the highest number of such enzymes ever observed in bacteria. The H37Rv genome revealed the presence of 22 such enzymes coded for in *M. tuberculosis* (Cole, 1998).

Studies into the binary fission process have highlighted the function of the *fts*Z gene in septum formation during cell division (Dziadek, et al, 2002). They also infer a link between the formation of what they call the "Z-ring" and the formation of clumps due to the modification of phospholipid synthesis and possible effect on septa formation. This is of significance as it is well known that clumping is more prominent in the virulent strains of *M. tuberculosis* when compared to the avirulent strains (Zhang , et al 1998).

1.5. Latent tuberculosis – the ability to persist

"Some PPD-positive individuals can develop active disease after an asymptomatic interlude" (Gedde Dahl, 1952). This statement made so many decades ago indicates the ability of the microorganism to undergo a period of dormancy and subsequent endogenous reactivation. In a study that combined epidemiological evidence with molecular fingerprinting data, reactivation of tuberculosis in an individual after 33 years of latent infection was convincingly demonstrated (Lillebaek, 2002). However, up till now the concept of clinical latency is still not fully understood. It is possible that this results from bacteria achieving a modified metabolic, non-replicative state. It is further possible that constant bacterial numbers result from a dynamic equilibrium between bacterial replication and elimination by the host (Parish, et al, 1998).

The idea that the bacteria can enter a dormant state during human infection was strengthened by studies where diseased tissues staining positive for acid-fast bacteria did not yield bacteria on culture or the bacteria grew very slowly. Dormant tubercle bacilli have been reported to remain inactive in the host tissue without any sign of infection, and most cases of *tuberculosis* are said to be due to the reactivation of dormant *Mycobacterium tuberculosis* bacilli rather than to recent infection (Smith & Moss, 1994). Because of prolonged periods of asymptomatic latent infection, it becomes critical to distinguish between replicating and non-replicating bacterial cells in specimens. Hellyer et al (1996) demonstrated that mycobacterial DNA was still detected in clinical specimens even a year after treatment.

In murine models, mycobacterial DNA was detectable during active disease when cells were culturable and also during the latent phase when cells were not detected under standard culture conditions. Different models of latency were established to study the pathogenesis pathways of *M. tuberculosis*. One such model involved mice that were infected by aerosol with a low dose of *M. tuberculosis* (5 to 10 colony forming units), and within 3 months the bacterial load stabilized at 3 to 4 log10. This is known as the low dose model (Orme, 1998(b)).

In this model the clinically inactive phase of the infection was maintained for 15 to 18 months, after which time the infection began to reactivate and the mice succumbed to the disease. This low-dose model is believed to resemble natural latency in the sense that it relies solely on the host immune response for control of the infection, but it differs from human infection by the high bacillary burden in the latent phase which is not found in human latent *M. tuberculosis* infection. (Orme, 1998(b)) Using a modified low-dose model of murine latent tuberculosis, it was demonstrated by Flynn and colleagues (1998) that reactive nitric intermediates (RNI) play an important role in preventing reactivation of the infection.

The second animal model of *M. tuberculosis* latency has been referred to as the Cornell model and was first described in the late 1950s (McCune and Tompsett, 1957). In the Cornell model, mice are inoculated intravenously with $1x\ 10^6$ to 3×10^6 viable bacilli with the H37Rv strain of *M. tuberculosis*. The resultant infection is treated for 12 weeks with the anti-mycobacterial drugs isoniazid (INH) and pyrazinamide (PZA). Treatment commenced within 20 minutes after infection. It appeared in this model that the anti-mycobacterial treatment was effective as there was no trace of culturable tubercle bacilli at the time of completion of the antibiotic

course. In addition it was reported that there is persistence of significant quantities of *M. tuberculosis* DNA throughout the various stages of the model and that this may represent dead bacilli, free DNA or dormant forms (De Wit, et al, 1995).

However 90 days after the anti-mycobacterial treatment ceased, culturable tubercle bacilli were once again detected. This drug-induced model of latent tuberculosis has the advantage of achieving very low or undetectable numbers of bacilli and maintaining those low levels for many weeks, analogous to latent infection in humans. Because this mode of latency is drug induced, it does not necessarily reflect the natural course of infection in humans. This is especially notable due the fact that many people, particularly in resource poor countries, may harbour mycobacteria for months or even years before seeking treatment. Recently there have been several investigations at the genomic level into expression profiles of *M. tuberculosis*, in an attempt to gain a more comprehensive knowledge of survival mechanisms and pathogenesis.

Fenhalls and colleagues (2002) detected transcripts of *M. tuberculosis* in human lung tissue that revealed differential gene expression in necrotic lesions. They described necrotic regions as follows: three distinct regions can be classified, the lymphocyte cut-off, the transition zone and the necrotic region. The transition zone is characterized by a depletion of oxygen and nutrients, two critical growth factors. Although they did not detect any gene expression in the necrotic region, they acknowledge that there could be an upregulation of genes not studied in their experiment, or even that their methods may not have been sensitive enough to detect low levels of expression under the restrictive conditions of the core of the necrotic granuloma. The necrotic granuloma model has been extensively scrutinized and it is widely believed that long

term survival of the tubercle bacilli is rooted in the ability of cells to survive in the core of such a structure. Bentrup and Russell (2001) explain how cells can survive using the fatty acids that are present as a substrate to fuel essential cellular processes. Latent infection is thus ultimately thought to result from major shifts in gene expression and metabolism, allowing persistence with minimal replication (Manabe and Bishai, 2000).

In 1933, survival of tubercle bacilli was reported in sealed liquid cultures held at 37^{O}C for as long as 12 years (Corper and Cohn, 1933). Variations were seen in the pH of these cultures, but only those between pH 6.1 and 7.6 yielded viable bacilli (Wayne and Sohaskey, 2001).

This was the first report of the microorganisms' ability to survive long term under challenging circumstances. One should note that the culture systems were sealed with no replenishing of nutrients resulting in redox potentials that are specific to anaerobic conditions. This is a significant observation as it highlights how resilient *M. tuberculosis* is and it substantiates our choice of culture model for this study.

1.6. The H37Rv Genome

In 1998 Cole and colleagues published the complete genome sequence of the laboratory strain H37Rv. This was a significant milestone in the pursuit of understanding tuberculosis. This paper was pivotal in the selection of the genes examined in this study. This section (1.6) is a discussion and summary of key points from this paper. The H37Rv genome comprises of 4.4 million base pairs which constitute approximately 4000 genes. Cole and colleagues offer a detailed discussion of the different coding regions of the genome with emphasis on lipid metabolism and a diverse array of potential pathways. The genome also consists of genes that have a role in anaerobic functions as well as a vast array of enzymes with various functions which are summarized below.

Approximately 10% of the genome consists of genes named the PE and PPE families. The names derive from the presence of conserved proline-glutamate (PE) or proline-proline-glutamate (PPE) residues near the N-terminus of the predicted proteins coded for by these genes (Glickman and Jacobs, 2001). A large portion of the *M. tuberculosis* genome is involved with lipid biosynthesis. One of the most important classes of genes is the homologues of the fatty acid β-oxidation system of *E. coli*. The *E. coli* fatty acid dehydrogenase (FAD) system consists of the *fad*A, *fad*B, *fad*C, and *fad*E genes. The *M. tuberculosis* genome contains in excess of thirty *fad*D and more than 30 *fad*E homologues.

The presence of multiple homologues of β-oxidation machinery suggests that *M. tuberculosis* assimilates fatty acids as important carbon sources. According to the authors (Cole, et al, 1998), mycobacteria further contain examples of every known lipid and polyketide biosynthetic

system, including enzymes usually found in mammals and plants as well as the common bacterial systems. In total, there are approximately 250 distinct enzymes involved in fatty acid metabolism in *M. tuberculosis* compared with only 50 in *E. coli*.

From the genome sequence it is clear that the tubercle bacillus has the potential to synthesize all the essential amino acids, vitamins and enzyme co-factors. The sequence data also revealed that *M. tuberculosis*, previously known to be a "strict aerobe", codes for several components involved in anaerobic electron transport as well as anaerobic pathways of metabolism. This supported observations made by Wayne in the late 1970's where he demonstrated the ability of the H37Rv strain to grow under a self-generated oxygen gradient developing extremely slowly under anaerobic conditions of growth. Components of several anaerobic phosphorylative electron transport chains are also present, including genes for nitrate reductase (*nar*GHJI), and fumarate reductase (*frd*ABCD).

Cole and colleagues report that based on their combined observations, "it can be deduced that horizontal transfer of genetic material into the free-living ancestor of the *M. tuberculosis* complex probably occurred before the tubercle bacillus adopted its specialized intracellular niche". Prior to the disclosure of the complete genome sequence of H37Rv, very little was known about the molecular basis of virulence of *M. tuberculosis*. This article has singularly catapulted molecular investigations into pathogenesis of *M tuberculosis*, and has been used in amongst other things the design of microarray studies that examine hundreds of genes simultaneously. Re-annotations of the genome have been reported by the same group of authors.

1.7. The Wayne Model

The Wayne model describes the growth of *Mycobacterium tuberculosis* under different conditions of oxygen tension. Wayne and Lin (1982) reported that when *M. tuberculosis* is grown in a detergent containing medium, and the culture is left undisturbed, equilibrium is established between the rate of replication, oxygen diffusion and the rate of bacilli settling at the bottom of the tube. Actively replicating bacilli die rapidly when they are abruptly derived of oxygen (Wayne & Diaz, 1967; Wayne and Lin, 1982). They have however been shown to shift into a stage of dormancy when they are allowed to adapt to gradually decreasing oxygen (Wayne, 1976). This can be linked to the action of the dormancy regulon (Voskuil, 2004).

In his study on "submerged" cultures of *M. tuberculosis*, Wayne (1976) showed that tubercle bacilli which have settled through a self-generated oxygen depletion gradient in unagitated culture tubes undergo an orderly metabolic shift. As they accumulate in the bottom of the tubes they enter a homogenous physiologic state of dormancy. The shift into first stage, designated non-replicating persistence (NRP) stage 1, occurs abruptly at a point when the dissolved oxygen level approached 1%. This micro-aerophilic stage is characterized by a slow rate of increase in turbidity without a corresponding increase in numbers of CFU or synthesis of DNA.

It was demonstrated by Wayne and colleagues, that abrupt transfer of vigorously aerated cultures of *M. tuberculosis* to anaerobic conditions results in rapid death, but gradual depletion of available oxygen permits expression of increased tolerance to anaerobiosis. Those studies used a model based on adaptation of un-agitated bacilli as they settle through a self-generated

oxygen gradient. This is a key concept in understanding the nature of the work presented in this dissertation, as it demonstrates that *M. tuberculosis* requires a "period of adaptation" to changing environmental conditions. The elucidation of the dormancy regulon (Voskuil, 2004) explains these observations. In this regulon a number of genes are selectively expressed in response to changes in environmental stimuli.

In contrast to the rapid depletion of oxygen in vigorously rotated cultures, the cultures subjected to slow magnetic stirring with no detectable disturbance of the surface of the medium had a much slower rate of equilibration with the air, and the headspace exhibited a much slower rate of oxygen depletion. When the oxygen content of the headspace air had been reduced by only 28%, an abrupt deflection in the turbidity curves occurs, and the rate of turbidity increase and headspace oxygen depletion paralleled one another until the oxygen was gone and the exponential increase in turbidity ceased (Wayne and Hayes, 1996). This means that cells were multiplying rapidly until approximately 30% of the oxygen was used up. The increase in turbity (biomass) thereafter was parallel with the oxygen depletion. At this point the turbidity (biomass) did not increase. This indicates that the number of new cells formed is equal to the number of existing cells that were dying. This is typical of the stationary phase in the typical bacterial growth curve.

Unexpectedly, this stage of slow increase in turbidity was not reflected in an increase in CFU. This means that there was an increase in biomass in the liquid culture but this was not detectable on aerobically incubated agar plates. This meant that the fraction of cells responsible for the increased biomass in the liquid media were not able to multiply on agar surfaces.

Wayne suggests that this period during which turbidity increased slowly without significant change in CFU counts, provides a model for studying the ability of tubercle bacilli to survive under conditions that do not support replication. This might be of value since it may resemble conditions encountered in inflammatory or necrotic tissue (Wayne, 1994). At the point where oxygen depletion showed a negative effect on replication, production of GDH (glycine dehydrogenase) began to rise, reaching a peak more than 10-fold higher than baseline and then declining to about half that level. It was previously postulated that this enzyme provides a mechanism for regenerating essential NAD as an adaptation to hypoxic conditions (Wayne and Sohaskey, 2001).

When the dissolved oxygen content of the culture dropped below about 0.06%, the bacilli shifted down abruptly to a stage, designated NRP stage 2, in which no further increase in turbidity was seen and the concentration of glycine dehydrogenase declined markedly.

The ability of bacilli in NRP stage 2 to survive anaerobically was shown to be dependent on having spent sufficient transit time in NRP stage 1. This relates back to the dormancy regulon. It has been suggested that the ability to shift down into one or both of the two non-replicating stages, corresponding to micro-aerophilic and anaerobic persistence, is responsible for the ability of tubercle bacilli to lie dormant in the host for long periods of time, with the capacity to re-activate and cause disease at a later time. A slow depletion of oxygen appears to permit the occurrence of adaptations that favour long-term non-replicating persistence of tubercle bacilli under micro-aerophilic conditions and also enhance the ability of the bacilli to survive under anaerobic conditions. This versatility could account for long-term latency of tuberculosis in the mammalian host (Wayne and Sramek, 1994).

Bacilli also persist in necrotic areas of host tissue which may be acellular and/or avascular and are thus exposed to a very wide range of physiologic conditions (Wayne, 1994). The model described here holds promise as a tool to help clarify events at the molecular level that permit the bacilli to persist under adverse conditions and to resume growth when conditions become favourable. It can also be used for screening drugs for the ability to kill tubercle bacilli in their different stages of non-replicating persistence.

In further studies using the settling bacilli culture model, Wayne and Sramek (1979) demonstrated the presence of antigenic components that were unique to the resting bacilli. In this study, protein extracts were prepared from aerobically growing cells of *M. tuberculosis* and presumed non-replicating cells derived from the sediments of non-agitated cultures. Although both preparations shared a number of antigens, the extracts of the non-replicating cells further contained components that were not present in the aerobic extracts. These antigens were then visualised using immunoaffinity chromatography and immunoelectrophoretic techniques.

These antigens are referred to as the URB (unique to resting bacilli) antigens. This demonstrates that the bacterial cells react differently due to the external conditions to which they are subjected. This is critical to understanding the pathogenesis of tuberculosis. Wayne's Model of culture was chosen as the basis for the experimental design for the project presented here. The advantages of the Wayne model are summarised by Dick T (2001) in his correspondence to the Journal of Antimicrobial Chemotherapy where he states that this model makes the molecular mechanisms of dormancy, experimentally accessible.

1.8. Understanding Anaerobiosis

The word “anaerobic” when directly translated means “without air”. This term is often interpreted as “without oxygen”. There are various terms that define differing states of oxygen availability. Anoxic refers to the absence of oxygen. Hypoxic refers to the presence of traces of oxygen insufficient for cells to function normally. Anaerobic generally refers to conditions where molecular oxygen is absent but it is still present in a bound form through sulphates and nitrates etc. The concept of varying oxygen tensions is extensively covered in studies on biofilms (Yu and Bishop, 1998).

1.9. Redox Potential

Redox chemistry is central to the energy conservation in both photosynthetic processes as well as cellular respiration. Substances vary in their ability to accept or donate electrons. The ability of a substance to accept or donate electrons is referred to as its redox potential. This is designated as the E_O value of the substance. A chemical oxidation is defined as loss of electrons while a reduction refers to a gain of electrons. A hydrogen atom consists of a single proton and a single electron. When it becomes oxidized, the hydrogen atom becomes a proton. However, electrons cannot exist in isolation and therefore redox reactions are also called half-reactions. Hydrogen is the reference standard against which all other redox potentials are measured.

Most molecules can serve as both electron donors and electron acceptors at different times, depending on what other substances they react with. The association between redox reactions and the environment in which cells function is reflected in the concept of the electron tower (Figure 1.1). The electron tower represents a range of redox potentials arranged from the most negative on the top, to the most positive at the bottom. Generally, extremely electropositive conditions favour aerobic processes and extremely electronegative conditions favour anaerobic processes. The reduced redox couple at the top has the greatest potential energy while that at the bottom has the least. The redox couple at the bottom however has the greatest tendency to accept electrons while the opposite holds true for the redox couple at the top. The effect of redox potential on bacterial growth is complicated and is summarized in the following paragraph.

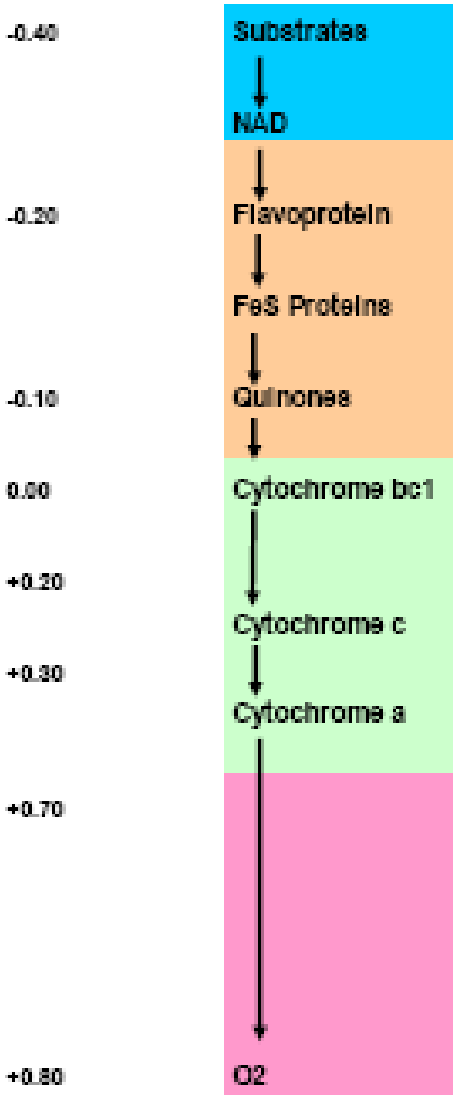


Figure 1.1: Electron Transport Redox Potential Tower (Modified from Brock and Madigan, Biology of Microorganisms, 6th Ed, pg 97)

1.10. ATP

The transfer of electrons between different chemical substances results in the formation of ATP (adenosine triphosphate). ATP is required to drive all major processes in the cell. Many different chemical processes take place at the cellular level e.g. proteins are formed, metabolites are released etc, all of which carry a relative charge. The combination of these different substances results in a net charge that influences the direction of redox reactions. Bacteria have the ability to adjust and counteract extremes in the environment that would adversely affect its survival. One such redox mediator is ferredoxin. It is proposed that the expression patterns of the ferredoxin gene differ in *M. tuberculosis* cells in different stages of metabolism.

Anaerobic conditions are favoured in environments that tend to be electro-negative. It has long been recognized that the anaerobic culture of bacteria has a high reducing character. Many obligate anaerobes will only grow in pre-reduced media. Others will grow in liquid culture with shorter lag periods if a compact inoculum is used and the medium is not disturbed until growth is well established. This is clearly demonstrated for *Mycobacterium tuberculosis* in the case of the Wayne Model. Facultative growth in bacteria implies that bacteria can grow under both reduced and oxidized conditions. Phenotypically, facultative bacterial growth in liquid culture is characterized by floccules that are present throughout the column of broth in a culture vial. This means that discrete levels of conditions exist within the tube that can create this situation of supporting aerobic and anaerobic growth simultaneously.

1.11. The Cell Membrane and Metabolism

1.11.1. Introduction

Free-living bacteria are usually flexible in the way in which they use energy and nutrient sources. Most species possess a range of electron transport chain components, which can be expressed according to environmental changes. Adaptable bacteria that can exploit various sources of

metabolic energy are able to assemble a diverse array of respiratory chains in response to the availability of electron acceptors (Rothery, et al, 2002). The electron transport chain is located in the cell membrane.

Bacterial electron transport chains are very similar in nature to those of mitochondria and photosynthetic systems. The electron transport chain consists of membrane associated electron carriers. They serve to accept electrons from electron donors and pass them on to electron acceptors while conserving some of the energy that is released in the process. There are several types of redox enzymes and proteins involved in electron transport (Brock and Madigan, 1996).

1.11.2. Electron Transport Mediators

The basic description of electron transport components given here has been adapted from Brock and Madigan (1996). NADH dehydrogenases are proteins that are bound to the inside surface of the cell membrane. They accept electrons from NADH and pass two high energy electrons to flavin mononucleotide (FMN) which is a flavoprotein. Flavoproteins contain a derivative of riboflavin. They are capable of accepting hydrogen atoms and donating electrons.

Iron sulphur proteins can vary in their iron content, resulting in varying redox potentials. A common iron sulphur protein is ferredoxin.

Cytochromes are proteins with iron containing porphyrin rings attached to them. They undergo reduction and oxidation through gain or loss of a single electron respectively.

Quinones are lipid soluble substances involved in electron transport. Like flavoproteins, they have the ability to accept hydrogen atoms and donate electrons.

Hydrogen atoms are passed from NADH to FMN, which is typically the first acceptor in the chain. The electron acceptors in the electron transport chain include FMN, ubiquinone (CoQ), and the closely related cytochromes. Cytochrome molecules accept only an electron from hydrogen, resulting in the formation of the positively charged protons. ATP Synthase translocates these protons back into the cell and ATP is formed in the process. Simultaneously, the electrons reunite with protons to form hydrogen atoms, and the chemical union of the hydrogen and oxygen produces water.

Oxygen is the final hydrogen acceptor in the electron transport system in aerobic systems. When no oxygen is available to accept the hydrogen atoms, the last cytochrome in the chain is stuck with its electrons. When that occurs, each acceptor molecule in the chain remains stuck with electrons, and the entire system is halted all the way back to NADH. As a result, no further ATPs are produced through this electron transport system. Most cells of complex organisms cannot live long without oxygen because the amount of energy they produce in its absence is insufficient to sustain life processes. Bacteria however, have existed from before the earth's atmosphere was able to sustain other life forms. Anaerobiosis would therefore have to be one of the earliest survival strategies amongst the prokaryotic kingdom.

1.12. Genes studied

As the biochemical systems involved in aerobic and anaerobic ATP production are complex and involve many different substances, the scope of the work presented here does not allow studying all of these. Therefore a choice needed to be made regarding the genes and their products that would be studied.

1.12.1. Ferredoxin *fdx*A

The *fdxA* gene codes for a ferredoxin product. Ferredoxins function exclusively as electron carriers and do not have classical enzyme functions. They have the ability to absorb and release negatively charged, sub-atomic particles but they do not act as biological catalysts. The term "ferredoxin" was first used by D.C.Wharton of the DuPont Company while describing the "iron protein" obtained from *Clostridium pasteurianum* (Mortenson, et al, 1962). Ferredoxins comprise of iron-sulphur proteins that contain several iron and sulphur atoms involved in electron transfer. Ferredoxin has been reported to function as an electron-mediating catalyst for the biological production or utilization of hydrogen by bacteria (Mortensen, et al, 1962; Mortensen, et al, 1963; Valentine et al, 1962, Valentine and Wolfe, 1963).

Early research on bacterial ferredoxin has helped to clarify the concept of low-potential electron transport. In this process, electrons from an electron donor are passed to oxidized ferredoxin with the aid of a specific dehydrogenase which converts oxidized ferredoxin to reduced ferredoxin. In ferredoxin assays performed by the Valentine group, this conversion was observed with a corresponding colour change from dark brown to colourless. Colourless ferredoxin donates its electron to the next molecule in the electron transport chain (ETC). In

this manner, electrons flow from the electron donor to ferredoxin and then to the electron acceptor. Many hydrogen-producing bacteria oxidize ferredoxin by the hydrogenase reaction and evolve large quantities of hydrogen gas (Valentine, et al 1963, Mortensen et al, 1962, Valentine, 1964).

Ferredoxins in *M. tuberculosis* H37Rv are involved in several reaction types both as a product and as a reactant. The following reactions are examples of some of the reported activity of ferredoxin in H37Rv (Biocyc A & B, 2008).

- **Glutamate degradation VII (to butyrate)** :

  oxidized ferredoxin + coenzyme A + pyruvate = **ferredoxin** + CO_2 + acetyl-CoA
- **Glycolysis III** :

  oxidized ferredoxin + coenzyme A + pyruvate = **ferredoxin** + CO_2 + acetyl-CoA
- **Pyruvate fermentation to acetate** :

  oxidized ferredoxin + coenzyme A + pyruvate = **ferredoxin** + CO_2 + acetyl-CoA
- **Sulphate assimilation III** :

  H_2O + oxidized ferredoxin + H_2S = 3 **ferredoxin** + SO_3^{-2}
- It may also be involved in reactions as a reactant.
- **Glutamate degradation VII (to butyrate)** :
- **Ferredoxin** + 2 H^+ = H_2 + oxidized ferredoxin
- **Reductive acetyl coenzyme A pathway** :

  5,10-methylene-THF + **ferredoxin** = 5-methyl-THF + oxidized ferredoxin
- Further to the above ferredoxin may be involved in reactions without pathways.

- oxidized ferredoxin + ammonia = 3 **ferredoxin** + NO_2^-
- 2 oxidized ferredoxin + 2 L-glutamate = 2 **ferredoxin** + α-ketoglutarate + L-glutamine

It has been reported that metronidazole is active against dormant cells of *M. tuberculosis*. Metronidazole is activated via the transfer of a single electron from ferredoxin to the nitro group of the drug, resulting in the formation of a free radical (Quon, et al, 1992). Therefore, metronidazole has targeted action against anaeroboes. It has no activity against actively dividing *M. tuberculosis* cells (Wayne and Sramek, 1994), suggesting the dormant state to be equivalent with anaerobic metabolism.

1.12.2. Naphthoate Synthase *menB*

This enzyme, coded for by *menB*, plays a key role in the production the respiratory quinones of bacteria. Bacterial respiratory quinones can be divided into two groups. Firstly benzoquinones, which are also known as ubiquinones (UQ) and secondly, the group contains the naphthoquinones which include menaquinone (vitamin K_2; MK or MK-*n*) and demethyl-menaquinone (DMK or DMK-*n*). Animal cells synthesize only UQ, while MK is obtained from the diet (Soballe and Poole, 1999). In prokaryotes, MK plays a role in the anaerobic biosynthesis of pyrimidines, in addition to its role in electron transport (Gibson & Cox, 1973).

Naphthoate synthase has been reported byTruligo and colleagues (2003) as a potential drug target. This key enzyme is involved in the pathway leading to the formation of the membrane soluble menaquinone (vitamin K). The potential use as a drug target is borne from the fact that humans do not have a vitamin K pathway. They obtain their vitamin K from the diet and through a mutualistic relation with intestinal flora. A drug that would inhibit the action of this enzyme would therefore potentially inhibit the electron transport process. Menaquinone is the sole quinone in mycobacteria making its production pathway extremely attractive as a drug target.

Some of the earliest work on menaquinone was conducted as nutritional studies in animals. The discovery of vitamin K biosynthesis in bacteria came from detailed studies of the nutrition of chickens. In the early 1930's McFarlane and colleagues (McFarlane et al, 1931) investigated the fat-soluble vitamin requirements in chicks. The basic foods studied were either fish (white non-oily fish) or meat (from which the fat was partially extracted). If the fat was extracted from

either food source, the animals demonstrated poor growth and, if injured, bled to death. The bleeding condition was most pronounced when ether extracted fish meal (rather than meat) was used. Almquist and Stokstad in 1935 reported further experiments with fish meal which had been moistened and allowed to putrefy, with similar findings to Halbrook's work.

The water-moistened fish was examined by Halbrook in 1935, who found that it prevented hemorrhagic symptoms in chicks. Dried water extracted nutrients had the same effect. However, if this powder was further treated with alcohol hemorrhagic symptoms did occur. Halbrook concluded that the protective action of water extracted fish meal "can only be explained by bacterial action, especially since mere moistening of the fish meal had a similar effect, whereas water extraction followed by moistening with ethyl alcohol to prevent bacterial action failed to prevent the symptoms from occurring." This work on chicks led to the discovery of vitamin K synthesis in bacteria (Bentley and Meganathan, 1982).

Most of the naphthoquinones found in nature are produced by plants and fungi, and the majority of these are derived by "'polyketide" pathways (Bentley, 1975). Some bacterial naphthoquinones are also produced in this way, e.g. a *Streptomyces* species (McGovern and Bentley, 1975). While research on Vitamin K in animals was being pursued, Twort and Ingram in their early work on the cultivation on *Mycobacterium paratuberculosis* (also known as Johne's Bacillus), recognized clearly that not all bacteria and not all strains of the same bacterial species were sources of "Essential Substance", now known as vitamin K (Twort and Ingram, 1912 and 1913). Production of this growth factor by *M. phlei* to stimulate the growth of *M. paratuberculosis* was found to be dependent on culture media components. For example,

glycerol-containing media were good substrates for Essential Substance production. Since each assay that requires growth of *M. paratuberculosis* takes about 2 months of incubation, progress was slow (Bentley and Meganathan, 1982).

These workers aimed at producing a vaccine for Johne's disease, a chronic, specific enteritis affecting cattle. They postulated that the acid fast bacillus (Johne's bacillus) present in the intestinal mucous membrane and mesenteric glands of infected animals was the causative agent. All attempts to cultivate the organism on ordinary, artificial laboratory media had failed. They considered that these failures must be due, either to some substance in the medium acting as an inhibitor, or to the absence of a substance essential for its vitality and growth (Bentley and Meganathan, 1982). Since related bacilli grew in ordinary laboratory media, the inhibitor option seemed highly improbable. They therefore concluded that the failure to grow the bacillus must be due to the absence of some necessary nutrient. When dead human tubercle bacilli were incorporated into an egg medium, Johne's bacillus was grown in culture for the first time. This result was announced by Twort in a preliminary fashion in 1911 (Bentley and Meganathan, 1982).

Extraction of *Mycobacterium phlei* cells with ethanol yielded a yellowish mass which supported growth. On further extraction with chloroform, the best stimulation was obtained with the material insoluble in chloroform. As Hanks has noted, this work provides the first discovery of a biological growth factor, a vitamin, for a microorganism (Hanks, 1966). The following pathway illustrates the formation of menaquinone and the role of the enzyme coded for by

*men*B in *M. tuberculosis* H37Rv. This pathway (Figure 1.2) was obtained online from the Biocyc Database (Biocyc C, 2008).

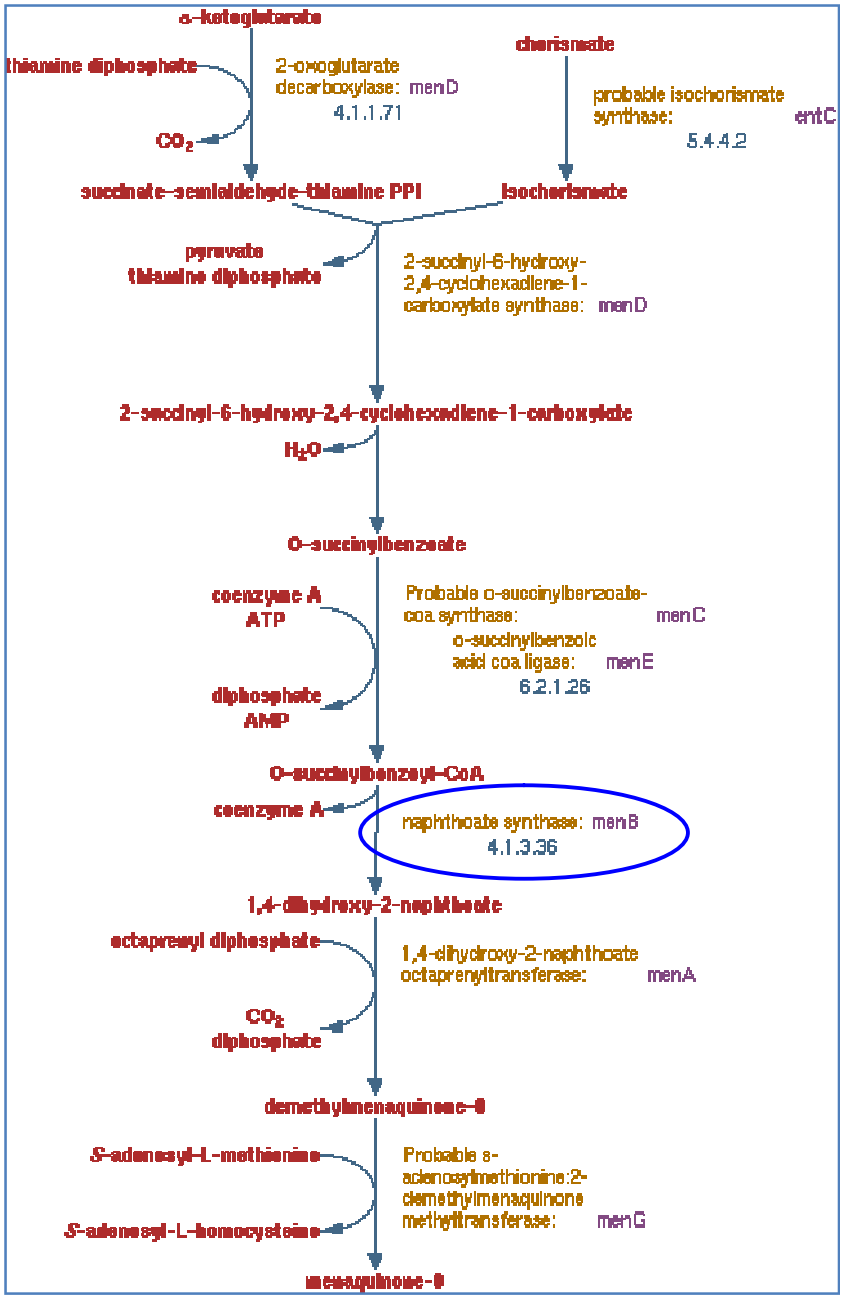


Figure 1.2: Pathway for the formation of menaquinone in *M. tuberculosis* H37Rv

1.12.3. Cytochrome B reductase subunit *qcr*B

As the name indicates, cytochromes are cellular pigments that absorb light at specific wavelengths via their heme cofactor. Since 1997, the application of spectroscopic techniques has resulted in the accumulation of a large amount of data on the biogenesis of cytochromes and cytochrome complexes (Thoney-Meyer, 1997).

Cytochromes are electron transfer proteins that carry heme as a prosthetic group. Their redox function is intimately related to the valence change of heme iron (Thoney-Meyer, 1997 Yamanaka, 1992). Most bacterial cytochromes function either in photosynthetic electron transport or in aerobic and anaerobic respiration, whereby ATP formation is coupled to the oxidation of reduced substrates such as organic substances, hydrogen, reduced sulphur, or metals. Since respiration takes place in the cytoplasmic membrane, cytochromes are often localized in this compartment. In addition, they are found in the periplasmic space, where their electron transfer function is connected with that of membrane-bound cytochromes. In many cases, cytochromes are assembled in multi-subunit enzyme complexes that may contain additional non-heme cofactors. However, they can also occur solitarily, e.g. when they function as mobile, periplasmic electron shuttles between other membrane-bound respiratory complexes and their reaction partners.

The cytochrome *bc*1 complex (quinol:cytochrome *c* oxidoreductase) is an integral membrane protein complex that functions as part of an electron transfer chain by passing electrons from quinol in the membrane to a *c*-type cytochrome. Coupled to electron transfer is the transport of protons across the membrane, and consequently, the enzyme contributes to the proton motive force. The complex has been isolated from mitochondria and several bacteria (Yu and Brun, 1998).

Cytochrome *b* is an integral membrane protein with nine predicted hydrophobic α-helices, of which eight appear to span the membrane bilayer, with two heme molecules bound non-covalently to conserved histidines in transmembrane helices II and IV. The last three transmembrane helices represent a protein domain that in chloroplast or cyanobacterial *bc*1-like complexes occurs as a separate subunit IV. The genes encoding bacterial *bc*1 complexes have been cloned and sequenced from a variety of species (Brand et al, 1992; Davidson and Daldal, 1987, Gabellini and Sebald, 1986; Kallas, et al, 1998). They are called *fbc*, *pet*, or *qcr* genes. The bacterial complex III genes are organized in one operon or, in the case of cyanobacteria, two operons, and they are present in different combinations. Various respiratory complexes consist of cytoplasmically active enzymes that are anchored to the membrane by one or two subunits which often are *b*-type cytochromes. These subunits connect the cytoplasmic redox reactions with electron transport to or from the quinone and quinol pool in the membrane.

1.12.4. Fumarate Reductase *frdA* and Succinate Dehydrogenase

These components are discussed together, as they have similar structure and related functions in terms of the TCA (tricarboxylic cycle). Succinate dehydrogenase and fumarate reductase are enzyme complexes similar both in composition and in subunit structure. *In vivo* however, they normally catalyze their enzymatic reactions in opposite directions (Ackrell et al, 1992). Succinate dehydrogenase plays an important role in cellular metabolism and directly connects the Krebs cycle with the aerobic respiratory chain (Hagerhall, 1997). Fumarate reductase catalyzes the final step in anaerobic respiration with fumarate as a terminal electron acceptor (Kroger A, 1978). These enzyme complexes are thus examples of the high evolutionary adaptation of organisms to specialized environments (Hagerhall, 1997).

There has been plenty of research on these components conducted in *E. coli*. Singh and Gosh (2006) reported that the biochemical reactions of the *E. coli* TCA cycle and that of *M. tuberculosis* were theoretically identical. It stands to reason then that findings in *E. coli* may shed some light on the metabolic mechanisms of *M. tuberculosis*. This is important to note as this central metabolic process is far easier to study in *E. coli* than the slow growing *M. tuberculosis*.

At the beginning of life the atmosphere was not able to support aerobic life forms. It is only due to the oxygen generated by the ancient cyanobacteria that the atmosphere eventually evolved to be able to support what we know today as higher life forms. Microorganisms that existed at the time were almost exclusively anaerobic (Margulis and Schwartz, 1998). After the accumulation of oxygen from this photosynthetic bacterial metabolism (Summons *et al.,* 1999; Brocks *et al.,*

1999), organisms began to use the citric acid cycle in the oxidative direction. Srinivasan and Morowitz (2006) show through deductive reasoning that the reductive TCA therefore did precede the classical oxidative TCA. Smith and Morowitz (2004) discuss the evolutionary path of metabolism with the reductive TCA as a central process from which other complex biological reactions could have developed.

The TCA cycle is also known as the Krebs cycle. This cycle is the foundation of most intermediary metabolism. It also has been identified as having a central role in microbial survival in hostile and difficult environments that are progressively nutrient-starved and oxygen-depleted (Gomez and McKinney, 2004; Voskuil *et al.*, 2004; Wayne and Sohaskey, 2001). The model that our study is based on is a reflection of such a situation, as the liquid media is sealed and left undisturbed over an extended period of incubation. This results in gradual starvation and oxygen depletion.

An organism that utilizes multiple energy sources must be able to maintain the balance of oxidative and reductive reactions in the metabolic scheme. Reducing agents involved in ATP formation are generated via aerobic conditions through glycolysis, the Krebs cycle, and β-oxidation pathways. These reducing agents could be re-oxidized efficiently through the electron-transport chain, terminating with electron transfer to oxygen. However, under oxygen-limiting micro-aerophilic and anaerobic conditions, severe restrictions may be imposed on the redox potential, leading to redox imbalance that results in accumulation of the reducing equivalents to toxic levels (Srinivasan and Morowitz, 2006). Because tuberculous lesions are enriched in CO_2 (Haapanen, et al,1959), the reductive TCA cycle, with its ability to obtain

carbon solely from CO_2, may allow these reducing equivalents to be used efficiently in the anabolic mode. Since *M. tuberculosis* has both the key enzymes for the reverse TCA (rTCA) cycle, a fully functional cycle in the reverse direction may be able to obtain carbon in the relatively reductive environment, maintaining replication at a low level that facilitates persistence anaerobically. It has been already reported that this may well be a common principle in survival strategies among bacteria that cause persistent infections like Salmonella and Helicobacter (Monack, et al, 2004; Srinivasan and Morowitz 2006).

In their review on carbon metabolism in intracellular bacteria Munoz-Elias and McKinney (2006) use *M. tuberculosis* to explain the role of the TCA in survival and persistence, with particular emphasis on the survival in macrophages. They also report an evolutionary trend with a limited role of the TCA in bacteria and a more extensive use of this process in eukaryotic cells. They give a detailed explanation of the role of the different enzymes in the cycle as well as the unique features of the TCA in *M. tuberculosis*. The ability of a bacterial species to use the processes of the TCA in both directions is significant in their ability to persist. In summary, the oxidative TCA generates intermediates that are used in other metabolic pathways. As an example, the α-ketoglutarate intermediate is required for the formation of the membrane soluble menaquinone, (see previous section on Naphthoate Synthase). Further to the above, hydrogen and hydrogenated reducing intermediates are formed and these intermediates drive the electron mediated formation of ATP. The reverse TCA produces storage fuels that can be used to form ATP under challenge situations.

Srinivasan and Morowitz have illustrated the processes in both directions in their paper on ancient genes in persistent microbial pathogens. The reverse reaction (fumarate to succinate) occurs in the reverse or reductive TCA and the reaction is catalyzed by fumarate reductase. Carbon dioxide is taken into the pathway and recycled to form storage fuel intermediates (Srinivasan and Morowitz, 2006). Anishetty, et al (2005) used advanced bioinformatics and theoretical modelling to investigate metabolic pathways of *M. tuberculosis.* They list fumarate reductase and succinate dehydrogenase as potential drug targets in *M. tuberculosis*. Fumarate reductase is particularly earmarked because of its role in anaerobic electron transport processes.

1.12.5. The Bacterial Ribosome: rrs gene

The number of 16S RNA gene copies per genome equivalent was shown to remain stable throughout different phases of *M. tuberculosis* growth *in vitro* (Desjardin, et al, 2001). Therefore, the number of copies of this gene per volume of culture medium or clinical specimen can be extrapolated into the concentration of bacterial cells. Shi, et al (2003) showed that 16S RNA gene copies showed a linear correlation with the number of colony forming units. The relationship between the increase in CFU and corresponding 16S transcripts was defined as a linear relationship defined by the formula : $y = 10457x + 3.1093$. The correlation co-efficient for this comparison (R^2) was reported as 0.9539.

1.13. Current Investigations, Techniques and Technologies

To the modern microbiologist a host of techniques are available to conduct studies at both the genomic and proteomic level. This allowed for the detection and analysis of mechanisms of resistance, the development of genotyping systems as well as the analysis of pathways of pathogenesis. Proteomic studies enable scientists to examine functional gene products that play a role in survival and pathogenesis of the tubercle bacilli. An early example of this is the study of Wayne and Sramek (1979) that identified what they termed the URB antigen, which was identified in cells cultured in broth incubated without shaking.

More recent proteomic investigations, like the work of Stark and colleagues (2004) show the differences in protein profiles of cells grown under different conditions. They show distinct differences between cells grown under aerobic and anaerobic conditions. Voskuil and colleagues (2004) described the expression profile of the dormancy regulon using microarray technology. The regulon consists of 48 genes that are strongly induced over a period of 20 days. They concluded that this dormancy regulon facilitates a long transition into the anaerobic state. The dormancy genes appear to encode functions previously described for the Wayne model as well as functions that may be useful during extended periods of dormancy. In addition to the hypoxia response, they also demonstrated that the dormancy regulon is induced by nitric oxide in the early adaptation phase to non-replicating persistence.

Gene expression studies are important in the analysis of cellular processes. Real-time reverse transcription PCR is a recognized technique for quantifying mRNA in biological samples. Benefits of this procedure over conventional methods for measuring RNA include its

sensitivity, large dynamic range, as well as the potential for high throughout and accurate quantification (Hugget, et al, 2005). While a normal PCR reaction provides information on whether a gene is present or not, it cannot be used to determine whether this was due to presence of intact cells, DNA from dead cells or free DNA. On the other hand, the detection of gene expression indicates the presence of viable cells. Gene expression analysis is a complex application that can be executed by detecting the mRNA transcript directly or by a 2-step process that involves extraction of RNA converting it to complementary DNA and then detection of the target DNA by real time PCR. This is the method applied in the study presented here.

Section 2

Methods

2. Methods

2.1. Objective

Mycobacterium tuberculosis is still widely accepted as a strict aerobe, despite the mounting evidence to dispute that assertion. Our objective is to determine if the infectious organisms *in vivo* are indeed in an anaerobic state of metabolism, and to determine what electron transport components add to its ability to persist under such challenge conditions.

2.2. Introduction

This study is composed of two main experiments. Firstly, we compared the number of *M. tuberculosis* organisms in clinical specimens that grew on solid media with those that grew in liquid media. Secondly, we studied the role of selected genes involved in metabolism during growth of *M. tuberculosis* under oxygen limiting conditions. In addition, we attempted to detect the expression of these genes directly in sputum. Figure. 2.1 summarizes these two experiments.

This section describes the methodology concisely, and detailed work instruction and protocols are available in Appendix A as ondicated below.

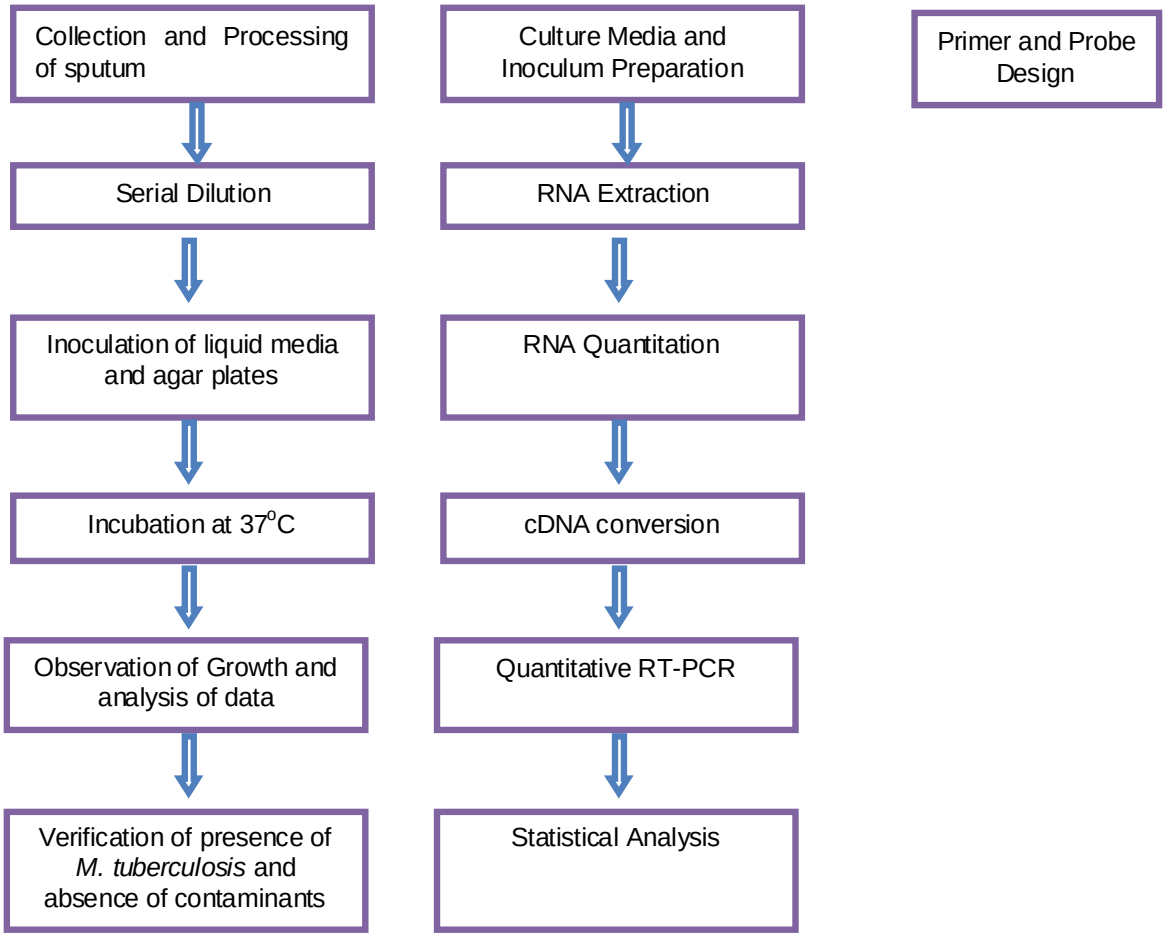


Figure 2.1 Schematic Overview of Methodology

2.3. Bacterial Strain and Culture

The laboratory strain *M. tuberculosis* H37Rv (ATCC 27294) was used for the in vitro gene expression studies. This strain's genome sequence has been annotated and compared with functional genome analysis of other bacterial species. It is the reference strain used in most experimental studies.

Middlebrook 7H9 supplemented with commercially available OADC (oleic acid, albumen-dextrose, catalase; Difco) according to the manufacturer's instructions and made selective by means of antibiotics. This was used in all experiments. Middlebrook 7H11 agar was prepared with similar antibiotics and supplements for assessment of sputum specimens (Appendix A Section 1).

2.4. Quantitative Sputum Cultures

2.4.1. Sputum Collection and Processing

Sputum specimens were collected from consenting patients enrolled in a clinical trial comparing 2 drug regimens for treatment of pulmonary tuberculosis. This trial was performed by the Clinical Trial Unit of the Medical Research Council, ethics approval no. T183. None of the patients were previously treated for tuberculosis. Five patients with positive Auramine stained smears were used for this experiment.

2.4.2. Serial Dilution Preparation

Specimens were homogenized by shaking with glass beads for 30 minutes. A commercially available ditheiotritol solution (Sputasol®, Oxoid) was added at a 1:1 ratio. This mixture was vortexed for 20 seconds following which 12 ten-fold serial dilutions were made in

Middlebrook 7H9 broth. From each dilution 200 µL aliquots were plated out on Middlebrook 7H11 agar. Middlebrook 7H9 broth cultures (10 mL in 15 mL tubes with a diameter of 10 mm, Fig 3.3) were also inoculated with 200 µL of each dilution. Agar and broth cultures were prepared in triplicate.

2.4.3. Incubation of inoculated media

Plates were sealed in gas permeable plastic bags while the tubes were tightly sealed with screw-caps. Both plates and tubes were incubated at 37^{o}C for 10 weeks. Plates were inspected for growth after 3, 6, 8 and 10 weeks of incubation. The liquid cultures were examined for growth at the same time points as the plates without disturbing the liquid.

2.4.4. Determination of Organism Density

The organism density in the sputum was determined according to Fisher and Yates (1963) using the following formula

$$\log \lambda = x \log a - k$$

Where:

x = mean fertile level (the total number of tubes or plates with growth divided by the number of replicates at each dilution level)

a = dilution factor = 10

k = constant derived from the published table

This method was used for both types of growth media.

2.4.5. Confirmation of the Presence of *M. tuberculosis* and Absence of Contaminants

To determine whether the growth observed was indeed *M. tuberculosis* a PCR for the detection of the IS*6110* insertion sequence (Eisenach et al. 1990) was used. Colonies from the Middlebrook 7H11 agar plates from each dilution showing growth were suspended in sterile phosphate buffered saline (PBS), pH 6.8 with 0.05% Tween 80 and glass beads. The suspensions were vortexed and DNA was extracted by heating 1 ml aliquots at 95^{O}C for 30 minutes. Aliquots of each broth culture showing growth were prepared in the same manner.

The agar plates were inspected for the presence of colonies with morphology not in keeping with *M. tuberculosis* while 200µL aliquots from each tube showing growth were inoculated into blood agar and incubated at 37^{o}C for 24 hours.

2.4.6. Ethics approval

Ethics approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (H125/06).

2.5. Gene Expression Studies

2.5.1. Inoculum Preparation

A log phase culture was established by inoculating 20mL of a culture of *M. tuberculosis* H37Rv into 180ml fresh Middlebrook 7H9 broth. This was incubated in a regular incubator for 10 days at 37^{o}C on a horizontally rotating plate. Of this log-phase culture, 2mL was added to each of three series of 15 tubes containing 8mL Middlebrook 7H9 broth. The tubes were placed in a regular incubator at 37^{o}C and left undisturbed till the time-point of RNA extraction.

RNA was extracted from the growth in 3 tubes at day 0 and after 3, 6, 8, 9, 10, 11, 12, 13, 14 and 15 weeks of incubation.

2.5.2. Primer and Probe Design

Primers and probes were designed for use with the ABI PRISM 7000SDS. For each selected gene the sequence was downloaded from three different databases (Research Tools, 2006; Tuberculist, 2006; Sanger, 2006) and compared in GeneDoc software (GeneDoc, 2006, Appendix A Section 2). Since the entire gene sequence was identical from each database, the entire gene sequence was uploaded in primer express software version 1.1 to generate primer-probe combinations. A section consisting of 200 bases was selected that included the region containing the highest ranking primer-probe combination. This was uploaded into Assay File Builder Software (Applied Biosystems Support, 2006) and sent to Applied Biosystems for optimal custom assay design. The sequences are shown in Table 1. Products of the genes investigated are represented in Figure 2.2.

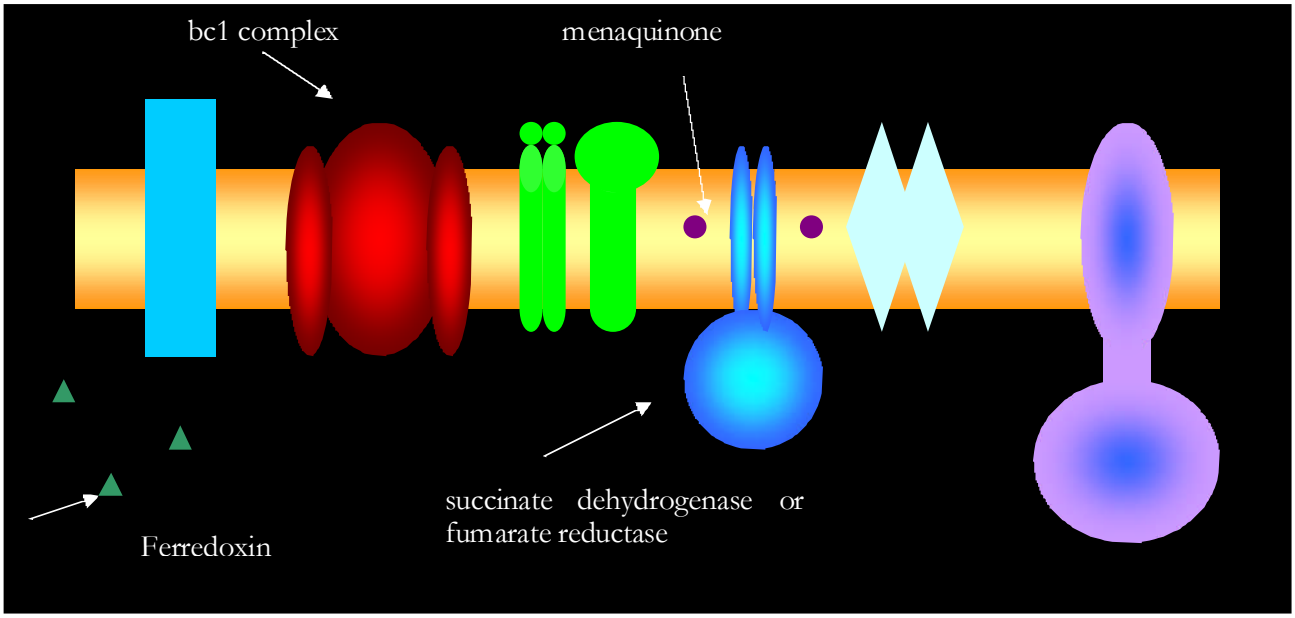


Figure 2.2: Electron transport components of interest in the cell membrane

Table 1: Primer and Probe Sequences

Sequences for *men* B	
Forward Primer	**CCGCCACGTCGACGAT**
Reverse Primer	**GCACTTCAGGGCGGTTGA**
Taqman MGB Probe	**ACGCCACCCGCACCGT**

Sequences for *fdx* A	
Forward Primer	**CGACGAGTGCGTGGATTGT**
Reverse Primer	**GGTAGATCGCCTTCCCAGTAGAT**
Taqman MGB Probe	**CCGGTTTGCACGCACC**

Sequences for *qcr* B	
Forward Primer	**CGTTGGTGTGGTTCCAGAAG**
Reverse Primer	**CGCCGACGACGTTGTG**
Taqman MGB Probe	**CACACCCAGTTCCC**

Sequences for *frd* A	
Forward Primer	**CCCAACACAACATCGTGGTTATC**
Reverse Primer	**GGAAACGATCGCCACATCCA**
Taqman MGB Probe	**ATTGCGATAGCCGAAACC**

Sequences for *sdhA*	
Forward Primer	**GCTGGATTTGGCTTTGACTCAA**
Reverse Primer	**CCGGTCGCTAGCTCG**
Taqman MGB Probe	**TGGCGTGAAAGACATGGATGT**

Sequences for 16S	
Forward Primer	**GGGTGACGGCCTACCAA**
Reverse Primer	**GCCGGACACCCTCTCA**
Taqman MGB Probe	**CCGGCTACCCGTCGTC**

2.5.3. RNA extraction, conversion to cDNA and quantitation of products

A combination of physical and chemical methods was used to extract the RNA from the mycobacterial cells. The RNA was then converted to cDNA for further evaluation. Broth cultures were centrifuged (20 min. 4500 x g) and after removal of the supernatant, the deposit was resuspended in 2 mL Trizol Reagent ® (Invitrogen, USA). The suspension was then transferred into an Eppendorf tube containing 300 µg silica beads with a diameter of 150-212 µm (Sigma, USA) and vortexed to disrupt the cells.

The efficiency of cell disruption was verified microscopically after Ziehl Neelsen staining (Fig. 3.4).

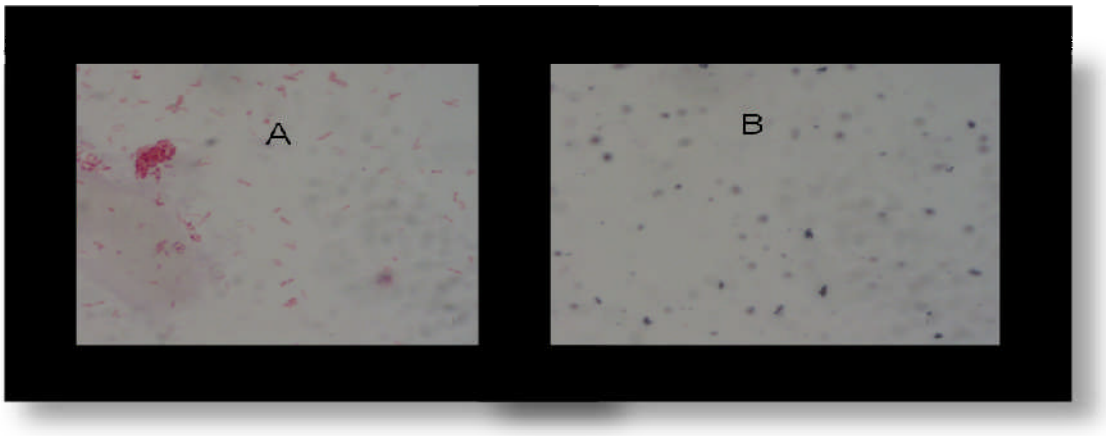


Figure 2.3a Ziehl Neelsen stain of vortexed H37Rv cells. And **Figure 2.3b Ziehl Neelsen Stain of H37Rv vortexed with glass beads and Trizol®.**

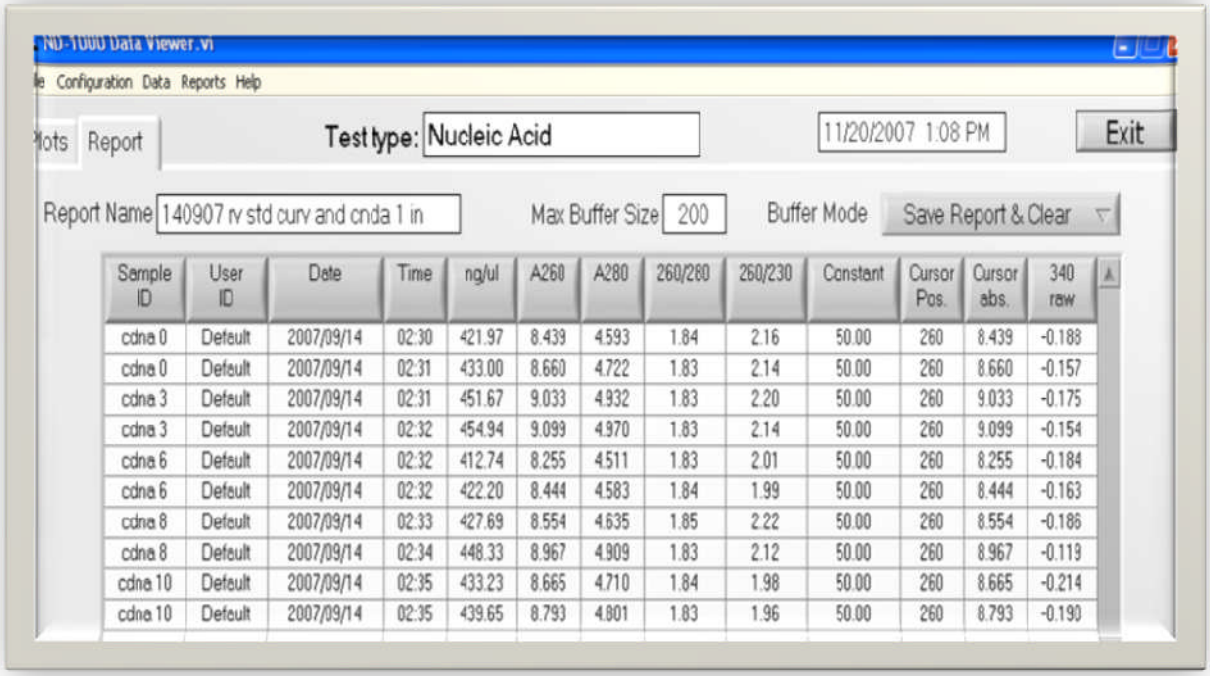
A detailed protocol for RNA extraction is available in Appendix A Section 3.

The extracted RNA was quantified by means of the Nanodrop-1000 (NanoDrop Technologies, Wilmingon USA). High-Capacity-cDNA Archive Kit (Applied Biosystems, Worrington, Cheshire, UK) was used to convert 2µg of RNA from each RNA extract into cDNA. PCR was conducted in 100µL reaction volumes and detailed steps are given in Appendix A Section 4. Quantities containing 2µg of RNA used for reverse transcription are given in Table 2.

Table 2: Quantities of RNA used to ensure 2ug conversion per reaction.

sample name	ng/ul Replicate 1	ul RNA required for 2ug	Nuclease free Water to make 50ul volume	ng/ul Replicate 2	ul RNA required for 2ug	Nuclease free Water to make 50ul volume	ng/ul Replicate 3	ul RNA required for 2ug	Nuclease free Water to make 50ul volume
DO	90.16	22.18	27.82	82.60	24.21	25.79	66.60	30.03	19.97
Wk3	102.69	19.48	30.52	88.62	22.57	27.43	121.64	16.44	33.56
Wk6	179.24	11.16	38.84	154.22	12.97	37.03	189.98	10.53	39.47
Wk8	153.66	13.02	36.98	182.54	10.96	39.04	155.55	12.86	37.14
Wk9	157.30	12.71	37.29	136.21	14.68	35.32	148.32	13.48	36.52
Wk10	397.78	5.03	44.97	485.62	4.12	45.88	422.27	4.74	45.26
Wk11	207.14	9.66	40.34	175.34	11.41	38.59	263.36	7.59	42.41
Wk12	161.96	12.35	37.65	144.64	13.83	36.17	178.30	11.22	38.78
Wk13	148.63	13.46	36.54	165.65	12.07	37.93	188.89	10.59	39.41
Wk14	186.16	10.74	39.26	199.30	10.04	39.96	241.10	8.30	41.70
Wk15	164.33	12.17	37.83	144.60	13.83	36.17	198.99	10.05	39.95

The efficiency of the cDNA conversion was evaluated by comparing the cDNA quantities.Figure 2.4 shows that the quantities were similar after the conversion. The quantity of cDNA was measured using the same technology (Figure 2.4: column 5).



Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
cdna 0	Default	2007/09/14	02:30	421.97	8.439	4.593	1.84	2.16	50.00	260	8.439	-0.188
cdna 0	Default	2007/09/14	02:31	433.00	8.660	4.722	1.83	2.14	50.00	260	8.660	-0.157
cdna 3	Default	2007/09/14	02:31	451.67	9.033	4.932	1.83	2.20	50.00	260	9.033	-0.175
cdna 3	Default	2007/09/14	02:32	454.94	9.099	4.970	1.83	2.14	50.00	260	9.099	-0.154
cdna 6	Default	2007/09/14	02:32	412.74	8.255	4.511	1.83	2.01	50.00	260	8.255	-0.184
cdna 6	Default	2007/09/14	02:32	422.20	8.444	4.583	1.84	1.99	50.00	260	8.444	-0.163
cdna 8	Default	2007/09/14	02:33	427.69	8.554	4.635	1.85	2.22	50.00	260	8.554	-0.186
cdna 8	Default	2007/09/14	02:34	448.33	8.967	4.909	1.83	2.12	50.00	260	8.967	-0.119
cdna 10	Default	2007/09/14	02:35	433.23	8.665	4.710	1.84	1.98	50.00	260	8.665	-0.214
cdna 10	Default	2007/09/14	02:35	439.65	8.793	4.801	1.83	1.96	50.00	260	8.793	-0.190

Figure 2.4 Screen capture of typical cDNA output data from Nanodrop1000 showing a similar quantity range between the different time points

2.5.4. Quantitative Real Time PCR

Genomic DNA was used to generate standard curves (Talaat et al, 2002). Quantities for the standard curve and a DNA extraction protocol are given in Appendix A, Sections 5 and 6 respectively. PCR reactions were performed in 25 µl volumes in a 96 well Optical Reaction Plate (Applied Biosystems, Worrington, Cheshire, UK) by adding 1.25 µl Primer-Probe-Assay reagent (Applied Biosystems, Worrington, Cheshire, UK) and 12.5 µl Universal Mastermix (Applied Biosystems, Worrington, Cheshire, UK) to 11.25 µl of a 1:5 cDNA solution. Each reaction was conducted in triplicate. Amplicon quantities were determined using a standard curve generated with genomic DNA of *M. tuberculosis* H37Rv. The real-time PCR reactions were conducted using an ABI-7000-SDS. Separate PCRs were performed with each primer-probe assay reagent shown in table 3.1. Gene expression levels were normalized against the 16S ribosomal RNA quantities at each time point. A detailed protocol with instruction on interpretation of graphical data from the ABI 7000 SDS is available in Appendix A section 7.

2.6. Sputum processing for gene expression studies

Specimens were digested and decontaminated by means of the standard NALC-NaOH method (Desjardin et al). The methods for isolation of RNA and for gene expression were the same as described above for bacterial cultures (2.4 and 2.5).

2.7. Statistical analysis

For the growth rate comparisons of clinical specimens on different growth media, the Fischer Yates method of estimating organism density was used (Fischer and Yates, 1963). Statistical

analysis was performed by means of GraphPad InStat V3 ® using the Mann Whitney and non-parametric ANOVA.

For the gene expression studies all tests were conducted in triplicate. Three cultures were used at each time point. Gene copy numbers were determined in SDS 7000 software. Statistical analysis was conducted using 9 datasets for each time point (i.e. 3 experimental sets of triplicate data). InStat V3 using the Tukey Kramer Test or Student-Newman-Keuls Multiple Comparisons Test and Repeated measures ANOVA. Additional statistical analysis was performed using one way ANOVA, and the Bartlett test when indicated. Statistical definitions are given in Appendix A Section 8.

Section 3

Results

3. Results

3.1. Growth Density Comparison in Clinical Specimens

We measured the density of *M. tuberculosis* in sputum of 5 patients with active tuberculosis and compared the values for growth obtained on the surface of agar plates incubated under aerobic conditions and those in standing broth cultures where the organisms grow in an increasingly oxygen depleted environment (Wayne and Hayes, 1996). The individual patient results are shown in Table 3. There was no statistically significant difference in inter-patient variation (P = 0.30) on the plates or in the broth cultures at any of the points of observation.

Table 3. Organism density in sputum from 5 patients measured on agar plates and in broth

	Week 3		Week 6		Week 8		Week 10	
	Plates	**Broths**	**Plates**	**Broths**	**Plates**	**Broths**	**Plates**	**Broths**
Patient 1	1.931	3.573	1.931	3.573	2.237	4.573	2.573	4.931
Patient 2	3.237	5.573	4.573	7.237	4.573	7.237	4.573	7.237
Patient 3	4.237	5.237	4.573	6.237	4.573	6.237	4.573	6.237
Patient 4	3.237	3.237	4.573	5.237	4.573	6.931	4.573	7.237
Patient 5	4.237	4.931	4.573	4.931	4.931	7.573	4.931	7.573

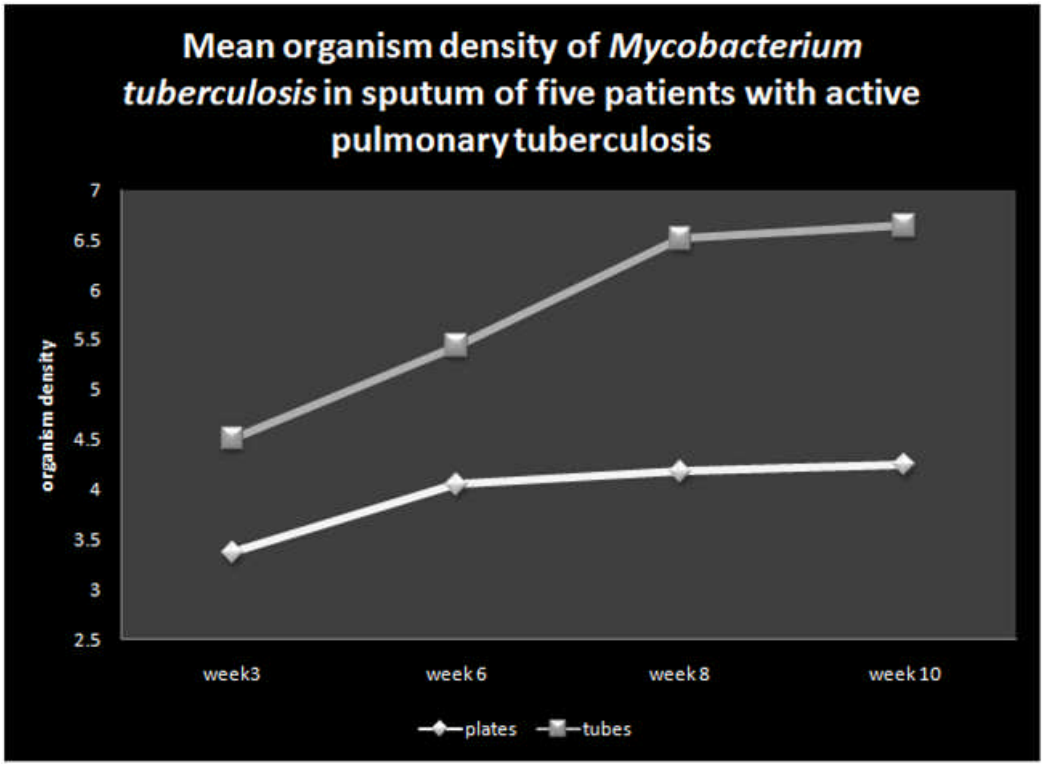


Figure 3.1 Mean Organism Density determined in clinical specimens by the Fischer Yates Method

Growth on the surface of the plates continued till week 6, but came almost to a standstill thereafter. Logarithmic growth in resting broth culture continued at least up till week 8. At the next point of measurement 2 weeks later, only a slight increase was found. This could reflect a further increase with a later decline or a stationary phase from week 8 onwards. Since broth cultures that remain undisturbed for 6 weeks are thought to be oxygen depleted from 1 week onwards, the continued growth in the broth is likely to represent growth of the anaerobic sub-population.

The Mann-Whitney test was used to determine if the difference in organism density between the plates and the broth cultures was statistically significant at each time point. The results are given in Table 4.

Table 4: P-values for significance in differences between the plates and broth cultures at each time point.

	Week 3	Week 6	Week 8	Week 10
Difference between organism density on plates vs broth	**Not Significant**	**Significant**	**Significant**	**Extremely significant**
P Value	**P= 0.0754**	**P=0.0476**	**P=0.159**	**P=0.004**

3.2. Individual Gene Expression Results

Representative graphs and results are given here, from an experimental replicate of *men*B and *qcr*B genes and the 16S quantities for the same run.

The standard curves for all genes in each experiment typically had an m value (gradient) of -3.3 with an R^2 of 0.99 indicating that the data obtained was suitable for quantitative analysis (detailed results for each gene including standard curves for each experiment shown in Appendix B). A typical standard curve is represented below in Figure 3.2.1. Results generated by the ABI 7000SDS are shown as sigmoidal amplification curves (Figure 3.2.2), or Threshold cycle relative to well position. (Figure 3.2.3). This type of graph shows the similarity in

threshold cycle of triplicate results and the equal spacing between threshold cycles of the standard curve are an indication of the suitability of the data for quantitative analysis.

A detailed explanation of these graph types is given in Appendix A Section 7. Detailed real-time PCR results and summary data are available in Appendix B. Detailed statistical analysis is available in Appendix C. Aliquots of PCR products from representative wells in each experiment were electrophoresed to ensure the visual confirmation of the presence of such products. A typical agarose gel of electrophoresed end products is shown below in Figure 3.2.4. and an example for each gene is given in Appendix D. This is not essential in real-ime PCR employing Taqman® Chemistry as this type of probe ensures increased specificity in the reaction. Agarose electrophoresis is a needed process in the event of using methods such as SYBR green, as this method reports a signal in the presence of any double stranded DNA formation including primer-dimer formation.

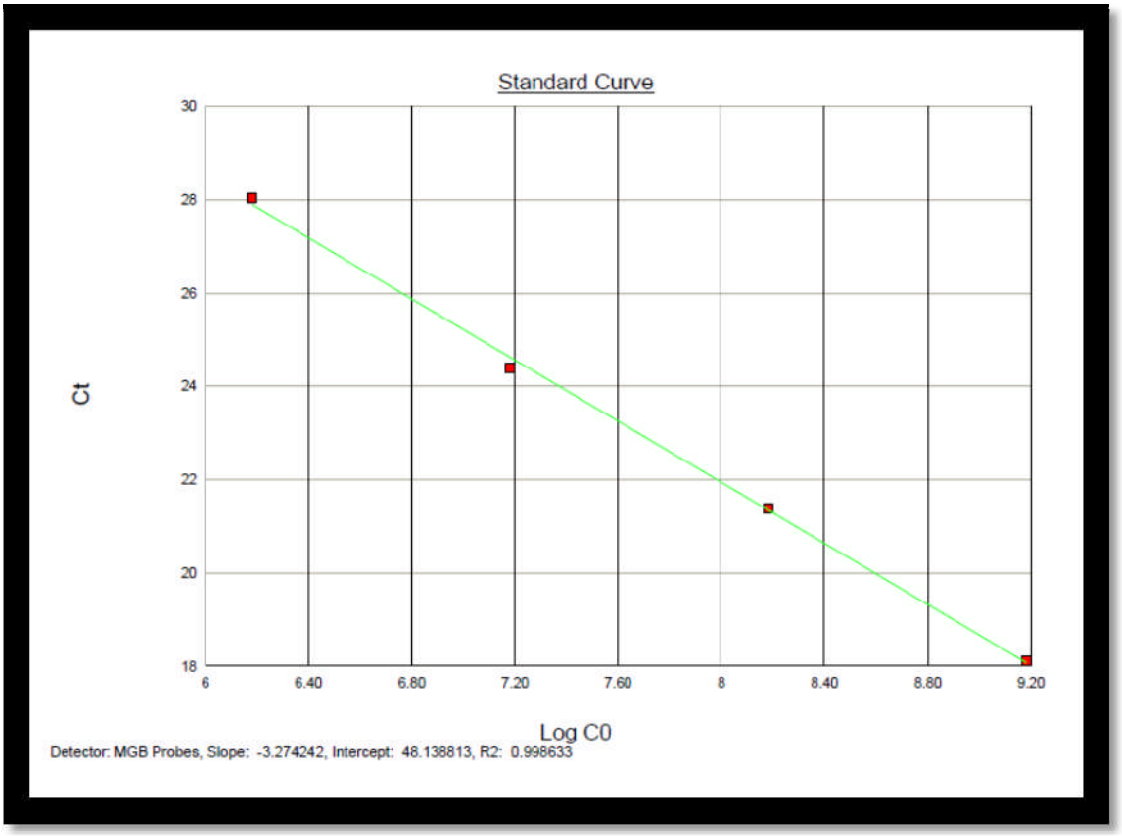


Figure 3.2.1: Representative standard curve generated in quantitative real-time pcr by the ABI 7000SDS

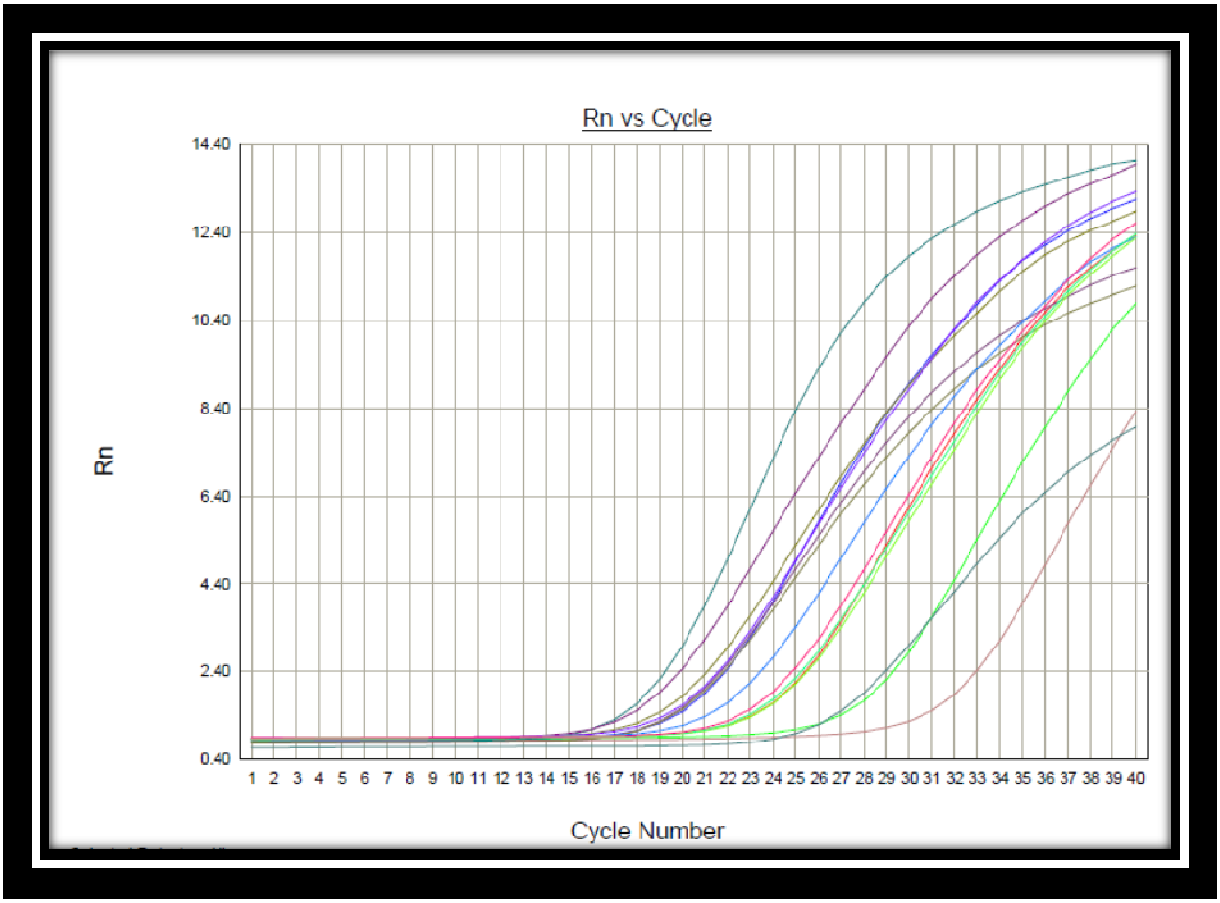


Figure 3.2.2: Amplification curve generated in quantitative real-time pcr by the ABI 7000SDS

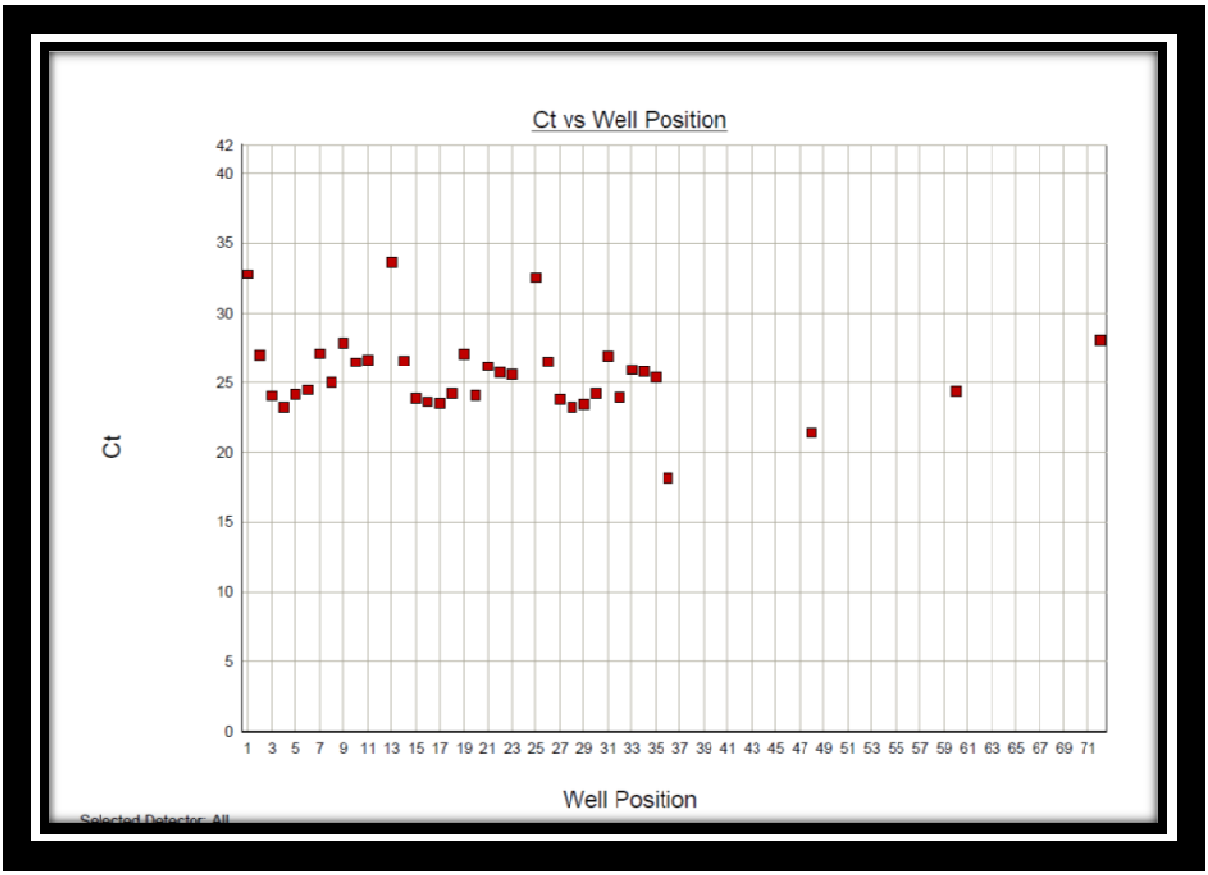


Figure 3.2.3: Representative scatter plot of threshold cycle vs well position generated in quantitative real-time PCR by the ABI 7000SDS

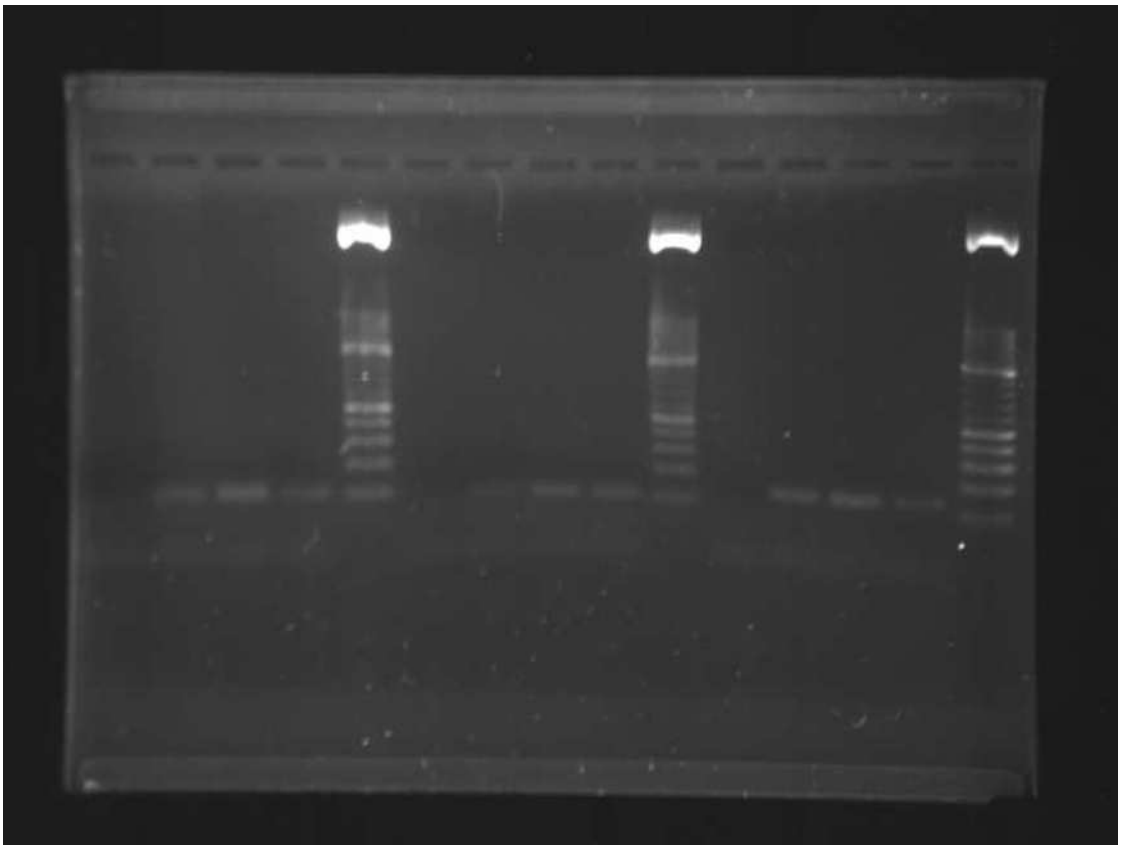


Figure 3.2.4: Agarose Gel (2%) showing the presence of PCR products

3.3. 16S (rrs gene)

The copy number of the 16sR-RNA gene was used to allow metabolic gene activity to be expressed per mycobacterial cell. Figure 3.3 shows the results as an average of 9 measurements at each time point with a 2-point moving average trend line indicated in orange. Summarized experimental data and statistical data is available in Appendices B and C respectively.

While there was a significant increase in the number of RNA copies over the first 9 weeks ($p < 0.0001$), this slowed down afterwards, indicating decreased replication rates. The expression of this gene represents the pattern of biomass of viable cells over the duration of this experiment. The data implies a prolonged stationary-type growth phase under these culture conditions.

Using repeated measures ANOVA it was determined that the P value is < 0.0001, considered extremely significant. The significance in the variance of the means is summarised in Table 3.

The statistical analysis indicates that the variation among the column means is significantly greater than expected by chance. In addition an assumption test showed that the matching was effective. This test uses a second value of F and a different P value. F = 122.48 = (MSindividual/MSresidual) as determined by the InStat Software. This P value is < 0.0001, considered extremely significant. Effective matching (or blocking) results in significant variation among means. With these data, the matching appears to be effective. Thus the variation in data represented in Table 5 is extremely significant.

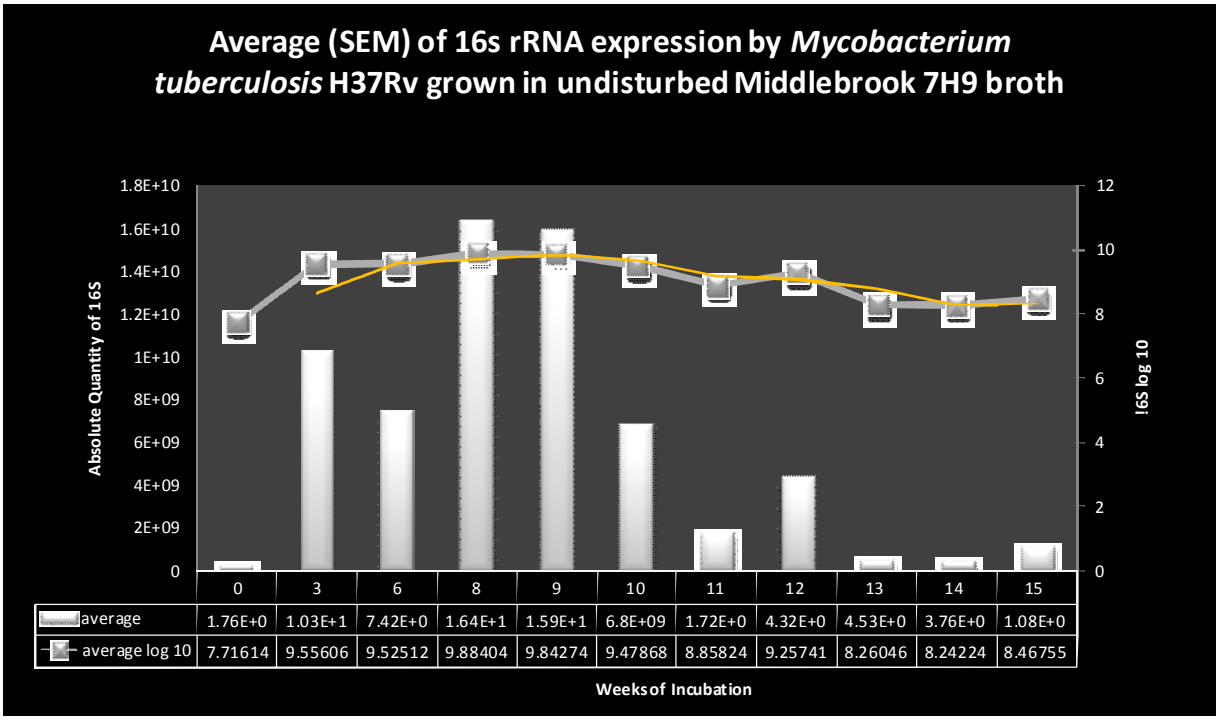


Figure 3.3: Absolute quantification of 16S RNA (log and linear data) as an average of 9 experiments

Table 5: Comparison of the P-values showing a significance in the variation of the means at each time point

	D0	Wk 3	Wk 6	Wk 8	Wk 9	Wk 10	Wk 11	Wk 12	Wk 13	Wk 14	Wk 15
D0		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Wk 3	<0.001		NS	<0.01	<0.05	NS	<0.001	<0.05	<0.001	<0.001	<0.001
Wk 6	<0.001	NS		<0.01	<0.01	NS	<0.001	NS	<0.001	<0.001	<0.001
Wk 8	<0.001	<0.01	<0.01		NS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Wk 9	<0.001	<0.05	<0.01	NS		<0.01	<0.001	<0.001	<0.001	<0.001	<0.001
Wk 10	<0.001	NS	NS	<0.001	<0.01		<0.001	NS	<0.001	<0.001	<0.001
Wk 11	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001	<0.001
Wk 12	<0.001	<0.05	NS	<0.001	<0.001	NS	<0.001		<0.001	<0.001	<0.001
Wk 13	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		NS	NS
Wk 14	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NS		NS
Wk 15	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NS	NS	

3.4. *fdx*A

The average ratio of expression of *fdx*A over 16s rRNA at baseline was -2.409. There was no significant difference in this ratio up till 9 weeks of incubation ($p > 0.05$; Figure 3.4). This is the period of rapid growth as shown in the 16s rRNA based growth curve (Figure 3.3). The *fdx*A/rRNA expression ratio increased significantly between week 9 and 10 ($p < 0.001$) to a level higher than those observed before this time point ($p < 0.001$). This was followed by a decrease to baseline values at week 11 ($p < 0.001$) and an uninterrupted increase up till week 15. The rapid increase of *fdx*A expression between week 9 and 10 coincides with the start of the decline phase in the mycobacterial growth curve (Figure 3.3) while the drop in *fdx*A expression in week 11 precedes a growth spurt at week 12 ($p < 0.001$; Figure 3.4). The 2-point moving average trend line, indicated by a solid black undulating line, indicates three cycles of dips and peaks with an increase in peak levels over time, the highest peak appearing at the last stages of incubation.

To reinforce the significance in the differences between the standard deviations an additional test was performed. ANOVA assumes that the data are sampled from populations with identical standard deviations. This assumption is tested using the Bartlett method. Bartlett statistic (corrected) = 69.973. The P value < 0.0001 obtained through the Bartlett's test suggests that the differences among the standard deviations are extremely significant.
The strong statistical data thus fortify that ferredoxin (*fdx*A) is an extremely significant component in *M. tuberculosis* survival under challenge conditions.

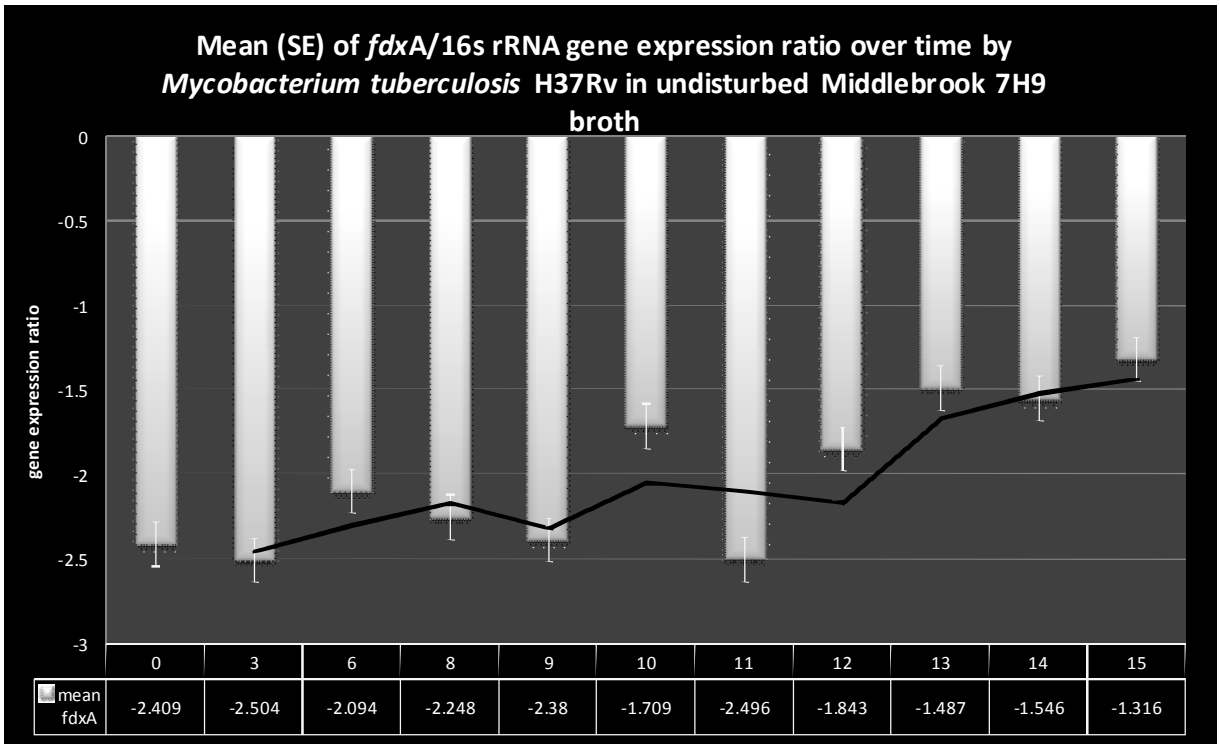


Figure 3.4: Mean gene expression ratios of *fdx*A/16S with 2-point moving average trend line.

Table 6: Comparison of the P-values showing a significance in the variation of the means at each time point

*fdx*A	DAY 0	WK 3	WK 6	WK 8	WK 9	WK 10	WK 11	WK 12	WK 13	WK 14	WK 15
DAY 0		NS	NS	NS	NS	<0.001	NS	<0.001	<0.001	<0.001	<0.001
WK 3	NS		<0.05	NS	NS	<0.001	NS	<0.001	<0.001	<0.001	<0.001
WK 6	NS	<0.05		NS	NS	<0.05	<0.05	<0.001	<0.001	<0.001	<0.001
WK 8	NS	NS	NS		NS	<0.001	NS	<0.05	<0.001	<0.001	<0.001
WK 9	NS	NS	NS	NS		<0.001	NS	<0.001	<0.001	<0.001	<0.001
WK 10	<0.001	<0.001	<0.05	<0.001	<0.001		<0.001	NS	NS	NS	<0.05
WK 11	NS	NS	<0.05	NS	NS	<0.001		<0.001	<0.001	<0.001	<0.001
WK 12	<0.001	<0.001	<0.001	<0.05	<0.001	NS	<0.001		NS	NS	<0.001
WK 13	<0.001	<0.001	<0.001	<0.001	<0.001	NS	<0.001	NS		NS	NS
WK 14	<0.001	<0.001	<0.001	<0.001	<0.001	NS	<0.001	NS	NS		NS
WK 15	<0.001	<0.001	<0.001	<0.001	<0.001	<0.05	<0.001	<0.001	NS	NS	

3.5. *men*B

The gene expression levels, determined from the absolute quantities of *men*B relative to the 16s rRNA at the different time points, are shown in Figure 3.5. There was an increase from baseline till week 15 following an undulating pattern.

The initial 4.3 fold increase between baseline and week 6 ($p < 0.001$) was followed by a plateau between week 6 and 10. This plateau was followed by a significant decrease over the next week ($p < 0.001$) and a subsequent rise between week 11 and 13 ($p < 0.001$) and another area of stability between week 13 and 15, hence showing an undulating pattern of expression in standing liquid culture over 15 weeks. The relative gene expression indicated that at week 15 there was an 85.8 fold increase relative to day 0. The rise in gene expression over the last two weeks of incubation was extremely significant relative to the earlier time points ($P<0.001$).

The 2-point moving average trend line represented as a blue dotted line, shows in a similar fashion to the *fdx*A gene, a pattern of peaks and dips that repeats over the course of incubation with the peak in absolute expression ratios seen in the final stages of incubation.

Both repeated measures ANOVA and one way ANOVA were conducted. The P value is < 0.0001 in both cases and thus extremely significant. Variation among column means is hence significantly greater than expected by chance. In addition the Bartlett statistic (corrected) = 60.150 and the P value is < 0.0001. Bartlett's test suggests that the differences among the SDs is extremely significant.

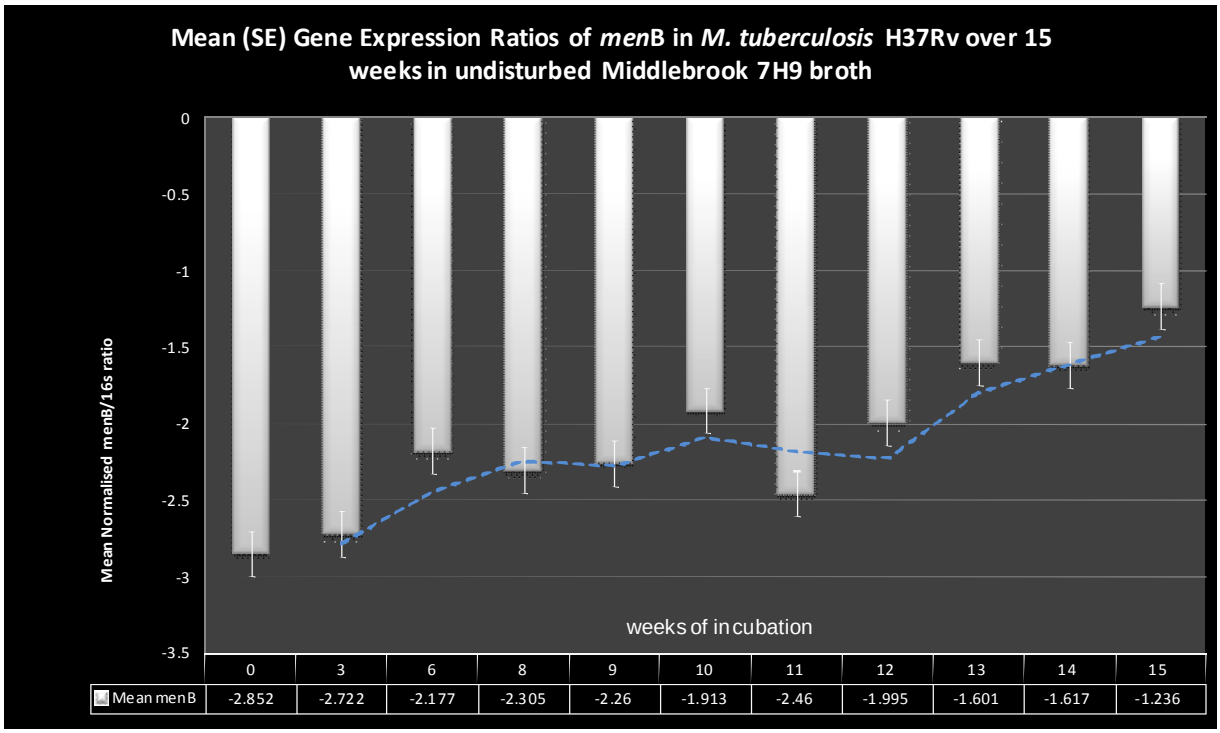


Figure 3.5 Absolute quantification of *men*B/16S RNA as an average of 9 experiments

Table 7: Comparison of the P-values showing a significance in the variation of the means (*men*B) at each time point

*men*B	DAY 0	WK 3	WK 6	WK 8	WK 9	WK 10	WK 11	WK 12	WK 13	WK 14	WK 15
DAY 0		NS	<0.001	<0.001	<0.001	<0.001	<0.05	<0.001	<0.001	<0.001	<0.001
WK 3	NS		<0.001	<0.05	<0.01	<0.001	NS	<0.001	<0.001	<0.001	<0.001
WK 6	<0.001	<0.001		NS	NS	NS	NS	NS	<0.001	<0.001	<0.001
WK 8	<0.001	<0.05	NS		NS	<0.05	NS	NS	<0.001	<0.001	<0.001
WK 9	<0.001	<0.01	NS	NS		NS	NS	NS	<0.001	<0.001	<0.001
WK 10	<0.001	<0.001	NS	<0.05	NS		<0.001	NS	NS	NS	<0.001
WK 11	<0.05	NS	NS	NS	NS	<0.001		<0.01	<0.001	<0.001	<0.001
WK 12	<0.001	<0.001	NS	NS	NS	NS	<0.01		<0.05	<0.05	<0.001
WK 13	<0.001	<0.001	<0.001	<0.001	<0.001	NS	<0.001	<0.05		NS	NS
WK 14	<0.001	<0.001	<0.001	<0.001	<0.001	NS	<0.001	<0.05	NS		<0.05
WK 15	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NS	<0.05	

3.6. *sdh*A and *frd*A

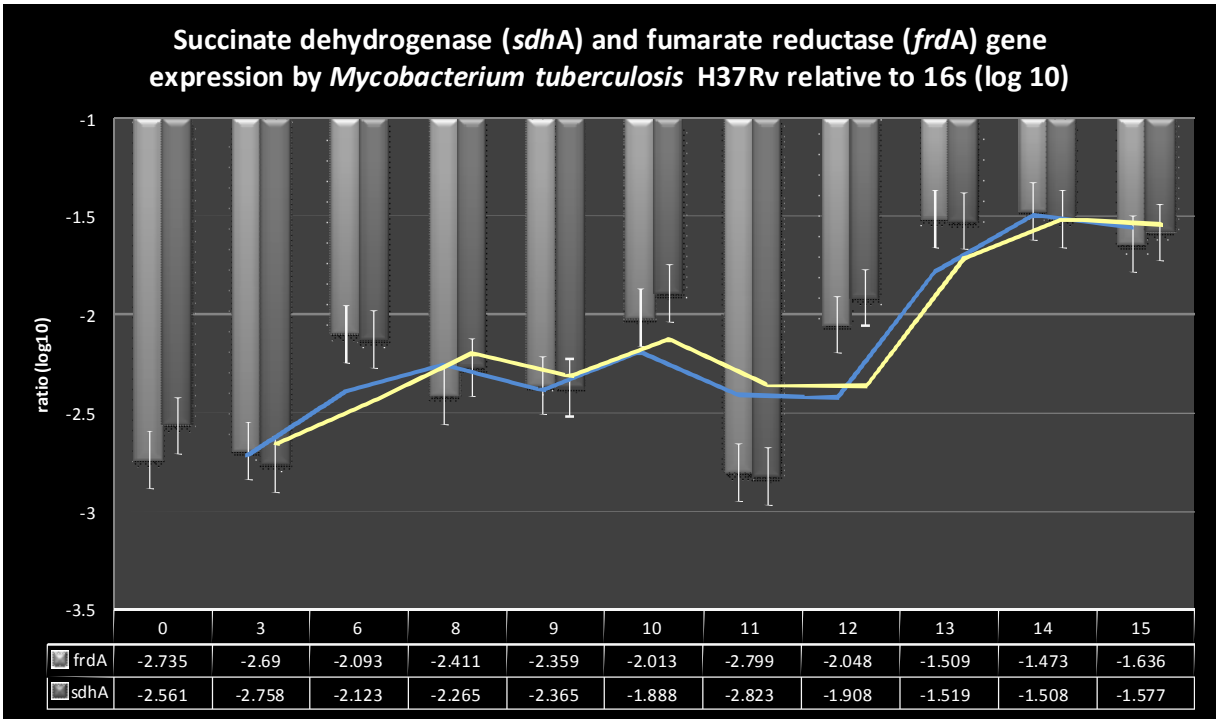


Figure 3.6: Gene Expression ratios of TCA components *sdh*A and *frd*A over 15 weeks

Figure 3.6 also shows the levels of expression of *sdh*A, *frd*A determined from absolute quantities of each gene as a ratio of the 16S quantity at each respective time point. The expression ratio of both TCA genes increased over time. The expression levels of these genes in the last period of incubation were significantly higher as compared to the early stages ($p < 0.001$). Within the first three weeks of incubation we see a decline in the expression levels of the aerobic *sdh*A and a slight increase in the anaerobic *frd*A. However throughout the rest of the incubation period, the expression patterns of each gene are nearly identical. The expression of both genes at week 6 increases significantly to week 3 although, no increased expression was observed between week 6 and 10, but there was a

significant decline in the next week ($p < 0.001$). A further significant increase in expression of both genes between weeks 13 and 15 was noted. At this late stage of incubation the absolute gene expression ratios are at their highest when compared to the rest of the incubation period. The two point moving average trend lines, shown as yellow (*sdh*A) and blue (*frd*A) solid lines, indicate a similar increase in undulating fashion as observed for the other genes.

In the case of *frd*A repeated measures ANOVA and the Student-Newman-Keuls Multiple Comparisons Test were used to determine the statistical significance of the difference in mean gene expression data between each time point. The repeated measures ANOVA indicates the P value is < 0.0001, considered extremely significant and variation among column means is significantly greater than expected by chance. Assumption test: To determine if the matching was effective another test using a second value of F and a different P value was run. F = 67.168 =(MSindividual/MSresidual) The P value is < 0.0001, considered extremely significant thus effective matching or blocking results in significant variation among

means. With these data, the matching appears to be effective.

The same statistical analysis was applied to the *sdh*A data (i.e. repeated measures ANOVA and Student-Newman-Keuls Multiple Comparisons Test). The repeated measures ANOVA once again designated that the P value is < 0.0001, and extremely significant. Variation among column means in this case is also significantly greater than expected by chance. Assumption test: The matching efficacy in this case, again using a test that uses a second F

value and a different P value. F = 38.783 = (MSindividual/MSresidual) the P value is < 0.0001, considered extremely significant. Effective matching (or blocking) results in significant variation among means. With these data, the matching appears to be effective.

The Bartlett test (corrected) = 3.696 with a P value of 0.9600, however suggests that the differences among the SDs is not significant in the case of *frdA*. In the case of *sdh*A, the Bartlett statistic (corrected) = 4.541 with a P value of 0.9196. Bartlett's test suggests that the differences among the SDs are again not significant.

Table 8 : Comparison of the P-values showing a significance in the variation of the means at each time point

frdA	DAY 0	WK 3	WK 6	WK 8	WK 9	WK 10	WK 11	WK 12	WK 13	WK 14	WK 15
DAY 0		NS	<0.001	<0.01	<0.001	<0.001	NS	<0.001	<0.001	<0.001	<0.001
WK 3	NS		<0.001	<0.05	<0.01	<0.001	NS	<0.001	<0.001	<0.001	<0.001
WK 6	<0.001	<0.001		<0.01	NS	NS	<0.001	<0.01	<0.001	<0.001	<0.001
WK 8	<0.01	<0.05	<0.01		NS	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001
WK 9	<0.001	<0.01	NS	NS		<0.01	<0.001	<0.05	<0.001	<0.001	<0.001
WK 10	<0.001	<0.001	NS	<0.001	<0.01		<0.001	NS	<0.001	<0.001	<0.001
WK 11	NS	NS	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001	<0.001
WK 12	<0.001	<0.001	<0.01	<0.01	<0.05	NS	<0.001		<0.001	<0.001	<0.001
WK 13	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		NS	NS
WK 14	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NS		NS
WK 15	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NS	NS	

Table 9 Comparison of the P-values showing a significance in the variation of the means at each time point

*sdh*A	DAY 0	WK 3	WK 6	WK 8	WK 9	WK 10	WK 11	WK 12	WK 13	WK 14	WK 15
DAY 0		NS	<.0.01	NS	NS	<0.001	NS	<0.001	<0.001	<0.001	<0.001
WK 3	NS		<0.001	<0.001	<0.01	<0.001	NS	<0.001	<0.001	<0.001	<0.001
WK 6	<.0.01	<0.001		NS	NS	NS	<0.001	NS	<0.001	<0.001	<0.001
WK 8	NS	<0.001	NS		NS	<0.01	<0.001	<0.05	<0.001	<0.001	<0.001
WK 9	NS	<0.01	NS	NS		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
WK 10	<0.001	<0.001	NS	<0.01	<0.001		<0.001	NS	<0.05	<0.01	NS
WK 11	NS	NS	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001	<0.001
WK 12	<0.001	<0.001	NS	<0.05	<0.001	NS	<0.001		<0.01	<0.01	<0.05
WK 13	<0.001	<0.001	<0.001	<0.001	<0.001	<0.05	<0.001	<0.01		NS	NS
WK 14	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001	<0.01	NS		NS
WK 15	<0.001	<0.001	<0.001	<0.001	<0.001	NS	<0.001	<0.05	NS	NS	

3.7. *qcr*B

The *qcr*B gene product shows a similar pattern of absolute gene expression over the incubation period as the rest of the genes, with a sharp increase in expression levels towards the end of incubation (Figure 3.7). The relative expression levels early in incubation are not statistically significant (P> 0.05) until week 10 (Table 10). This is surprising as the *qcrB* gene is reported to code for a product that has a function in aerobic respiration, which would presumably be dominant over the early stages of incubation.

Both one way and repeated measures ANOVA capitulate a P value is < 0.0001, considered extremely significant thus again, the variation among column means is significantly greater than expected by chance. ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett. Bartlett statistic (corrected) = 111.68. The P value is < 0.0001. Bartlett's test suggests that the differences among the SDs is extremely significant.

Table 10: Comparison of the P-values showing a significance in the variation of the means at each time point

qcrB	DAY 0	WK 3	WK 6	WK 8	WK 9	WK 10	WK 11	WK 12	WK 13	WK 14	WK 15
DAY 0		NS	NS	NS	NS	<0.001	NS	<0.001	<0.001	<0.001	<0.001
WK 3	NS		<0.01	NS	NS	<0.001	NS	<0.001	<0.001	<0.001	<0.001
WK 6	NS	<0.01		NS	NS	NS	NS	NS	<0.05	<0.001	<0.001
WK 8	NS	NS	NS		NS	NS	NS	NS	<0.001	<0.001	<0.001
WK 9	NS	NS	NS	NS		NS	NS	NS	<0.001	<0.001	<0.001
WK 10	<0.001	<0.001	NS	NS	NS		<0.001	NS	NS	NS	<0.001
WK 11	NS	NS	NS	NS	NS	<0.001		<0.01	<0.001	<0.001	<0.001
WK 12	<0.001	<0.001	NS	NS	NS	NS	<0.01		NS	NS	<0001
WK 13	<0.001	<0.001	<0.05	<0.001	<0.001	NS	<0.001	NS		NS	NS
WK 14	<0.001	<0.001	<0.001	<0.001	<0.001	NS	<0.001	NS	NS		<0.05
WK 15	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0001	NS	<0.05	

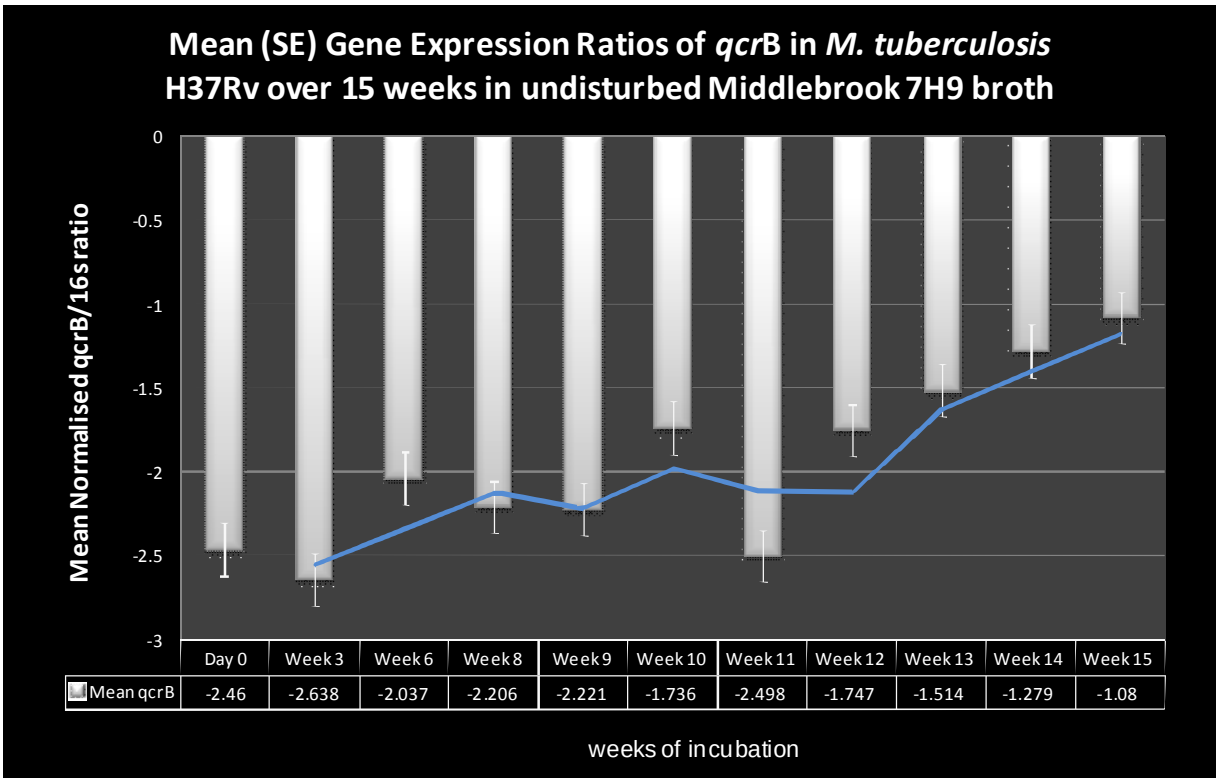


Figure 3.7 Summary of *qcr*B gene expression

3.8. Summary of results and trends of all genes

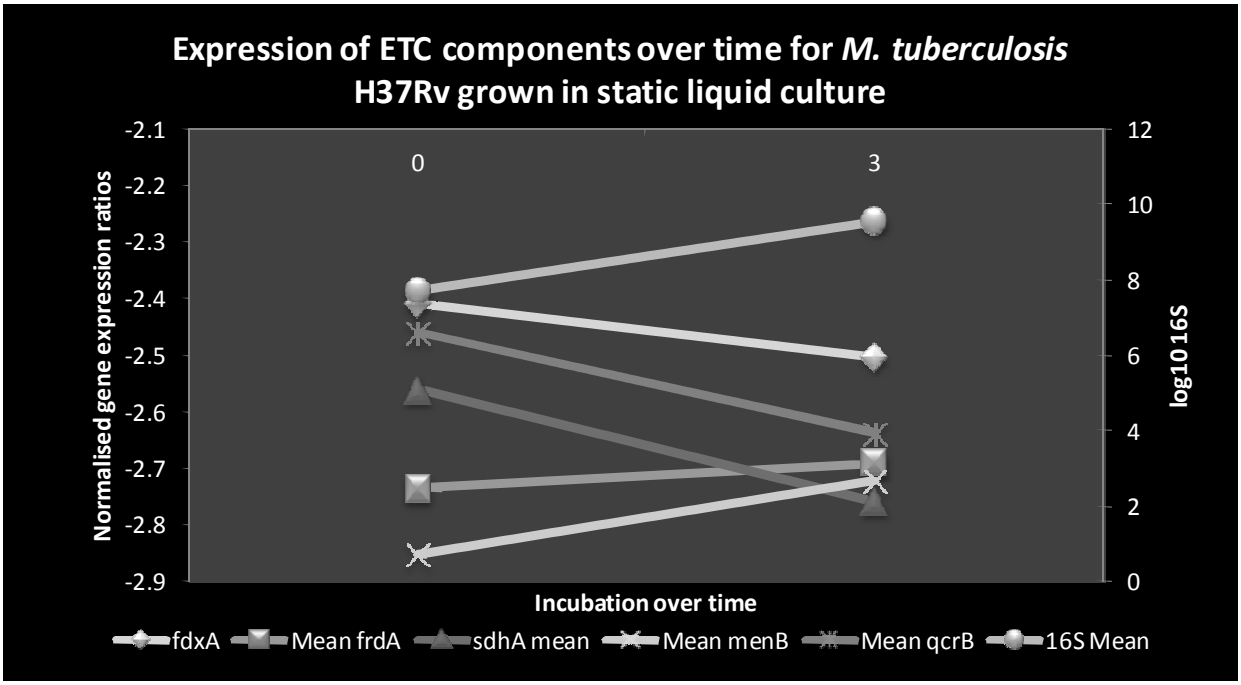


Figure 3.8.1 Gene expression pattern over 3 weeks of incubation.

In Figure 3.8.1 above, we see the the gene expression trend over 3 weeks in standing liquid culture. At this stage it appears that some genes are distinctly upregulated, while others are conversely downregulated. It implies that anaerobic genes are upregulated and aerobic genes are downregulated.

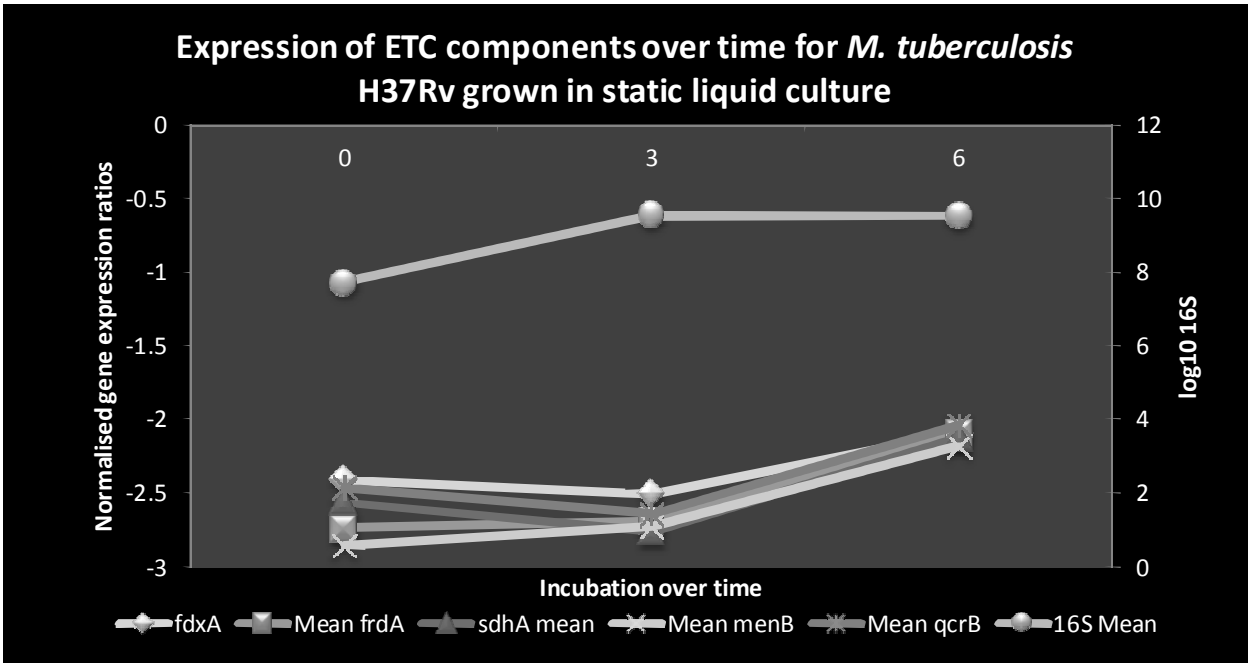


Figure 3.8.2: Gene expression pattern over 6 weeks of incubation.

Figure 3.8.2 illustrates that at 6 weeks however, this trend is subdued by an upregulation in the expression of all these genes.

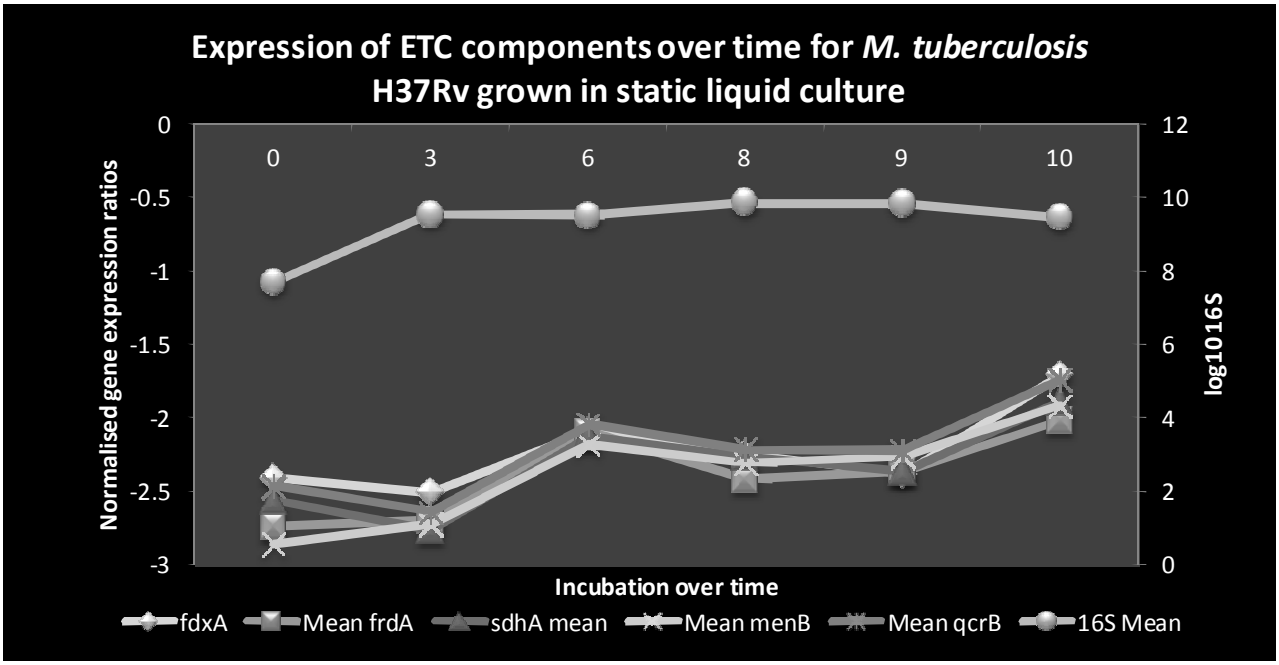


Figure 3.8.3 Gene expression patterns over 10 weeks of incubation.

At 10 weeks in sealed culture we expect that the cells will be shutting down due to an accumulation of toxic metabolites and depleted oxygen. However we see an unexpected upregulation of all genes expressed (Figure 3.8.3). At this point all gene expression levels are highly significant relative to day 0 and week 3. It is noteworthy that an earlier experiment over 10 weeks of incubation showed an identical trend of extremely significant upregulation at week 10.

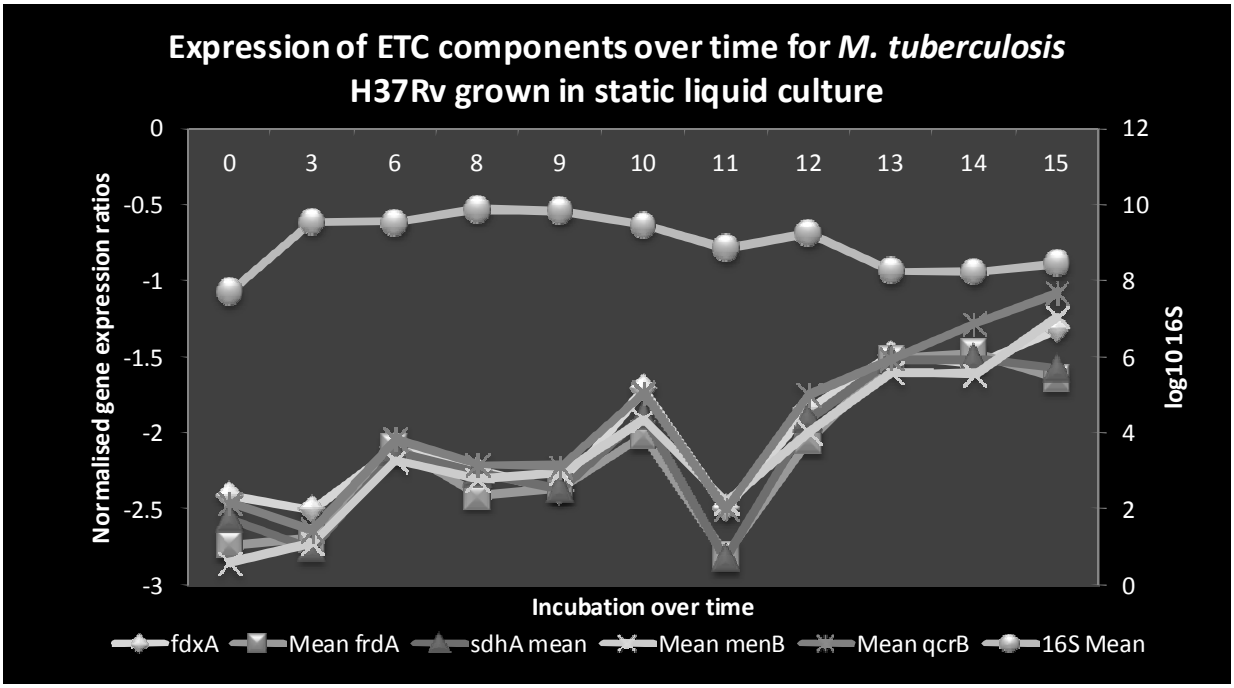


Figure 3.8.4 Complete summary of experimental data.

On further examination, we see a sharp drop in expression levels between weeks 10 and 11 for all selected genes. .The decrease is extremely significant and it precedes an increase in 16S quantities between weeks 11 and 12, that is extremely significant ($P<0.001$). The expression of these genes at weeks 13, 14 and 15 is extremely significant relative to the earlier timepoints ($P<0.001$). In addition the decrease in 16S between weeks 12 and 13 is statistically significant while there is no statistical significance in the changes of the 16S quantites thereafter.

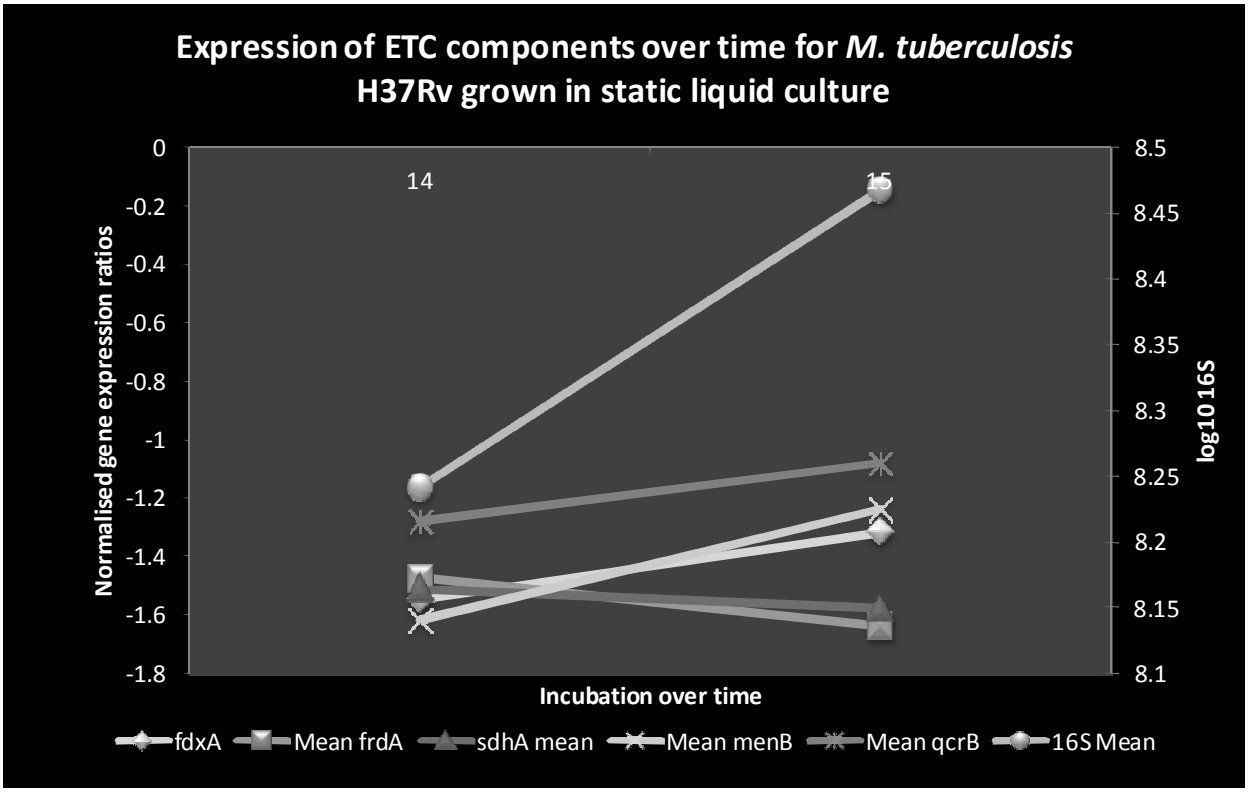
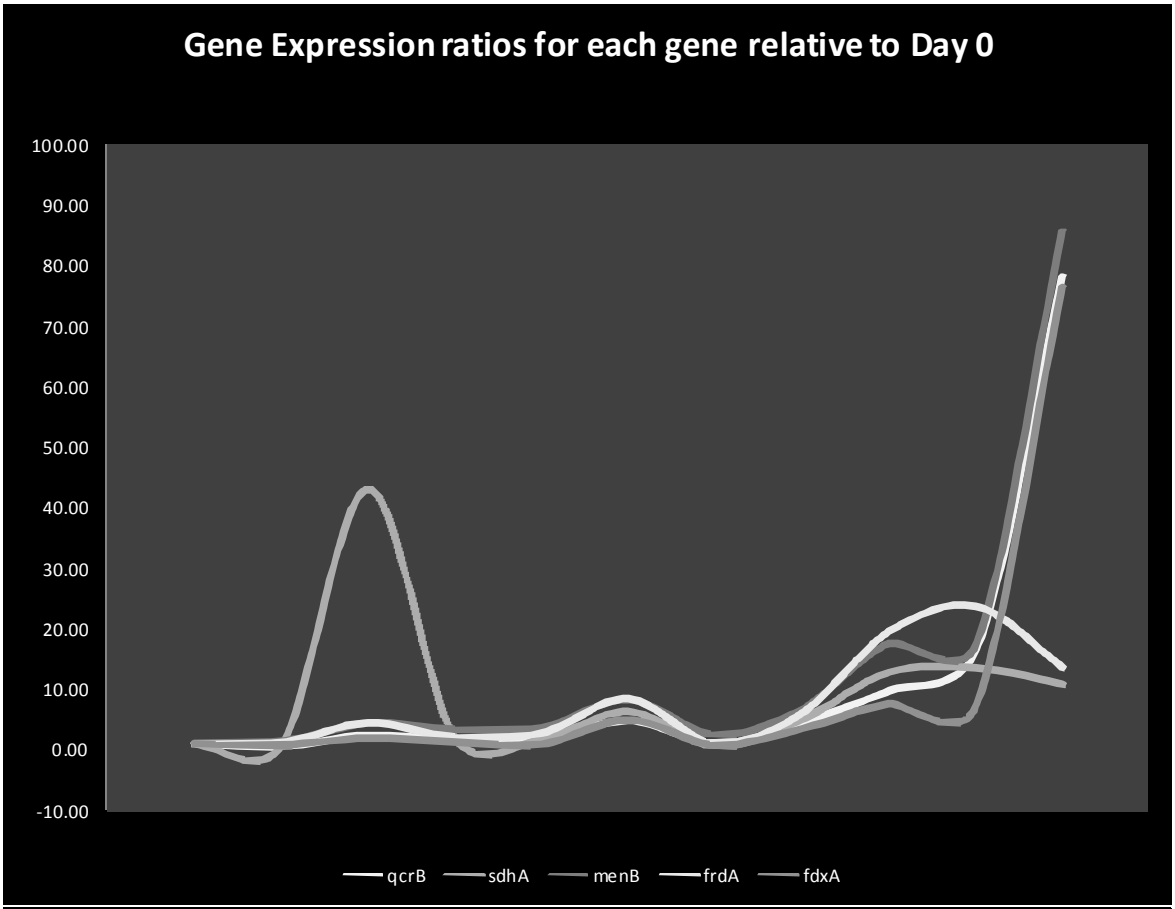


Figure 3.8.5: Comparison of Gene expression ratios between weeks 14 and 15

A closer look at weeks 14 and 15 shows that there exists a similar situation relative to weeks 0 and 3, where there is a difference in the up and downregulation of selected genes. This implies the start of yet another cycle of increased expression of ETC components as a means to survive.

The relative gene expression results presented in Figure 3.8.6 were obtained by dividing the gene expression ratio at each time point by the gene expression ratio obtained at day 0 for that gene. This shows the amount of expression relative to its first recorded time point.



	D0	Wk 3	Wk 6	Wk 8	Wk 9	Wk 10	Wk 11	Wk 12	Wk 13	Wk 14	Wk 15
qcrB	1.00	0.61	2.49	1.72	1.57	4.87	1.05	4.65	9.79	16.37	78.03
sdhA	1.00	0.73	43.09	2.04	1.76	6.34	0.56	5.07	12.80	13.57	10.85
menB	1.00	1.26	4.30	3.26	3.59	8.14	2.34	6.63	17.49	17.21	85.78
frdA	1.00	1.17	4.47	2.10	2.80	8.39	1.03	6.06	19.48	23.67	13.63
fdxA	1.00	0.76	2.04	1.32	0.97	5.00	0.77	3.51	7.61	7.19	76.46

Figure 3.8.6 Relative quantitation of each gene relative to its own day 0

The relative expression graphs visually clarify why the Bartlett test for *sdh*A and *frd*A indicated that the variation in the means was not as significant as observed for the other 3 genes. The extreme upregulation of the *fdx*A gene is noted with a 76.46 fold increase relative to its day 0

expression levels. The *men*B and *qcr*B genes also showed similar extremes in upregulation at week 15 with an 85.78 and a 78.03 fold increase respectively.

3.9. RNA quantitation and 16S RNA gene detection in sputum specimens

Table 10 shows the quantities of RNA in five sputum specimens of different patients. The amounts obtained ranged from 129 to 434 **ng/µL**. The variation in the concentration of cDNA obtained from identical amounts of RNA (2µg) indicates inhibition of the reverse transcription process.

Table 11: RNA obtained from sputum and conversion to cDNA

Clinical Specimens	Total RNA (ng/µL)	Volume used for conversion containing 2µg	cDNA (ng/µL)
1	434.78	4.60	98.80
2	204.08	9.80	133.50
3	270.27	7.40	113.40
4	285.71	7.00	111.40
5	129.87	15.40	215.50

The amounts of 16s RNA as well as the amounts of mRNA coding for the different components of the metabolic pathways studied are shown in Table 12 as number of amplicons/mL. As to be expected, broad variation between specimens is seen with all.

Table 12 : Amplicon quantities per 1000 µL

Specimen	16s	*frd*A	*fdx*A	*men*B	*qcr*B	*sdh*A
1	88.89	358406.22	21294.22	86345.78	88.89	88.89
2	552.89	456078.22	53247.11	108077.33	53426.67	3750.22
3	88.89	17068.44	2728.89	22289.78	31487.11	135.11
4	88.89	88.89	6448.89	4255.11	88.89	1331.56
5	344.00	88.89	11915.56	16402.67	188.44	88.89
Average	232.71	166346.13	19126.93	47474.13	17056.00	1078.93

Assuming that the level of inhibition was similar for all primer sets, the amount of amplicons obtained can be compared with each other. The highest number was found with *fdx*A, *frd*A and *men*B amplicons while *sdh*A showed the lowest numbers. Although the average for *qcr*B was similar to *fdx*A, this value is skewed as the expression was high in one of the five specimens only.

Section 4

Discussion

4. Discussion

The claim that *M. tuberculosis* is a facultative instead of a strict aerobic species is based on observations that the organism is susceptible to metronidazole and that its growth is enhanced by agitation in subcultures of the bacteria but not clinical specimens. Furthermore, the genes coding for anaerobic pathways have been identified in the H37Rv genome. Metronidazole is a pro-drug that is activated via the transfer of a single electron from ferredoxin to the nitro group of the drug, resulting in the formation of a free radical (Quon D et al, 1992). Therefore, metronidazole is exclusively active against microbes that use the fermentative pathway of metabolism. Metronidazole has been found to kill dormant cells of *M. tuberculosis* in vitro but it has no activity against actively dividing *M. tuberculosis* cells (Wayne and Sramek, 1994), suggesting the dormant state to be equivalent with anaerobic metabolism.

The work presented in this thesis shows that the number of organisms in sputum of patients with active pulmonary tuberculosis capable of growing in oxygen depleted Middlebrook broth outnumbers those that grow on the surface of Middlebrook agar plates in the presence of oxygen. It also shows variations in gene expression when grown under oxygen limiting conditions during a prolonged incubation period. These results support the claim that *M. tuberculosis* is a facultative organism.

Wayne and Hayes (1996) observed in undisturbed sealed broth cultures that the organisms went from aerobic metabolism through a micro-aerophilic stage into anaerobic metabolism in which no replication was observed. This differs from our first set of experiments with sputum from patients with active pulmonary tuberculosis. Wayne and Hayes inoculated mycobacterial

cultures grown aerobically into the broth while we inoculated the broths directly with sputum. Our results indicate that a significant part of the mycobacterial population in the lungs of patients with TB is formed by an anaerobic fraction that is replicating. Our observations based on quantitative growth of *M. tuberculosis* with and without oxygen directly from sputum, are corroborated by the gene transcription experiments in sputum. The genes with highest level of transcription were the genes coding for components involved in anaerobic metabolism.

Oxygen saturation has shown to approach 0 % at approximately 8.3 days (200 hrs) of incubation (Wayne and Hayes, 1996). From this it follows that at our first point of measurement (21 days) there was full oxygen depletion. This suggests that the observed organism density in broth resulted from anaerobically metabolizing organisms only. The observed non-replicating state (NRP1 and NRP2) of *M. tuberculosis* has been clearly demonstrated in vitro but this seems not to correlate with *in vivo* conditions. Heifets, et al (2005) reported on the effect of drugs on organisms in the non-replicating state in vitro. Their study assumes that the NRP state reflects dormancy *in vivo*. Our work on sputum specimens indicates that this might not be the case.

The methodology used to quantify growth from sputum makes a day 0 observation futile since in vitro growth has not occurred as yet. However, the graph (Figure 3.1) suggests the presence of large numbers of *M. tuberculosis* cells metabolizing anaerobically at that point. At all time points the number of anaerobically metabolizing cells outnumbered the aerobic ones although, as to be expected, the replication is slower anaerobically than aerobically. This difference became statistically significant at week 6 ($p=0.0476$) and increased in signicance at week 10 ($p= 0.004$).

Dhillon , et al (2004) conducted similar research, where they compared the growth density in broth and the growth on plates in tissue from mice with a 7-day old acute *M. tuberculosis* infection with that in mice with a chronic 10-month old infection. The ratio of growth in broth over that on plates was significantly higher in the latter group. Our results in the culture experiments with clinical specimens, were quantitatively similar to the results obtained from the murine experiments conducted by Dhillon, et al (2004) in which they demonstrated the difference in organism densities using acute and chronic infectious organisms. The observations of Dhillon, et al (2004) concur with our findings. Our patients are likely to harbour microorganisms that are similar in physiological state to those in the mice with a 10-month old infection.

This part of our study indicates that in patients with active pulmonary tuberculosis, a large fraction of *M. tuberculosis* cells is anaerobically metabolizing. These organisms replicate *in vivo* in oxygen depleted conditions and are therefore not identical with dormant *M. tuberculosis* as defined in terms of hypoxic conditions conferring non-replicating persistence.

If a proportion of mycobacteria in active tuberculosis are in the anaerobic mode, metronidazole could be a useful adjunct to the treatment, particularly when therapeutic choices are limited as in cases with extensive drug resistance. However, the therapeutic effect of metronidazole is difficult to measure. Treatment of tuberculosis in humans is by means of a combination of a minimum of 3 drugs. Studies that measure the effect of single drugs over a period of 2 months are therefore impossible. The Early Bactericidal Assay (EBA) measures the effect of metronidazole and its derivatives in human pulmonary tuberculosis over a period 3 to 5 days

and has been accepted as a predictor of therapeutic effect when used in multidrug regimens. This test measures the decline of the rapidly multiplying fraction of mycobacteria (Jindani, et al, 1980). As this fraction has a down regulated *fdx*A expression, the EBA cannot be used to measure the effect of metronidazole and its derivatives.

The results from the first set of experiments suggest that *in vitro* dormancy models are not representative of the *in vivo* conditions in patients with tuberculosis. Therefore, gene expression studies should preferably be done on bacteria in clinical specimens without growing them first in vitro. However, there is still value in studying gene expression in cultures containing organisms that do not replicate. This can tell us whether these nonreplicating cells are metabolically active or in a non-metabolic state like bacterial spores. We therefore studied expression levels of a selected number of genes coding for products known to play a role in aerobic as well as anaerobic metabolism. We used the H37Rv strain grown in standing liquid cultures, as described in the Wayne Model.

The Wayne model of nonreplicating persistence shows that, when *M. tuberculosis* is grown in unagitated liquid culture media, it adjusts to a state of equilibrium, where cells remain viable but are not actively dividing. He rationalizes this model of gradual oxygen depletion, by explaining that *M. tuberculosis* in the host will initially be exposed to oxygenated alveoli, and subsequently to conditions that become increasingly hypoxic (Wayne and Lin, 1982). Thus the organism would have to undergo a gradual adjustment to increasing oxidative stress. The un-agitated liquid culture model serves to represent such conditions of gradual oxygen depletion and is

widely reported on as a demonstration of the organism's resilience. The genes we studied were *fdx*A, *men*B, *frd*A, *sdh*A and *qcr*B.

The ferredoxin coding gene *fdx*A is in *M. tuberculosis* part of the DosR regulon, also known as the dormancy regulon, which is upregulated by oxygen depletion under in vitro growth conditions (Voskuil, 2004). Our *fdx*A expression results (Fig 3.4) show that the bacteria produce minimal amounts of ferredoxin while in the phase of rapid replication. Since the culture media were left undisturbed in a sealed tube, there was a systematic deterioration of conditions for aerobic growth, due to depletion of nutrients and oxygen and accumulation of toxic metabolites. As a result, the culture went from the log phase into the stationary phase after 3 weeks followed by the decline phase starting at week 9. The bacteria responded to this by a sudden increase in the expression of ferredoxin as measured at week 10. The growth spurt observed at week 12 (Figure 3.3) was preceded by a decline in ferredoxin expression. This underscores the notion that ferredoxin expression is associated with a decline in the speed of replication. It seems that *fdx*A expression is reciprocally associated with speed of replication. In facultative bacterial species the speed of replication is associated with aerobic and anaerobic metabolism with the slowest replication under anaerobic circumstances. Our results suggest that this is also the case with *M. tuberculosis* since *fdx*A expression is associated with anaerobic metabolism.

The decrease in multiplication rate at week 11 precedes an increase in the *fdx*A expression which from there increased slowly but steadily till the end of the incubation period. During that time the number of bacterial cells remained low. This contradicts the assumption that the cells

arrest all activity under circumstances where there is a systematic depletion of oxygen and nutrients. This challenges the concept that the organism becomes dormant like bacterial spores. It seems to merely change to anaerobic metabolism which results in a decrease in multiplication. Since the species multiplies under aerobic conditions once per 18 to 24 hours, a multiplication event under anaerobic conditions will take several days. As a result, increase in cell numbers under anaerobic conditions will be difficult to measure.

Upregulation of the *fdx*A gene should render the microbe susceptible to metronidazole. This susceptibility has been shown in vitro, but has not been observed in the mouse aerosol or the mouse hollow fiber model (Klinkenberg , et al, 2008). Information on activity of metronidazole on *M. tuberculosis* in the human host is not available. The consistent presence of the *fdx*A mRNA in the sputum of all five patients studied suggests that this drug must be able to contribute to successful treatment of patients with tuberculosis.

Other support for the hypothesis that *M. tuberculosis* can switch to anaerobic metabolism comes from our observations on *men*B transcription. The enzymes involved in the menaquinone pathway are not produced by human cells and therefore naphthoate synthase has already been identified as a potential drug target (Truligo, et al, 2003). In un-agitated broth cultures, the bacteria undergo multiple challenges due to changes in the media over the incubation period. The cells face a gradual depletion of oxygen as the tubes remain sealed throughout the experiment. In addition there is a depletion of nutrients and an accumulation of toxic metabolic intermediates as media is not replenished. Under these conditions, one would expect the cells to systematically shut-down their metabolic processes. However, there is an increase in relative gene expression of *men*B with the highest expression levels shown in weeks 13-15. Since the

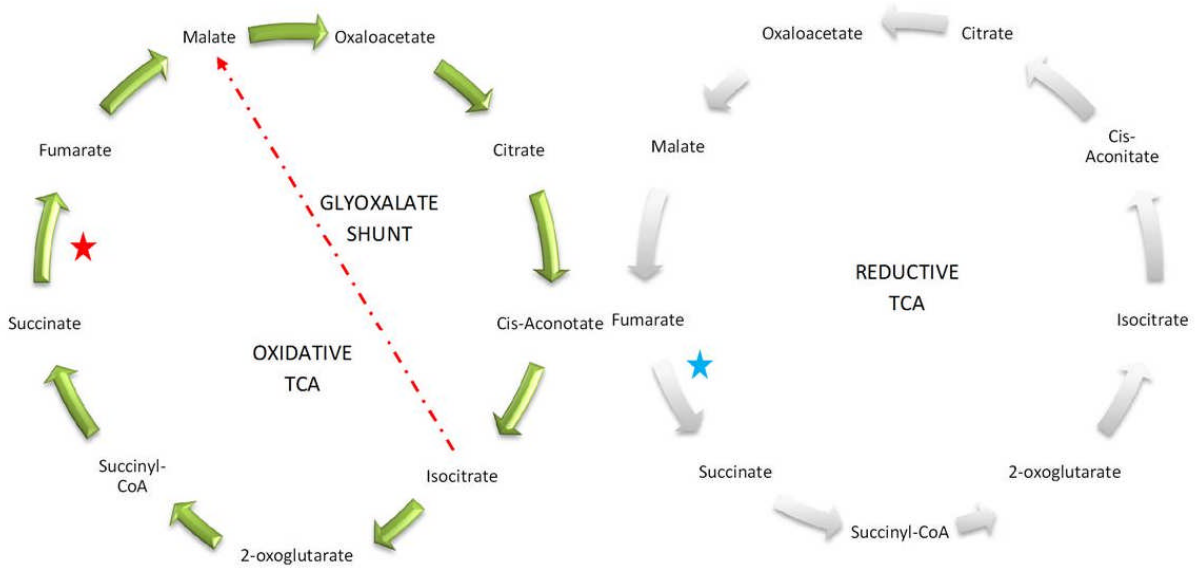
enzyme activity is normalized against ribosomal RNA gene expression, the data reflect increased enzyme expression at cellular level and is not a reflection of the increase in cell mass.

The decrease of *men*B expression at week 11 may be due to the depletion of succinyl benzoyl-CoA which is the substrate for naphtoate synthase (Figure 1.3). It is also possible that feedback inhibition occurs due to the accumulation of the end product menaquinone. The steady increase in *men*B expression over time appears to serve the purpose of adjusting to a corresponding increase in oxidative stress. Our observations therefore further reinforce the concept that *M. tuberculosis* shifts to an anaerobic mode of metabolism when depleted of oxygen and should therefore be classified as a facultative rather than a strict aerobic organism. Further exploration of the *men*B gene product as drug target is thus warranted. Naphthoate synthase is a key enzyme in the production of menaquinone. It has been reported that menaquinone is predominant under anaerobic culture conditions in *E. coli*, with the presence of little ubiquinone, and the opposite is found under aerobic conditions (Soballe and Poole, 1999). In prokaryotes, it also plays an important role in the anaerobic biosynthesis of pyrimidines (Gibson and Cox, 1973).

The TCA cycle is a core intermediary metabolic pathway and plays an important role in many biosynthetic processes by providing intermediates for other reactions. Although the TCA cycle is predominant in eukaryotic cells, it seldom presents in bacteria. Gene sequencing has revealed that *M. tuberculosis* possesses a complete set of genes for the tri-carboxyl acid (TCA) cycle (Cole, et al, 1998). While little is known about the metabolic mechanisms employed by *M. tuberculosis in vivo*, there have been reports on modifications to the TCA cycle that might

explain its persistence in macrophages (Munoz-Eliot and Mckinney, 2006). In mycobacteria, the TCA cycle functions in either reductive or oxidative processes, depending on the availability of nutrients and energy sources. It has been shown that isocitrate lyase and glyoxylate dehydrogenase, two enzymes of the glyoxylate shunt, are activated during adaptation to oxygen limited conditions (Wayne and Lin, 1982), and isocitrate lyase expression is enhanced during intracellular metabolism in macrophages (Honer et al, 1999).

The TCA cycle plays a central role in microbial survival in hostile environments that are nutrient-starved and oxygen-depleted (Hagerhall, 1997; Voskuil et al, 2004; ,Wayne and Sohaskey, 2001). We report on the expression of the succinate dehydrogenase (*sdh*A) and fumarate reductase (*frdA*) genes in *M. tuberculosis* undergoing gradual starvation and oxygen depletion.



Conversion of Succinate to Fumarate in the Forward/Oxidative TCA by Succinate Dehydrogenase

Conversion of Fumarate to Succinate in the Reverse/Reductive TCA by Fumarate Reductase

Direct Conversion of Isocitrate to Malate indicating the Glyoxalate Shunt

Figure 4.1 Schematic Overview of the forward and reverse TCA cycle with the Glyoxalate Shunt in the oxidative cycle

Fig. 4.1 shows the forward and reverse cycles of the TCA cycle. Succinate dehydrogenase and fumarate reductase are key enzymes in this cycle. They are protein complexes that are similar both in composition and in subunit structure. The former catalyzes the conversion of succinate into fumarate while the latter catalyses the reverse reaction (Ackrell et al, 1992). Succinate dehydrogenase plays a role in the aerobic respiratory chain (Hagerhall, 1997) and fumarate reductase catalyzes the final step in anaerobic respiration (Kroger, 1978). These enzymes therefore allow adaptation to specific environments. These enzymes thus represent potential markers of aero- and anaerobiosis *in vivo*.

As *sdh*A expression is associated with the aerobic, oxidative direction of the TCA pathway, one would expect an opposite trend as compared to *frd*A. This was the case in the first 3 weeks of the experiments (Figure 3.6 and Figure 3.8.1). However, later on the results show near equal levels of gene expression indicating similar conversion rates of succinate to fumarate and vice versa. At the first point of measurement the media is already depleted of oxygen (Wayne and Hayes, 1996). This suggests that in *M. tuberculosis* in the absence of free oxygen, there is still a need for succinate dehydrogenase. Since it is know that fumarate can serve as an electron acceptor under anaerobic conditions, the action of the succinate dehydrogenase enzyme may indeed be required.

It was demonstrated that in *Escherichia coli* under anaerobic conditions, the succinate dehydrogenase complex functionally replaces the fumarate reductase complex (Makalashina et al, 1998). It was found that under anaerobic circumstances both enzyme complexes were similarly expressed. Thus, the similar function of succinate dehydrogenase under anaerobic

conditions in *M. tuberculosis* is not unique. This further implies that this gene pair does not function as a suitable marker for aero- and anaerobiosis *in vivo* as they both show similar expression patterns under oxygen depletion.

Our observations suggest that in the absence of oxygen, *M. tuberculosis* cells go through phases of high metabolic activity with deceleration in between. Cells in older cultures up till week 15 show higher metabolic activity than cells in younger ones. Our data indicate that succinate dehydrogenase may have a dual role as described for *E. coli*. Further to the above, a component of the fumarate reductase has been theoretically indicated as drug target in *M. tuberculosis (*Anishetty et al, 2005*)*. Although the expression pattern in vitro indicates that succinate dehydrogenase may have a dual role, the *in vivo* detection of the TCA transcripts identifies *frd*A as a potential drug target. The Bartlett statistics indicate that the differences in the standard deviations for *sdh*A and *frd*A are, in statistical terms, not more significant than can be expected by chance. This implies that it has a more consistent role in cellular process than the other genes investigated. While there is an increase over time, the level of increase is not as significant as observed for the *men*B, *fdx*A and *qcr*B genes.

The operation of the glyoxalate bypass (Figure 4.1) implies that the enzymatic conversions of isocitrate to malate in the TCA can be circumvented (Wayne and Lin, 1982). However it appears that in *M. tuberculosis* the direct conversion from isocitrate to malate may occur in parallel with the enzymatic conversions in between, giving rise to a two-dimensional TCA. Further studies into the role of the TCA in mycobacterial survival and persistence are warranted.

The *qcr*B gene is reported to code for a product that is involved in aerobic processes. The experimental data (Figure 3.7) indicates a similar pattern of expression to that of the other genes with a peak in expression towards 15 weeks of incubation, when conditions within the culture vessel would have been most hostile. One would have expected to see a decrease in the expression of the *qcr*B gene over time, with the corresponding increase in that of *men*B. These results therefore indicate that that the *qcr*B gene may not function exclusively under aerobic conditions.

While expression of *sdh*A and *frd*A is part of the same pathway, the *men*B and *qcr*B genes are not directly linked. The *men*B gene codes for an enzyme that leads to the formation of the membrane found menaquinone, while the *qcr*B gene codes for the cytochrome component of the membrane bound ubiquinone cytochrome B reductase complex. Little is currently known about the particular role of this complex in mycobacteria. Matsoso and colleagues reported in 1998 that *M. smegmatis* strains with mutations in the *bc*1 complex and in subunit II of cytochrome *c* oxidase were found to be profoundly growth impaired, confirming the importance of this respiratory pathway for mycobacterial growth under aerobic conditions. However, even when knockout experiments on the *qcr*B and related genes to establish the role of other cytochrome components in aerobic growth were carried out, growth did not completely cease. This implies that if the cytochrome *b* reductase complex is inhibited under anaerobic conditions, there are alternative means of electron transport, and thus growth of mycobacteria under these conditions. This supports the switch from aerobic to anaerobic metabolism.

Early work indicates that there may be a link between the *b*-type cytochromes and succinate dehydrogense. In a study on the relationship between the iron containing enzymes of *Corynebacterium diphtheriae* and diphtheria toxin, Pappenheimer and Hendee (1947) obtained evidence that cytochrome b was "directly concerned in the oxidation of succinate". They were unable to separate succinate dehydrogenase from cytochrome *b* by differential centrifugation. Structural genes encoding quinol-cytochrome c reductase (*Qcr*) were cloned and sequenced from the *Rhodococcus rhodochrous*. The genes q*cr*C and *qcr*A encode diheme cytochrome c and the Rieske Fe–S protein, respectively, while the *qcr*B product is a diheme cytochrome b. These amino acid sequences are similar in *Corynebacterium* and *Mycobacterium* (Sone, et al, 2003). Although it seems that the *qcr*B gene product in mycobacteria is not a potential drug target, it may still have other applications. It is used as a phylogenetic marker, though mainly in eukaryotic cells, where it is found in the mitochondria. Since there is an evolutionary link between bacteria and mitochondria, there may be scope for this type of application with mycobacteria. This concept has not yet been explored, but there are reports on the use of b-type cytochromes for the rapid identification of mycobacterial species using real-time PCR and DNA-microarrays (Tobler, et al, 2006).

Our gene expression studies in the un-agitated broth culture model differ from other studies in that the incubation time is significantly prolonged. However, Corper and Cohn reported as early as 1933 that viable cells of *M. tuberculosis* were detected in a sealed standing culture tube after 12 years. By comparison the upregulation of electron transport genes at 13-15 weeks is hardly stirring. However, we still need to be able to explain the dynamic behind this growth

phenomenon. Since these observations are unique to liquid culture, the following discussion focuses on the chemical nature of water.

It has been reported that due to the clumping tendency of H37Rv, the number of colony forming units on solid agar may be artificially reduced (Zhang, et al, 1998).

They also report that the phenomenon of clumping is far more prevalent in the H37Rv strain when compared to the H37Ra. In order for a bacterial clump to be visible to the naked eye, it must consist of millions of cells compacted to form what is perceived as floccules suspended in culture. It is possible that the clumping of these cells is a frontline survival strategy of the tubercle bacilli.

Theoretically, the single cell of *Mycobacterium tuberculosis* is reported to be approximately 2-4um in length with a cylindrical diameter of 0.2-0.5µm. A visible clump of, hypothetically, 2mm diameter would have a total surface area of 12571429 µm as determined by the formula $4\pi r2$. The surface area of a bacterium of average length and diameter of 3µm and 0.35µm respectively would be 7.37µm; determined by the formula $2\pi r2 + 2\pi rh$. That would make the clump consist of just fewer than 2 million cells. While it is assumed by many that the cells having gone through several orders of binary fission are all similar in physiological state, this cannot theoretically be the case. Figure 4.2 illustrates a clump that was observed in a cell suspension that was vortexed for 30 seconds on maximum speed, and provides a visual demonstration of the clumping concept. While clumping in culture is visible to the naked eye, the aggregate illustrated was only visible under oil emersion.

Within a biofilm, different microorganisms co-exist in equilibrium with one another, each having a different function in maintaining the homeostatic nature of the film. The cells at the core of the biofilm are protected from exposure to its outermost conditions. The nutrient content is regulated and of course the redox potential will differ from the core of the biofilm moving outwards. To expand this concept even further, cells at the core of a clump are protected from physical contact with antibiotics and further potentially toxic intermediates. Even in the host when being engulfed by phagocytes, the outermost cells in a clump may well be able to communicate to cells at the core, inducing factors (known and unknown) to be expressed that aid in its survival. While this exercise (visualizing clumps) is simplistic in its design and nature, it emphasizes a key concept in further decrypting the survival mechanisms of *M. tuberculosis*.

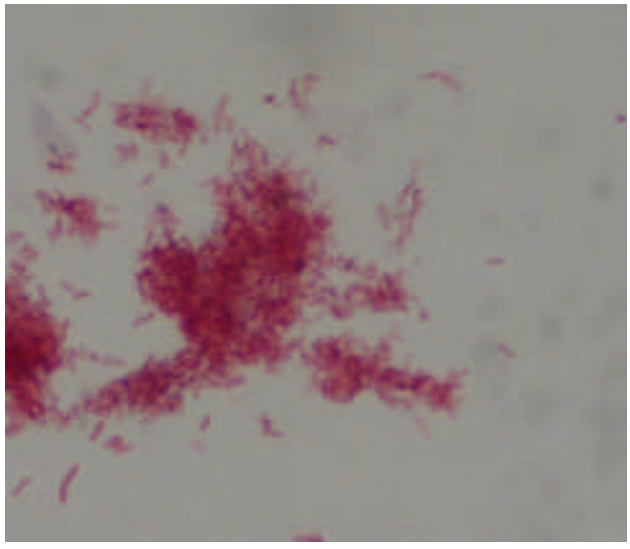


Figure 4.2: Ziehl Neelsen Stain showing clump formation in *M. tuberculosis* H37Rv

The co-expression of aerobic and anaerobic components may thus be a very deliberate survival strategy of the microorganism. Gause's law of competitive exclusion, states categorically that where two species coexist in the same environment, there will ultimately be a dominance of the

one species over the other. In other words one species will out-compete the other for essential nutrients etc. This however refers to different species. It is possible that a similar intra-species situation would occur within a sealed tube, where you would see a dominance of the anaerobic fraction over the aerobic. Based on both the phenotypic and genomic observations in liquid culture under gradual oxygen depletion, there appears to be a type of co-operation between the different populations that ultimately forms a formidable survival strategy for the organism.

It is thus possible that the dynamic within the clump can offer cells a level of protection in the host. This protective phenomenon is observed elsewhere in nature as is the case for bacteria within a biofilm. This phenomenon can be theoretically compared to the cellular dynamic within a biofilm. *Pseudomonas syringae* for example, is reported to have augmented survival on leaf surfaces in aggregates when compared to individual cells (Monier and Lindow, 2003). It is also known that spatial distribution of cells, specifically in aggregates, is invariably influenced by the conditions of the environment, with particular emphasis on survival of cells in clumps (Wilson et al, 2004). While the concept of cellular survival by aggregation or biofilm formation is well known, this concept has not been studied sufficiently in *Mycobacterium tuberculosis*. It is noted however that Thornton and colleagues (1996) describe mucus formation in the respiratory tract as a biofilm.

Bull reported as early as 1915, that aggregation of cells s of typhoid and dysentery bacilli agglutinate more immediately upon entering the circulatory system of rabbits (Bull, 1916). Virulent pneumonococci did not agglutinate upon introduction into the rabbit circulatory system, however they did form aggregations after intravenously introducing specific immune

sera (Bull, 1916). Although they state that clumping *in vivo* ensures rapid elimination of the cells from the bloodstream, they imply that these clumps may actually accumulate in organs due to their rapid removal from circulation. Contrary to the role of the clump in virulence though, they state that single cells are actually more likely to persist as it may be easier for single cells to evade phagocytosis. However, single cells may be more prone to the action of complement activation. It is well conceptualized that cells at the core of a biolfim formation are safely shielded from harsh conditions in the environment, including a shift in conditions and lethal substances.This may explain why differential gene expression results are seen in experiments are carried out on the laboratory strain H37Rv. Further to the above, it appears that this stratification in physiological states is a mechanism of survival. The cells operate in a synergistic manner, possible supplying other cells with essential components and facilitating long term survival by providing a protective effect. The biofilm concept can be further extended to include sputum specimens. The glycopeptide structure of sputum is comparable to the matrix of a biofilm. The laws of thermodynamics, tell is that in an isolated system the total amount of energy is conserved though it may change from one form to another. Even in chemical reactions, with the transfer of energy, the number of atoms and sub-atomic particles is precisely conserved on either side of a reaction. In the application of this law to biological systems, the impact at the cellular level is not that obvious. Many reactions generate heat as well as chemical products. Since it is a closed system, over time, the total energy in the system is conserved and that is what ultimately enables the organisms to survive. There may be an issue of nutrient depletion, but that energy is still present in the cellular environment, in the form of ready made enzyme complexes (possibly from dead cells) and cellular components that may be

assimilated into existing viable cells. In other words, the cells that persist over time show an ability to utilize energy in forms that were not necessary for initial growth in a closed system.

After studying the expression of genes whose products could differentiate between aerobiosis and anaerobiosis, an attempt was made to support the observations from the first set of experiments by quantifying expression of the same genes directly in sputum. It was hoped that we could measure gene expression normalized for the number of organisms present in the specimen. However, this could not be achieved since there was hardly any 16s RNA detectable. This is likely on the account of inhibition of the reversed transcriptase PCR reaction by inhibitors in the sputum. Despite these inhibitors, there were relatively large amounts of *fdx*A, *frd*A and *men*B. This supports the findings of the culture based experiments that the lungs of patients with pulmonary tuberculosis harbour large amounts of mycobacterial cells in the anaerobic metabolic mode. The low 16S transcripts may imply the presence of nonreplicating bacilli.

Measuring gene expression of *M. tuberculosis* in sputum can be used as a proxy for bacterial load. Therefore, such tests can be used to measure response to treatment if this is done in consecutive specimens of patients on anti-TB treatment. This has potential application in molecular diagnostics.

We found large variations in the quantities of RNA per microlitre in sputum. The total RNA measured is a combination of RNA from host cells and from bacteria. Since these specimens were decontaminated, it is not likely that contaminating organisms contributed to the quantities noted. Differences in the number of inflammatory cells, i.e. host factors, could account for the

differences in total RNA, In addition, the alkaline environment created by the decontamination process could also destroy RNA in an un-standardised way. The conversion of RNA to cDNA from the same amount of RNA was also erratic. Sputum is rich in highly glycosolated protein mucin and such molecules have been identified as potent PCR inhibitors. The amount of mucus present in sputum specimens varies between patients and between specimens obtained from the same patient. Despite the decontamination and the presence of inhibitors, we were still able to detect transcription products of all the genes looked for directly in sputum specimens.

Our results indicate that the "dormancy" achieved in the oxygen depletion model equates anaerobic metabolism likely in combination with a low level of multiplication. The question remains whether this is also the case *in vivo*. Since the redox potential in human tissue and on mucosal surfaces is too low to allow oxidative processes, facultative bacterial species largely would have to apply anaerobic metabolism while in the human host. This might also be the case with *M. tuberculosis*. The dormant state during latent infection however, might be different from this.

Cunningham and Spreadbury (1998) showed that there is a thickening of the cell wall under anaerobic conditions. Perhaps this is due to the over expression of electron transport components as demonstrated in our in vitro study. Although depletion of free oxygen occurs in liquid cultures, oxygen is always present due to the molecular structure of the water molecule. This oxygen can always become available through enzymatic hydrolysis. However, the resulting release of protons will affect the redox potential in the immediate vicinity of the cells and this interferes with the transfer of electrons to oxygen.

Further studies with organisms grown under strict anaerobic conditions are warranted. Studies using the Wayne model should incubate for a prolonged period of time.

In summary, all three components of this study support the facultative nature of *M. tuberculosis*. It raises the important question whether dormancy in *M. tuberculosis in vivo* is real dormancy in which the organism stops its metabolism or a change to anaerobiosis which is combined with a slow multiplication rate. If the latter is true, we will likely be able to find or design drugs that are able to eradicate these "dormant" organisms. In addition the work presented here forms the basis of a potential application in molecular diagnostics for drug efficiency, where multiple markers can be used to track bacterial loading in specimens.

REFERENCES

1. **Ackrell B.A.C., Johnson M.K., Gunsalus R.P., & Cecchini G.** (1992) Structure and function of succinate dehydrogenase and fumarate reductase. 229–297. *In* F. Muller (ed.), Chemistry and biochemistry of flavoenzymes, vol. III. CRC Press, Inc., Boca Raton, Fla.

2. **Actor J. K., Leonard, C.D., Watson V.E., Wells A., Jagannath C., Hunter R. L., Jr & Dasgupta, A**. (2000) Cytokine mRNA expression and serum cortisol evaluation during murine lung inflammation induced by *Mycobacterium tuberculosis*. Comb Chem High Throughput Screen **3**:343–351.

3. **Af-Gay Y. & Everett M.** (2002) The eukaryotic-like Ser/Thr protein kinases of *M. tuberculosis*. Trends in Micro **8(5):** 238-244

4. **Af-Gay & Davis J** (1997) Components of eukaryotic-like signalling pathways in *Mycobacterium tuberculosis*. Microbiol. Comp. Genom. **2:** 63-73

5. **AImquist H. J., & Stokstad E.L.R**. (1935) Hemorrhagic chick disease of dietary origin. J. Biol. Chem. **111:**105- 113.

6. **All-Party Parliamentary Group** (2007) Tuberculosis in the UK. http://www.appg-tb.org.uk/documents/TBinUK-final.pdf Last accessed 4 January 2010

7. **Anishetty S., Pulimi M. & Pennathur G.** (2005) Potential drug targets in *Mycobacterium tuberculosis* through metabolic pathway analysis. Comput Biol Chem. **29**:368–378

8. **Applied Biosystems Support** (2006) http://www6.appliedbiosystems.com/support/software/filebuilder/ Last accessed 28 February 2006

9. **Behling C.A, Perez R.L., Kidd M.R., Staton G.W. Jr & Hunter R.L.** (1993) Induction of pulmonary granulomas, macrophage procoagulant activity, and tumor necrosis factor-alpha by trehalose glycolipids. Ann Clin Lab Sci **23:**256–266.

10. **Bentley R.** (1975) Biosynthesis of quinones. Biochemistry **3**:181-246.

11. **Bentley R. & Meganathan R.** (1982) Biosynthesis of Vitamin K (Menaquinone) in Bacteria. Micro Rev. 241-280

12. **Bentrup K.H. & Russell D.G.** (2001) Mycobacterial persistence: adaptation to a changing environment. TRENDS in Microbiol, **9**:597—605.

13. **(A) Biocyc (2008)** *Mycobacterium tuberculosis* H37Rv Polypeptide Ferredoxin .http://biocyc.org/MTBRV/NEW-IMAGE?type=ENZYME&object=FERREDOXIN-MONOMER Last accessed 15 February 2008

14. **(B) Biocyc (2008)** *Mycobacterium tuberculosis* H37Rv Polypeptide Oxidized Ferredoxin. http://biocyc.org/MTBRV/NEW-IMAGE?type=ENZYME&object=OX-FERREDOXIN Last Accessed 15 February 2008

15. **(C) Biocyc (2008)** *Mycobacterium tuberculosis* H37Rv Pathway 1,4 dihydroxy-2-Napthtoate biosynthesis 1. http://biocyc.org/MTBRV/NEW-IMAGE?type=PATHWAY&object=PWY-5837&detail-level=2&detail-level=1 Last accessed 11 January 2008

16. **Bloch H.** (1950) Studies on the virulence of tubercle bacilli. Isolation and biological properties of a constituent of virulent organisms. J Exp Med **91:** 197–218.

17. **Bloch H. & Segal W. (**1955) Viability and multiplication of vaccines in immunization against tuberculosis. Am. Rev. Tuberc. Pulm. Dis. **71:**228–248.

18. **Bloom B. R. & C. J. L. Murray. (**1992) Tuberculosis: commentary on a reemergent killer. Science **257:**1055–1064.

19. **Boshoff H.I.M & Barry CE.** (2005) Tuberculosis, respiration and metabolism in the absence of growth. Nat. Rev. Micro**. 3**:70-80

20. **Brand S. N., Tan X. &. Widger W. R. (**1992) Cloning and sequencing of the *petBD* operon from the cyanobacterium *Synechococcus* sp. PCC 7002. Plant Mol. Biol. **20:**481–491.

21. **Brennan P.J. & Nikaido H**. (1995) The envelope of mycobacteria. Annu Rev Biochem **64:** 29–63.

22. **Brocks J. J., Logan G. A, Buick R. & Summons R. E.** (1999) Archean molecular fossils and the early rise of eukaryotes. Science **2(85)**:1033–1036

23. **Brock T.D. & Madigan M.T**. (1991) Biology of Microorganisms. Edition 6. p96 Prentice Hall Publications

24. **Bull C.G.** (1916) Further observations on the agglutination of bacteria *in vivo*. J Exp Med. 1;24(1):25-33.

25. **Chatterjee D. & Khoo, K.H** (1998) Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. Glycobiology **8:** 113–120.

26. **Chow K.**D., **Stokes R & Johnson P.** (1994) Protein tyrosine phosphorylation in *Mycobacterium tuberculosis*. FEMS Micro. Letters. **124:**203-207

27. **Cole S. T., Brosch R.,. Parkhill J, Garnier T., Churcher C., Harris D., Gordon S.V., Eiglmeier K., Gas S., Barry III C.E., Tekaia F., Badcock K., Basham D., Brown D., Chillingworth T., Connor R., Davies R., Devlin K., Feltwell T., Gentles S., Hamlin N., Holroyd S., Hornsby T., Jagels K., Krogh A., McLean J., Moule S., Murphy L., Oliver K., Osborne J.,. Quail M.A,. Rajandream M.-A, Rogers J., Rutter S., Seeger K., Skelton J., Squares R., Squares S., Sulston J.E., Taylor K., Whitehead S.&. Barrell B. G.** (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393**:537-544

28. **Cox R.A. (2003)** Correlation of the rate of protein synthesis and the third power of RNA:protein ratio in *Escherichia coli* and *Mycobacterium tuberculosis*. Microbiology **14:** 729-737

29. **Corper HJ & Cohn ML**. (1933) The viability and virulence of old cultures of tubercle bacilli: studies on twelve-year broth cultures maintained at incubator temperature. Am Rev Tuberc **28**:856–874

30. Crowe L. M., Spargo B.J., Ioneda T., Beaman B.L. & Crowe, J.H. (1994) Interaction of cord factor (a,a9-trehalose-6,69-dimycolate)with phospholipids. Biochim Biophys Acta **1194**, 53–60.

31. **Cunningham A.F & Spreadbury C.L. (1998)** Mycobacterial Stationary Phase Induced by Low Oxygen Tension: Cell Wall Thickening and Localization of the 16-Kilodalton α-Crystallin Homolog. J. Bact. **180(4):** 801-8

32. **Davidson E. & Daldal F.** (1987**)** Primary structure of the *bc*1 complex of *Rhodopseudomonas capsulata*. Nucleotide sequence of the *pet* operon encoding the Rieske, cytochrome *b* and cytochrome *c*1 apoproteins. J. Mol. Biol. **195**:13–24

33. **Desjardin L.E, Hayes L.G., Sohaskey C.D., Wayne L.G., Eisenach K.D.** (2001) Microaerophilic Induction of the Alpha-Crystallin Chaperone Protein Homologue (*hspX*) mRNA of *Mycobacterium tuberculosis*. J. Bacteriol. **183,** 5311–5316.

34. **De Wit D., Wooton M., Dhillon J. & Mitchison D.A.** (1995) The bacterial DNA content of mouse organs in the Cornell model of dormant tuberculosis. Tubercle Lung Dis **76**:555-62

35. **Dhillon J., Lowrie D.B. & Mitchison D.A. (2004)** *Mycobacterium tuberculosis* from chronic murine infections that grows in liquid but not on solid medium. BMC Infect Dis **17:**4:51

36. **Dick T.** (2001) Dormant tubercle bacilli. The key to more effective TB chemotherapy? JAC **47**:117-118

37. **Dziadek J., Madiraju M.V.V.S., Rutherford S.A., Atkinson M.A.L. & Rajagopalan M.** (2002) Physiological consequences associated with overproduction of *Mycobacterium tuberculosis* ftsZ in mycobacterial hosts. Microbiology. **148**:961-971

38. **Fenhalls G., Stevens L., Moses L., Bezuidenhout J., Betts J.C., van Helden P., Lukey P.T. & Duncan K. (**2002) In Situ Detection of *Mycobacterium tuberculosis* transcripts in human lung Granulomas, reveals differential gene expression in Necrotic Lesions. Infection and Immunity.. **70(11)** :6330-6338

39. **Fisher R.A. & Yates F.** (1963) Statistical tables for biological, agricultural and medical Research, Oliver and Boyd 6^{th} *ed.* :66 Table $VIII_2$

40. **Filliol I., Driscoll J.R., van Soolingen D., Kreiswirth B.N., Kremer K., Valétudie G., Duc Anh D., Barlow R, Banerjee D, Bifani P.J., Brudey K., Cataldi A., Cooksey R.C, Cousins D.V., Dale J.W., Dellagostin O.A, Drobniewski F., Engelmann G., Ferdinand S., Gascoyne-Binzi D.,Gordon M., Gutierrez M.C., Haas W.H.,Heersma H., Kassa-Kelembho E. , Ly H.M., Makristathis A., Mammina C.,Martin G., Moström P, Mokrousov I., Narbonne V., Narvskaya O., Nastasi A., Niobe-Eyangoh S.N., Pape J.W., Rasolofo-Razanamparany V., Ridell M, Rossetti M.L, Stauffer F., Suffys P.N., Takiff H., Texier-Maugein J, Vincent V., de Waard J.H., Sola C., & Rastogi N.** (2003) Snapshot of moving

and expanding clones of *Mycobacterium tuberculosis* and their global distribution assessed by spoligotyping in an international study. J Clin Microbiol; **41:** 1963 – 70

41. **Fletcher H.A., Donoghue H.D., Tayor G.M., van der Zanden A.G.M. & Spigelman M.** (2003) Molecular analysis of *Mycobacterium tuberculosis* DNA from a family of 18th century Hungarians. Microbiology. **149**:143-151

42. **Flynn J.L., Scanga C.A., Tanaka D.E., & Chan J. (**1998) Effects of aminoguanidine on latent murine tuberculosis. J. Immunol. **160:**1796–1803

43. **Forgarty International Center** (2007) Congressional Justification. http://www.fic.nih.gov/about/testimony/2007cj.htm Last accessed 4 January 2010

44. **Gabellini N. & Sebald W. (**1986) Nucleotide sequence and transcription of the *fbc* operon from *Rhodopseudomonas sphaeroides*. Eur. J. Biochem. **154:**569–579

45. **Gedde-Dahl T.** (1952) Tuberculous infection in the light of tuberculin matriculation. Am. J. Hyg. **56:**139–214

46. **GeneDoc** (2006) http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html Last accessed 15 January 2006

47. **Gibson F. & Cox G. B.** (1973) The use of mutants of *Escherichia coli* K12 in studying electron transport and oxidative phosphorylation. Essays Biochem **9**:1-29.

48. Glickman M.S. & Jacobs W.J. Jr (2001) Microbial Pathogenesis of *Mycobacterium tuberculosis:* Dawn of a Discipline. Cell. **104(4):** 477-485

49. **Gomez J.E. & McKinney J. D.** (2004) *M. tuberculosis* persistence, latency and drug tolerance. *Tuberculosis* **84**:29–44

50. **Haapanen J H., Kass I., Gensini G. & Middlebrook G.** (1959) Studies on the gaseous content of tuberculous cavities. Am. Rev. Respir. *Dis.* **80**:1–5

51. **Halbrook E. R.** (1935) The occurrence of hemorrhages and gizzard ulcers in chicks and their relation to the diet used. M.S. thesis, Graduate Division of the University of California, Berkeley. (Deposited in University Library, February 1936.)

52. **Hagerhall C. (**1997) Succinate:quinone oxidoreductases; variations on a conserved theme. Biochim. Biophys. Acta **1320:**107–141.

53. **Hanks J. H**. (1966) Host-dependent microbes. Bacteriol. Rev. **30:**114-135.

54. **Heifets L., Simon J & Pham V** (2005) Capreomycin is active against non-replicating *M. tuberculosis.* Annals Clin Micro Antimic. **4**:6

55. **Hellyer T.J., Fletcher T.W., Bates J.H., Stead W.W., Templeton G.L., Donald Cave M., Eisenach K.D.** (1996) Strand dis-placement amplification and the

polymerase chain re-action for monitoring response to treatment in patients with pulmonary tuberculosis. J.Infec. Dis. **173**: 934-41

56. **Herrera L., Valverde A., Saiz P., Sáez-Nieto J., Portero J., Soledad Jiménez** (2004) Molecular characterization of isoniazid-resistant Mycobacterium tuberculosis clinical strains isolated in the Philippines International Journal of Antimicrobial Agents, **23(6):** 572-576.

57. **Hoffmann C., Leis A., Niederweis M., Plitzko J.M. & Engelhart H.** (2008) Disclosure of the mycobacterial outer membrane L Crypto-electron tomography and vitreous sections reveal the lipid bilayer structure. Proc Nat Acad Sci USA. **11(105-10):** 3963–3967

58. **Honer Z., Bentrup K. A., Miczak D. L., Swenson & Russell. D. G. (**1999) Characterization of activity and expression of isocitrate lyase in *Mycobacterium avium* and *Mycobacterium tuberculosis*. J. Bacteriol. **181:**7161-7167

59. **Hugget J.,** et al. (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes and Immunity. 1–6

60. **Hunter S.W. & Brennan P.J.** (1990) Evidence for the presence of a phosphatidylinositol anchor on the lipoarabinomannan and lipomannan of *Mycobacterium tuberculosis*. J Biol Chem **265:** 9272–9279.

61. **Indrigo J., Hunter R,L, Jr & Actor J.K.** (2003) Cord factor trehalose 6,69-dimycolate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages Microbiology **149**:2049–2059

62. **Jindani A., Aber V.R., Edwards E.A. & Mitchison D.A.** (1980) The early bactericidal activity of drugs in patients with pulmonary tuberculosis , Am Rev Respir Dis **121** : 939-949

63. **Kallas T., Spiller S., and Malkin R. (**1988) Primary structure of cotranscribed genes encoding Rieske Fe-S and cytochrome *f* proteins of the cyanobacterium *Nostoc* PCC 7906. Proc. Natl. Acad. Sci. USA **85:**5794–579

64. **Kaufmann S.H.E.** (2003) Immune response to tuberculosis: experimental animal models. Tuberculosis. 83(1-3):107-111

65. Klinkenberg L.G., Sutherland L. A., Bishai W.R. & KaraKousis P.C. (2008) Metronidazole Lacks Activity Against *Mycobacterium tuberculosis* in an *in vivo* Hypoxic Granuloma Model of Latency. J. Infec. Dis. **198(15):** 275-83

66. Koch R. (1905) Nobel Lecture. The current state of the struggle against tuberculosis. http://nobelprize.org/nobel_prizes/medicine/laureates/1905/koch-lecture.html Last accessed 12 February 2010

67. Kochi A. (1991) The global tuberculosis situation and the new control strategy of the World Health Organization. Tubercle **72:**1–6

68. **Kroger A. (**1978) Fumarate as terminal acceptor of phosphorylative electron transport. Biochim. Biophys. Acta **505:**129–145.

.

69. **Lillebaek T**., **Dirksen A., Baess I., Strunge B., Thomsen V.O. & Andersen A.B.** (2002) Molecular evidence of endogenous reactivation of *Mycobacterium tuberculosis* after 33 years of latent infection. J. Infect. Dis. **185(3)**:401–4

70. **Manabe Y.C. & Bishai W.R**. (2000) Latent *Mycobacterium tuberculosis* persistence, patience, and winning by waiting. *Nat. Med.* **6**:1327–29

71. **Maklashina E.**, **Berthold D.A., Cecchini G.** (1998) Anaerobic Expression of *Escherichia coli* Succinate Dehydrogenase:Functional Replacement of Fumarate Reductase in the Respiratory Chain during Anaerobic Growth. J Bact. **180(22):**5989-5996

72. **Margulis L., and K. V. Schwartz. 1998**. Five Kingdoms—An Illustrated Guide to the Phyla of Life on Earth. 3rd edition. W. H. Freeman, NewYork. 5

73. **Matsoso L.G., Kana B.D., Crellin P.K., Lea-Smith D.J., Pelosi A., Powell D., Dawes S.S., Rubin H., Coppel R.L. & Mizrahi V** (2005) Function of the

cytochrome bc1-aa3 branch of the respiratory network in mycobacteria and network adaptation occurring in response to its disruption. J Bacteriol. **187(18)**:6300-8.

74. **McLean K. J., Marshall K. R., Richmond A., Hunter I. S., Fowler K., Kieser T., Gurcha S. S., Besra G. S. & Munro A. W.** (2002) Azole antifungals are potent inhibitors of cytochrome P450 mono-oxygenases and bacterial growth in mycobacteria and streptomycetes. *Microbiology* **148**:2937-2949

75. **McCune R. M. & Tompsett R. (**1957) Fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. I. The persistence of drug-susceptible tubercle bacilli in the tissues despite prolonged antimicrobial therapy. J. Exp. Med. **104:**737–762.

76. **McFarlane W. D., Graham W.R. & Hall G.E.** (1931) . The fat-soluble vitamin requirements of the chick. The vitamin A and vitamin D content of fish meal and meat meal. Biochem. J. **25:**358-366.

77. **McGovern E. P. & Bentley R**. (1975) Biosynthesis of flaviolin and 5,8-dihydroxy-2,7-dimethoxy-1,4-naphthoquinone. Biochemistry **14:**3138-3143.

78. **Mclane B. A., Mietzner T.A., Dowling, J.N. and Phlillips A. B.** (1999) Microbial Pathogenesis._2:31_http://books.google.co.za/books?id=vFIJge-

DVJ8C&printsec=frontcover#v=onepage&q&f=false Last accessed 10 February 2010

79. **Mitchison D.A& Nunn A.J.** (1986) Influence of initial drug resistance on the response to short-course chemotherapy of pulmonary tuberculosis**.** Am Rev Respir Dis **133**:423–430.

80. **Monack D. M., Mueller A. & Falkow S. (**2004) Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat. Rev. Microbiol. **2:**747–765.

81. **Monier J.M. & Lindow S.E.** (2003) Differential survival of solitary and aggregated bacterial cells promotes aggregate formation on leaf surfaces. Proc Natl Acad Sci USA. **100:**15977-15982;

82. **Mortensen, L. E., Valentine RC and Carnahan JE**. (1962) An electron transport factor from *Clostridium pasteurianum*. Biochem. Biophys. Res. Commun. **7:**448-452.

83. **Mortensen, L.E**. **Valentine RC and Carnahan JE**, (1963) Ferredoxin in the phosphoroclastic reaction of pyruvic acid and its relation to nitrogen fixation in *Clostridium pasteurianum.* J. Biol. Chem**. 238**:794-801.

84. **Munoz-Elias E.J. & Mckinney J.D.**. (2006) Carbon metabolism of intracellular bacteria. Cellular Microbiology **8(1)** :10–22

85. **Nathan C. & Shiloh M.U. (**2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc. Natl. Acad. Sci. USA**. 97:**8841–8848.

86. **Nikaido H**. (2001) Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Semin Cell Dev Biol **12:** 215–223.

87. **(A) Orme I. M.** (1988) Induction of nonspecific acquired resistance and delayed type hypersensitivity, but not specific acquired resistance, in mice inoculated with killed mycobacterial vaccines. Infect. Immun. **56:**3310–3312.

88. **(B) Orme I. M.** (1988) A mouse model of the recrudescence of latent tuberculosis in the elderly. Am. Rev. Respir. Dis. **137**:716–718.

89. **Outsouring-pharma.com** (2005) Alliance starts human TB drug testing. http://www.outsourcing-pharma.com/Preclinical-Research/Alliance-starts-human-TB-drug-testing Last accessed 11 December 2009

90. **Toungoussova O., Bjune G., Caugant D.**(2006) Epidemic of tuberculosis in the former Soviet Union: Social and biological reasons *Tuberculosis,* **86(1)**:1-10

91. **Pappenheimer A. M. & Hendee E. D.** (1947) Diptheria Toxin. A Comparison between the diphterial succinoxidase system and that of beef heart muscle. A J. biol. Chem. **171**: 597-609

92. **Parakh P. R.** (2009). Paediatric Thoracic Surgery :Pulmonary Tuberculosis.Springer London**. 2(10)** :129-143

93. **Parkinson J. S**. (1993) Signal transduction schemes of bacteria. Cell **73:**857-871

94. **Parrish N.M., Dick J.D. & Bishai W.R.** (1998) Mechanisms of latency in *Mycobacterium tuberculosis*. Trends Microbiol. **6:**107–12

95. **Perez R.L., Roman J., Roser S., Little C., Olsen M., Indrigo J., Hunter R. L. & Actor J. K.** (2000) Cytokine message and protein expression during lung granuloma formation and resolution induced by the mycobacterial cord factor trehalose-6,69-dimycolate. J Interferon Cytokine Res **20:** 795–804.

96. **Pillay M. & Sturm A.W.** (2007) Evolution of the extensive drug resistant (XDR) F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in KwaZulu-Natal. Clinical Infectious Diseases. **45**:1409-1414

97. **Quon D.V.K., d'Oliviera C.E. & Johnson P.J** (1992) Reduced transcription of the ferredoxin gene in metronidazole resistant *Trichomonas vaginalis*. Proc. Nati. Acad. Sci. USA. **89:**4402-4406

98. **Rao A.R. & Vijaya S**. (1995) An approach for studying mediators in pathogenesis in *M tuberculosis*. J. Biosci**., 21 (3):**413-421

99. **Research Tools** (2006) http://www.nih.go.jp/~jun/research/ Last accessed 16 February 2006

100. **Rothery R.A., Kalra N., Turner R.J. & Weiner J.H.** (2002) Sequence Similarity as a Predictor of the Transmembrane Topology of Membrane-Intrinsic Subunits of Bacterial Respiratory Chain Enzymes. J. Mol. Microbiol. Biotechnol. **4(2):** 133–150

101. **Russell D.G. (**2001) *Mycobacterium tuberculosis*: here today, and here tomorrow. Nat. Rev. Mol. Cell Biol. **2:**569–577

102. **Salo W.L., AufderHeide AC, Buikstra J and Holcomb TA (1994)** Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. Proc. Nat. Acad. Sc. **91:** 2091-2094

103. **Sanger** (2006) http://www.sanger.ac.uk/resources/downloads/bacteria/mycobacterium.html Last accessed 8 February 2008

104. **Sharma and Mohan (2004)** Extrapulmonary tuberculosis. Ind J Med res **120:**316-353

105. **Singh V.K., & Gosh I.** (2006) Kinetic modeling of tricarboxylic acid cycle and glyoxylate bypass in *Mycobacterium tuberculosis*, and its application to assessment of drug targets. Theor Bio Med Mod. **3:**27

106. **Shi L.Y., Jung Y.J., Tyagi S., Gennaro M.L. & North R.J**. (2003) Expression of Th1-mediated immunity in mouse lung induces a *Mycobacterium tuberculosis* transcription pattern characteristic of nonreplicating persistence. Proc. Natl. Acad. Sci. USA **100:**241–246

107. **Siddiqi S., Takhar P., Baldeviano C., Glover W. & Zhang Y**. (2007) Isoniazid induces its own resistance in non-replicating Mycobacterium tuberculosis. Antimic Agents Chemo. **51(6):**2100-2104

108. **Smith E. & Morowitz H. J.** (2004) Universality in intermediary metabolism. *Proc. Natl. Acad. Sci. USA* 101:13168–13173.

109. **Smith P. G. & Moss A. R. (**1994) Epidemiology of tuberculosis. *Tuberculosis*: *Pathogenesis, Protection and Control*. Edited by B. R. Bloom. Washington, DC: American Society for Microbiology.

110. **Soballe B and Poole RK.** (1999) Microbial ubiquinones: multiple roles in respiration, gene regulation and oxidative stress management. *Microbiology***. 145**:1817–1830

111. **Sone N., Fukuda M., Shigetaka K., Aki J., Mie S., Noguchi S., Sakamoto J.** (2003) *qcr*CAB operon of norcardia-form actinomycete *Rhodococcus rhodochrous* encodes cytochrome reductase complex with diheme cytochrome cc subunit. Biochimica et Biophysica Acta **1557:** 125–131

112. **Spargo B. J., Crowe L. M., Ioneda T., Beama, B. L. & Crow, J. H.** (1991) Cord factor (a,a-trehalose 6,69-dimycolate) inhibits fusion between phospholipid vesicles. Proc Natl Acad Sci USA **88:**737–740.

113. **Somoskovi A., Parsons L.M & Salfinger M.** (2001. The molecular basis of resistance to isoniazid, rifampicin and pyrazinamide in *Mycobacterium tuberculosis*. Respir Res. **2**:164–168

114. **Srinivasan V. & Morowitz H.J.** (2006) Ancient genes in contemporary Persistent Microbial Pathogens. *Biol. Bull*. **210:** 1-9

115. **Starck J**., **Kallenius G, Marklund BI, Andersson, DI Akerlund T.** (2004) Comparative proteome analysis of *Mycobacterium tuberculosis* grown under aerobic and anaerobic conditions Microbiology. **150:**3821–3829

116. **Summons R. E.,. Jahnke L. L Hope J. M. and Logan G. A. (**1999) 2-Methylhopanoids as biomarkers for cyanobacterial oxygenic photosynthesis. Nature **400**:554–557

117. **Talaat A.M., Howard S.T., Hale W .4th, Lyons R., Garner H. & Johnston S.A.** (2002) Genomic DNA standards for gene expression profiling in *M. tuberculosis.*Nucleic Acids Research. **30:(20)**104

118. **Thoney-Meyer L.** (1997) Biogenesis of Respiratory Cytochromes in Bacteria Micro and Molec Bio Reviews.. 337–376

119. **Thornton D.J., Carlstedt I., Howard M., Devine P.L., Price M.R. & ShehaanJ.K.** (1996) Respiratory Mucins: identification of core proteins and glycoforms. Biochem. J. **316:** 967-75

120. **Tobler N.E., Pfunder M., Katrin H., Frey J.E. & Altwegg M**. (2006) Rapid detection and species identification of *Mycobacterium* spp. using real-time PCR and DNA-Microarray Journal of microbiological methods **66(1):** 116-124

121. **Truligo J.J., Theis K., Feng Y., Gajda R., Machutta C., Tonge P.J. & Kisker C.** (2003) Crystal Structure of *Mycobacterium tuberculosis men*B, a Key Enzyme in Vitamin K2 Biosynthesis. J. Biol. Chem **278(43):** 42352-4236

122. **Tuberculist** (2006) http://genolist.pasteur.fr/TubercuList/ Last Accessed 12 February 2006

123. **Twort F. W. & Ingram. G.L.Y** (1912) A method for isolating and cultivating the *Mycobacterium* enteritidis chronicae pseudotuberculosae bovis, Johne, and some

experiments on the preparation of a diagnostic vaccine for pseudo-tuberculous enteritis of bovines. Proc. R. Soc. London Ser. B **84:**517-542.

124. **Twort F. W. & Ingram G.L.Y**. (1913) A monograph on Johne's disease. Bailliere, Tindall and Cox, London.

125. **Valentine R.C. , Jackson R.L. & Wolfe R.S. (**1962) Role of ferredoxin in hydrogen metabolism of *Micrococcus lactilyticus*. Biochem. Biophys. Res. Commun. **7:**453-456.

126. **Valentine R. C. & Wolfe R.S.** (1963) Role of ferredoxin in the metabolism of molecular hydrogen. J. Bacteriol. **85:**1114-1120.

127. **Valentine R. C., Mortenson L. E., & Carnahan J. E. (**1963) The hydrogenase system of *Clostridium pasteurianum*. J. Biol. Chem. **238:**1141-1145.

128. **Valentine C.** (1964) Bacterial Ferredoxin. Bacteriological Reviews. **28(4):**497-517

129. **Verma A. & Maurelli, A.T.** (2003) Identification of two eukaryote like Serine/Threonine kinases encoded by *Chlamydia trachomatis* serovar L2 and Characterisation of Interacting partners of Pkn1 Infect Immun. **71(10):** 5772–5784.

130. **Voskuil M.I., Visconti K.C. & Schoolnik G.K.(** 2004) *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. Tuberculosis **84:** 218-227

131. **Wade M.M & Zhang Y**. (2004) Anaerobic conditions enhance the activity of pyrazinamide. J Med Microbiol **53** :769-773

132. **Wayne L.G.** (1976) Dynamics of submerged culture growth of *Mycobacterium tuberculosis* under aerobic and microaerophillic conditions. Am. Rev. Respir. Dis **114:**807-811

133. **Wayne L. G**. (1994) Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur. J. Clin. Microbiol. Infect. Dis. **13:**908–914

134. **Wayne L.G. & Diaz G.A.** (1967) Autolysis and secondary growth of *Mycobacterium tuberculosis* in submerged culture. J Bacteriol. **93(4)**:1374–1381

135. **Wayne L.G. & Sohaskey C.D. (**2001) Nonreplicating persistence of *Mycobacterium tuberculosis*. Annu. Rev. Microbiol**. 55:**139–163.

136. **Wayne L. G. & Hayes L. G. (**1996**)** An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. Infect Immun **6:** 2062-2069.

137. **Wayne L.G. & Lin K.Y**. (1982) Glyoxalate metabolism and adaptation of *Mycobacterium tuberculosis* to anaerobic conditions. Infect Immun 1042-1049

138. **Wayne L.G. & Sramek H.** (1979.) Antigenic differences between extracts of actively replicating and synchronised resting cells of *M. tuberculosis.* Infec Immun **24(2):** 363-370

139. **Wayne L.G & Sramek H**. (1994) Metronidazole is bactericidal to dormant cells of *M. tuberculosis.* Ant Micr Agents & Chemo. **38(9):** 2054-2058

140. **Weinstein E. A., Yano T., Lin-Sheng L., Avarbock D., Avabock A., Helm D., McColm D.K., Lonsdale J.T. and Rubin H.** (2005) Inhibitors of type II NADH:menaquinone oxidoreductase present a class of antitubercular drugs**.** Proc Natl Acad Sci USA**.102(12):** 4548-53

141. **Wilson S., Hamilton M.A., Hamilton G.C., Schuman M.R. & Stoodley P**. (2004) Statistical Quantification of Detachment Rates and Size Distributions of Cell Clumps from Wild-Type (PAO1) and Cell Signalling Mutant (JP1) *Pseudomonas aeruginosa* Biofilms. App and Env Micro **70(10):**5847-52

142. **Voskuil M.I**, **Visconti K.C. & Schoolnik G.K.** (2004) *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. Tuberculosis **84:** 218-227

143. **World Health Organisation**. (2010) Fact Sheet, No 104, Nov 2010. http://www.who.int/mediacenter/factsheets/fs104/en/ Last Accessed 03 March 2011.

144. **Yamanaka T. (**1992) The biochemistry of bacterial cytochromes. Japan Scientific Societies Press, Tokyo

145. **Yu J. & Brun** N.E.L. (1998) Studies of the Cytochrome Subunits of Menaquinone:Cytochrome *c* Reductase (*bc* Complex) of *Bacillus subtilis* **273(15):** 8860–8866

146. **Yu T. & Bishop T.L.** (1998) Stratification of microbial metabolic processes and redox potential change in aerobic biofilm studied using microelectrodes. Wat. Sci. Tech. **37(4-5):**195-198

147. **Zhang Y.** (2004) Isoniazid, p. 739-758. *In* W. N. Rom and S. M. Garay (ed.), Tuberculosis, 2nd ed. Lippincott Williams & Wilkins, New York, NY

148. **Zhang M. J., Lin Y. & Barnes P.F.** (1998) Growth of virulent and avirulent strains of *M. tuberculosis* strains in human macrophages. Infec and immun. 794-799.

149. **Zimhony O., Cox J. S., Welch J. T., Vilcheze C. &. Jacobs, W. R. Jr (**2000) Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of *Mycobacterium tuberculosis*. Nat. Med. **6:**1043–1047.

APPENDIX A

APPENDIX A

Methodology

Classical Culture: Serial Dilution of Specimens

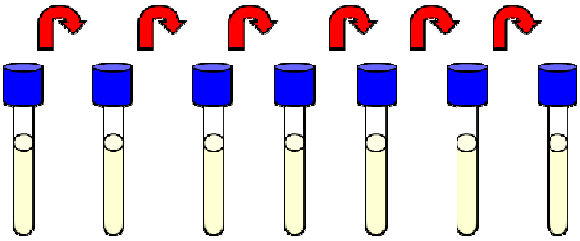


Figure A1: Serial dilution schematic and 10ml screw-cap tube showing growth in Middlebrook 7H9 Liquid media

1. Classical Culture techniques

1.1. 10-fold serial dilution of homogenized sputum specimens.

1.1.1. Add 1ml of homogenized sputum to 9ml of PBS

1.1.2. Pipette repeatedly with gentle action to prevent aerosol formation, but enough to ensure homogeneity of the media and sputum.

1.1.3. Have the number of required tubes for the dilution series prepared with 9ml media.

1.1.4. Add 1ml of sputum mixture to the next tube in the dilution series.

1.1.5. Mix as directed in 1.1.2.

1.1.6. Repeat serial dilution to the level required for the experiment.

1.1.7. Label caps with permanent ink.

1.1.8. 10-fold dilutions can be made as follows according to different experimental requirements

1.1.8.1. 1ml of sample into 9ml of media

1.1.8.2. 0.5ml sample into 4.5ml media

1.1.8.3. 2ml sample into 18ml of media

1.2. MEDIA PREPARATION

1.2.1.1. Middlebrook 7H9 liquid media (From Internal Protocols Dept Medical Microbiology)

1.2.1.2. Middlebrook 7H9 dehydrated media (Difco, USA) [9.14g]

- Distilled water [1800ml]
- Glycerol [4ml]
- Suspend dry ingredients in distilled water. Add Glycerol
- Autoclave at 121^{O}C for 15 minutes
- Allow to cool in a water bath to 60^{O}C
- Aseptically add 200ml of OADC (Difco) supplement
- Add antibiotics as follows (see in-house protocol).
- Carbenicilline 0,05
- Trimethoprim 0,02

- Amphotericin B 0,01
- Polymyxin B 200,000 units
- Aseptically dispense liquid media into tubes as required.

1.2.1.3. Middlebrook 7H11 Agar. (From Internal Protocols Dept Medical Microbiology)

- Middlebrook 7H11 Agar (Difco, USA) [42g]
- Casitone [2g]
- L-Asparagine Monohydrate [10g]
- Glycerol [10ml]
- Distilled Water [1800ml]
- Suspend dry ingredients in distilled water. Add Glycerol
- Autoclave at 121^{O}C for 15 minutes
- Allow to cool in a water bath to 60^{o}C
- Aseptically add 200ml of OADC (Difco) supplement
- Add antibiotics as indicated for MB7H9 above

- Aseptically dispense agar into plates as required and aliquots of liquid media into sterile tubes as required.
- Take note of manufactures instructions at all times, and between batches.

2. Primer and Probe Design

2.1. Download gene sequences from 3 databases

2.2. Checked for conserved regions in Genedoc The figures below show an example based on the 16s ribosomal rna geme sequence. Random "x's" were used to replace certain bases for illustrative purposes. The software highlight where the sequences do not align (grey coloration) emphasized with red rings'

2.3. Chose a region of the gene where there is complete congruence between the sequences.

2.4. Upload that gene segment into Primer Express Software.

2.5. Run the Automatic Primer and Probe search.

2.6. A list of primer and probe combinations will be generated and listed from highest ranking to lowest.

2.7. The higher the ranking (ie lower penalty score) the better the primer and probe combination.

2.8. High penalties are of significance in the event that you plan to run multiplex PCR

2.9. It is not recommended that you run Multiplex PCR on genes that you are testing for the first time.

2.10. If you want the sequence design to be outsourced, you still need to pick a region for the probe.

2.11. Choose an area in the gene segment where the top 10 probes are located. Once your primer and probes are received – ensure that they are aliquoted into smaller volumes to avoid unnecessary freezing and thawing.

2.12. If a different PCR system is to be employed, ensure that primers and probes are designed for optimal use on that particular system. This is particularly important for genes that have not been studied before,or tested under novel experimental conditions.

2.13. .

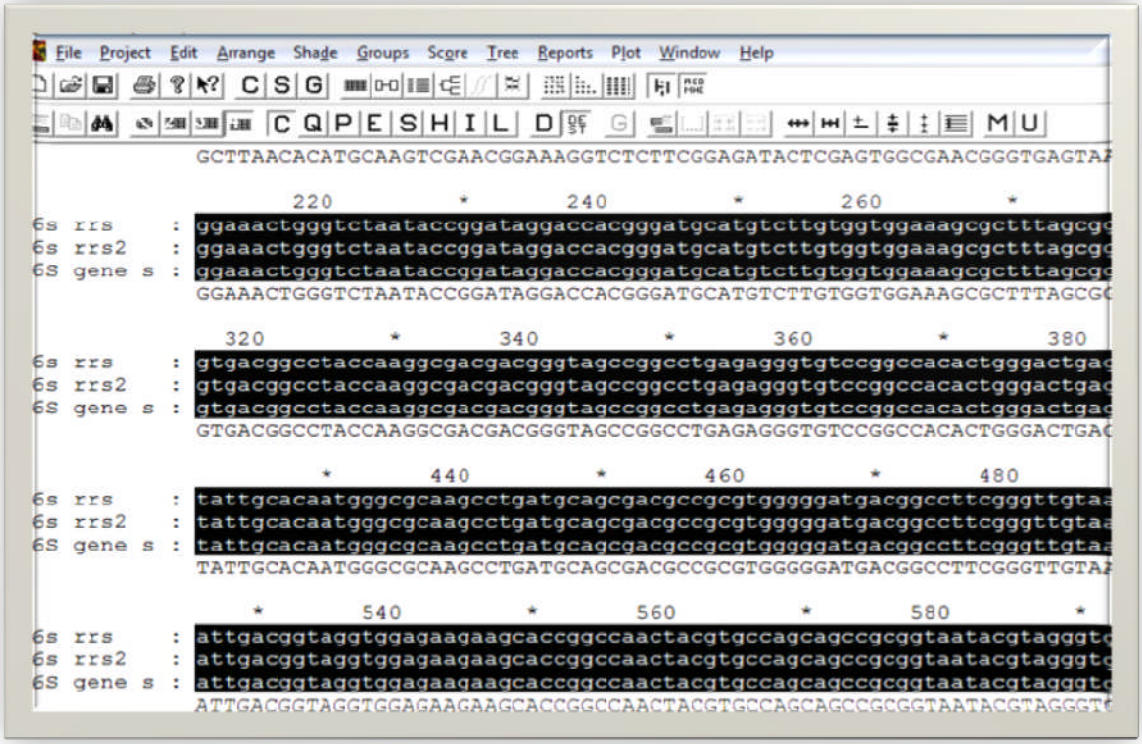


Figure A2.1 Screen Capture of GeneDoc with 16S rrs gene sequences from different databases

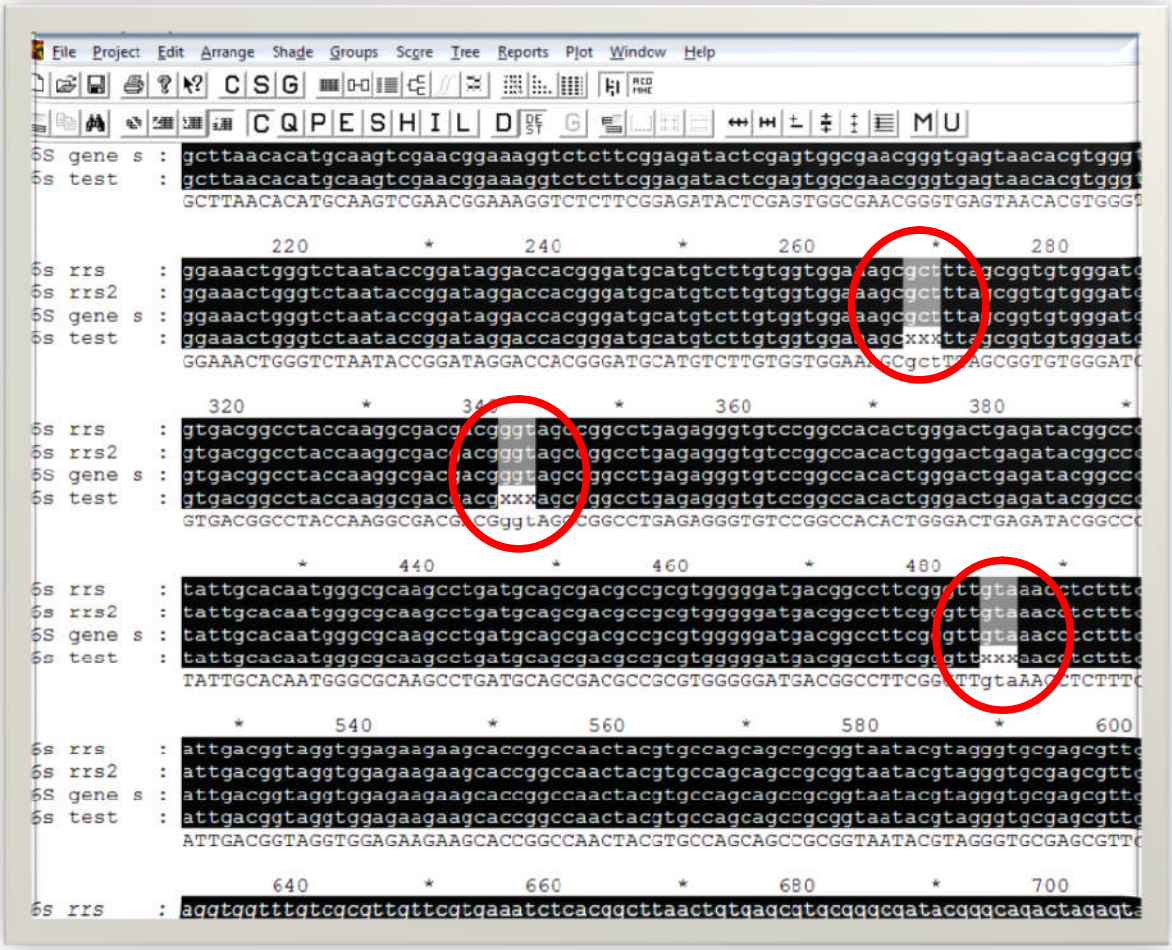


Figure A2.2 Screen capture of GeneDoc software with 16S gene sequence alignment and deliberate "x"s added in to illustrate highlighting of unconserved regions

3. RNA Isolation from H37Rv

3.1. Prepare culture tubes by inoculating log phase cells into liquid culture. Prepare sufficient tubes for experimental replicates as well as extra tubes for use in optical density determination.

3.2. If a time series is involved, inoculate extra tubes for the early time points to increase the yield if necessary. *(To convert OD600 to cell number use the conversion of 3 x10^7 cells/ml for OD600 = 1)*

3.3. Centrifuge cultures at 4500xg for 40 minutes (the supernatant must be visually clear of all floccules, repeat centrifugation if necessary to ensure that all floccules have pelleted.)

3.4. Aspirate media using a 1ml pipette

3.5. Add 0.5ml Trizol to each pellet and mix by repeated pippetting (if the pellet does not completely dissolve, split the mixture and treat as two samples, then pool the RNA at the end to ensure a cleaner product)

3.6. Transfer bacterial culture to a pre-chilled microcentrifuge tube containing 300µg glass beads (150-220µm) (it is better to use 2ml tubes for the initial stages instead of 1.5ml tubes)

3.7. Vortex tubes 5 times for 30 seconds at a time.

(Initially, Microscopically analyse lysates for verification of cell disruption (Ziehl Neelsen Staining Technique)

3.8. Incubate at room temperature for 5 minutes.

3.9. Add 0.1 ml cold chloroform and mix by shaking the tube vigorously by hand for 15 seconds.

3.10. Incubate at room temperature for 2-3 minutes.

3.11. Centrifuge the samples at 12,000 x g for 15 minutes at 4°C.

3.12. Transfer ~400 µl of the colourless, upper phase containing RNA to a fresh tube. DO NOT let the pipette tip come into contact with the opaque layer separating the organic and aqueous phases (It is better to use a 1.5ml tube with a pointed bottom to reduce the surface area for the RNA and therefore improve the dissolution process).

3.13. To remove any contaminating gDNA use DNAse 1 as follows

i. To aqueous phase in clean tube, add:

1. 10x Reaction buffer with MgCl2 : 10µl
2. DNAse 1 (1U/µl) : 10µl

ii. Incubate at 37^{O}C for 60 minutes

iii. Add 5µl EDTA and incubate for 15 minutes at 65^{O}C to inactivate DNAse Activity.

(It is better to use a dry heat method eg heating block instead of a water bath to reduce the risk of cross contamination)

3.14. Add 0.4 ml cold isopropanol to the aqueous phase to precipitate RNA. Mix by inverting the tube.

3.15. Incubate at room temperature for 10 minutes.

3.16. Centrifuge at 15,000 x g for 10 minutes at 4°C.

3.17. Remove the supernatant carefully without disturbing the RNA pellet

3.18. Resuspend the pellet in 0.8 ml 75% ethanol. Mix well by vigorously shaking by hand for 15 seconds.

3.19. Centrifuge at 7,500 x g for 5 minutes at 4°C.

3.20. Aspirate the ethanol carefully and allow all traces of ethanol to evaporate, i.e.: Air-dry the RNA pellet.

3.21. Resuspend the RNA pellet in 30µl DEPC treated water

3.22. Dissolve the RNA pellet by heating at 60-65^{O}C for 10 minutes *(The importance of this is shown by Nanodrop evaluation as shown below in figure 1*

3.23. Store RNA immediately at -80^{O}C

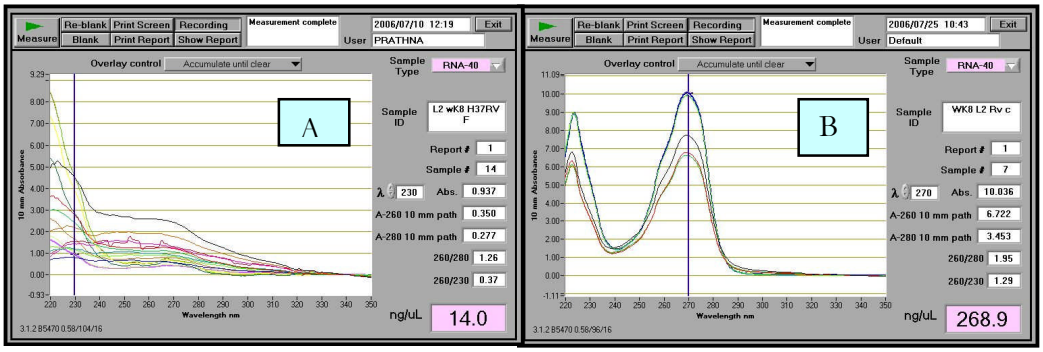


Figure A3. Nanodrop-1000 readings for RNA dissolved at 55°C (A) and the same sample after heating at 65°C (B)

4. REVERSE TRANSCRIPTION USING THE HIGH CAPACITY CDNA ARCHIVE KIT

4.1. Allow the kit components to thaw on ice

4.2. It is useful to aliquot each solution into sterile nuclease free tubes. This reduces the number of freeze-thaw cycles that the components undergo. Aliquot volumes should be enough to supply for an entire experiment with 2 reactions to spare.

4.3. Determine the volume of components needed according to the manufactures recommendations as follows

Kit Component	Reaction volume µl
10X Reverse Transcription Buffer	10
25X dNTPs	4
10X Random Primers	10
50U/ml Reverse Transcriptase	5
Nuclease free H_20	21
Total volume	50

4.4. Add 50µl of Master mix to 50µl of RNA in a 200µl PCR tube

4.5. Briefly spin tubes to eliminate air bubbles

4.6. Place tubes in a GeneAmp 9700 Thermal Cycler (Applied Biosystems)

4.7. Run the reverse transcription cycle under the following conditions which are optimized for use with this kit at 100µl reaction volume

Step (1) 25^{O}C 10 minutes**:**

Step (2) 37^{O}C 120 minutes

4.8. cDNA should be stored at -80^{O}C for long term. cDNA is more stable than RNA, therefore one should minimize the time between RNA extraction and cDNA conversion and store cDNA rather than RNA for long term use.

4.9. Although it is not essential, it is useful to determine the quantities of cDNA usinghte Nanodrop system as this will indicate if there was inhibition or failure of any kit components during the conversion process.

5. DETERMINING QUANTITY OF DNA FOR STANDARD CURVE

It is a standard practice in gene expression assays to use genomic DNA to establish a standard curve. The following description of establishing such a standard curve is based on an in-house method for the extraction of DNA and the formula derivation of quantities was obtained through Applied Biosystems Technical Literature.

The standard curve will be established based on the number of gene copies and mass of the genome of *M. tuberculosis*. The genome size for any organism can be obtained from the following website:

http://www.cbs.dtu.dk/databases/DOGS/index.html

The following formula was used to calculate the mass of the genome

Derivation of DNA Mass Formula

$$m = [\ n\ ]\left[\frac{1.096e\text{-}21\ g}{bp}\right]$$

FORMULA 1

The formula above was derived as follows

$$m = [\ n\ ]\left[\frac{1\ mole}{6.023e23\ molecules\ (bp)}\right]\left[\frac{660\ g}{mole}\right] = [\ n\ ]\left[\frac{1.096e\text{-}21\ g}{bp}\right]$$

where:
n = DNA size (bp)
m = mass
Avogadros number = 6.023e23 molecules / 1 mole
Average MW of a double-stranded DNA molecule = 660 g/mole

150.

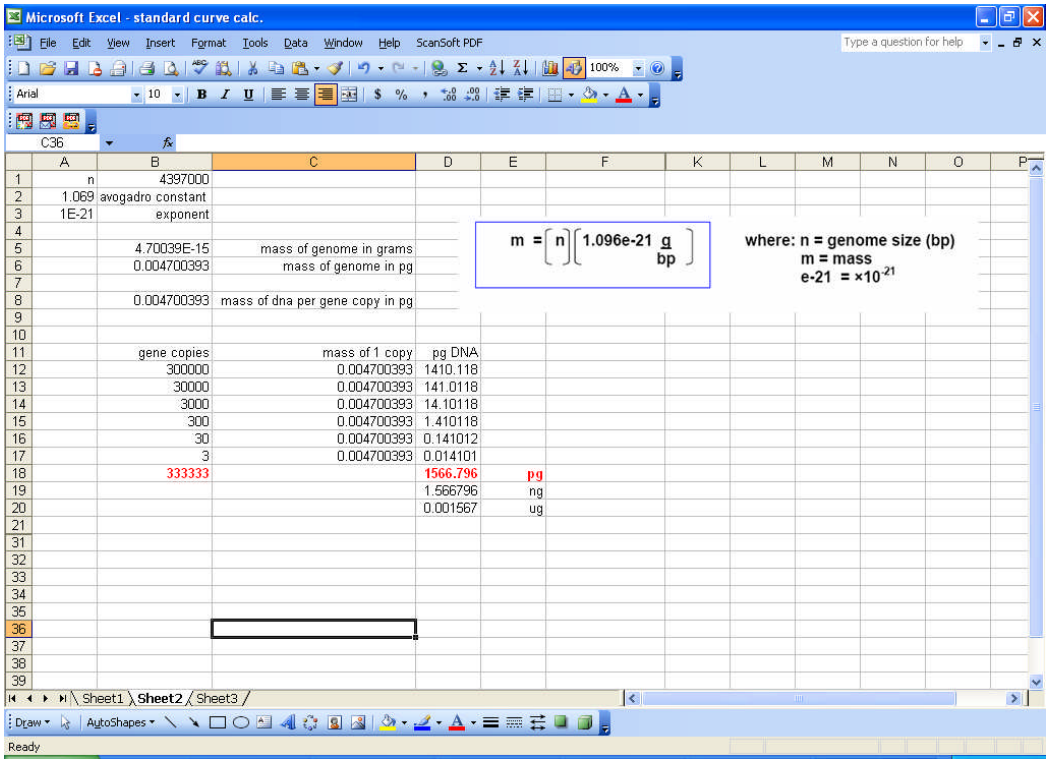
151.

Based on the mass of the DNA and genome size the number of gene copies can be derived for a specific volume of sample using the standard volume/concentration formula

$$C_1V_1 = C_2V_2$$

FORMULA 2

The volume loaded into each of the reactions of gDNA for the PCR is 11.25μl. Using the formula above and genome information from the above named website – it was determined that the volume of 100µl containing 333333 gene copies will require a concentration of 1.6µg. Quantitation parameters for the standard curve will be determined using Formula 2 indicated above.



	A	B	C	D	E
1	n	4397000			
2	1.069	avogadro constant			
3	1E-21	exponent			
4					
5		4.70039E-15	mass of genome in grams		
6		0.004700393	mass of genome in pg		
7					
8		0.004700393	mass of dna per gene copy in pg		
9					
10					
11		gene copies	mass of 1 copy	pg DNA	
12		300000	0.004700393	1410.118	
13		30000	0.004700393	141.0118	
14		3000	0.004700393	14.10118	
15		300	0.004700393	1.410118	
16		30	0.004700393	0.141012	
17		3	0.004700393	0.014101	
18		333333		1566.796	pg
19				1.566796	ng
20				0.001567	ug

Figure A5: Screen Capture of typical gene copy quantity determination for application in standard curves

Since it is nearly impossible to measure DNA of such a minute quantity, one obtains a mass of DNA and uses the ratio of DNA to gene copy number to determine the values of the standard curve. Using basic ratio and proportion, one can determine the number of gene copies per mg of DNA using the ratio of 1 gene copy: 0.0047pg as determined in the calculation shown above.

6. DNA EXTRACTION FOR STANDARD CURVE

6.1. Aliquots of 30 ml of MB7H9 culture were added to 50ml Sterilin centriguge tubes.

6.2. Mycobacterial cells were pelleted by centrifugation for 30mins at 3000xg.

6.3. The media was carefully removed by aspiration without disturbibg the pellet

6.4. The pellet was then re-suspended in 2ml of 1xTE buffer.

6.5. 500 µL aliquots were transferred to a 2ml Eppendorf tube.

6.6. The cells were heat treated for 20 minutes at 95^{O}C in a water bath.

6.7. The cells were vortexed for 30 seconds

6.8. Tubes were left on the bench at room temperature for 3 minutes to allow the beads to settle.

6.9. The supernatant was transferred to sterile Eppendorf tubes.

6.10. 50µl of 10mg/ml lysozyme was added to each tube.

6.11. The tubes were incubated in a water bath for 60mins at 37^{O}C.

6.12. 75 µl Proteinase K / 10% SDS solution was added

6.13. The tubes were incubated for 20 minutes in a water bath at 65^{O}C.

6.14. 50µl of RNAse (DNAse-free) molecular grade (Fermentas) was added

6.15. The tubes incubated at room temperature for 20 minutes.

6.16. 300µl 5M NaCl was added along with 300µl of prewarmed (65^{O}C) CTAB/NaCl.

6.17. The tubes were vortexed for 15 seconds and incubated in a waterbath for 30 minutes at 65^{O}C.

6.18. Thereafter 750µl of Chloroform/Isoamyl Alcohol (in a 24:1 ratio) was added and vortexed.

6.19. The tubes were then centrifuged for 20 minutes at 12000g.

6.20. The tubes after centrifugation presented 2 distinct phases separated by a creamy interphase that had a fatty texture.

6.21. The upper phase was transferred to a sterile 1.5ml Eppendorf tube using a 200µl pipette.

6.22. 500µl of chilled isopropanol was added

6.23. Tubes were incubated overnight at -20^{O}C to allow the DNA to precipitate.

6.24. The DNA was pelleted by centrifugation for 20 minutes at 12000g.

6.25. The isopropanol was carefully removed from the tubes without disturbing the pellet.

6.26. The pellet was washed 3x by adding 1ml of chilled 70% ethanol followed by centrifugation for 20 minutes at 12000xg.

6.27. The ethanol was removed by aspiration followed by air drying.

6.28. The pellet was re-suspendend in 40µl of 1XTE buffer.

6.29. The DNA was allowed to dissolve overnight at 4^{O}C.

6.30. DNA samples were pooled into a single tube and the concentration was then determined using the NANODROP 1000.

6.31. The following figures illustrate quantities from an extraction and corresponding graphical output from the Nanodrop-1000 as well as genomic DNA visualized by electrphoresis in 2% agarose.

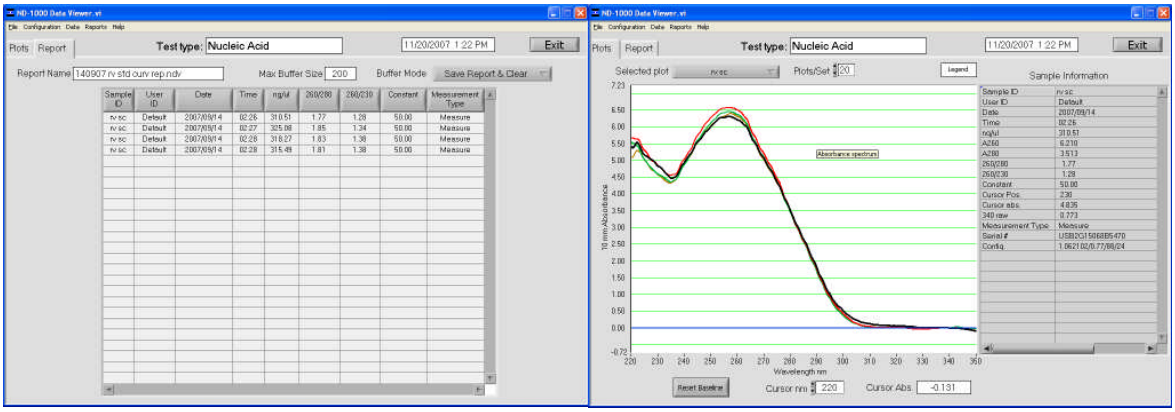


Figure A6.1: Screen Capture of genomic DNA quantitation data from Nanodrop 1000

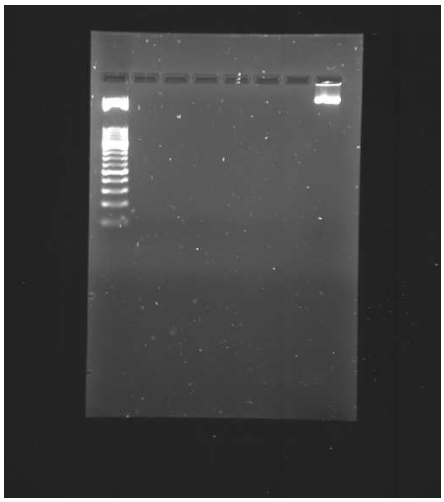


Figure A6.2: DNA in agarose gel

- Once a stock solution of DNA is established, it is essential to split the DNA solution into sinlge use aliquots, to avoid the freeze/thaw cycles on the same tube. This will contribute to the degradation of nucleic acids and affect the quantification.

7. PCR

The real-time PCR will be carried out in 96-well optical reaction plates designed for the ABI Prism 7000 sequence detection system. The genes of interest will be investigated in triplicate for each time point and in three separate runs. The software determines the gene copy numbers for each gene and these results will be normalized against the 16S RNA gene copy numbers at each respective time point. This is a standard practice in gene expression assays. The number of 16S copies is thought to represent the number of cells present at the time of the extraction. This serves to neutralize any effects of inconsistency that may be present in cell numbers at the time of extraction.

7.1 Relative Quantitation and Analysis of Raw Data

The ABI 7000SDS equipment gives a comprehensive report of each real-time PCR in each run including gene copy number of each respective gene. The gene copy numbers were divided by the copy number of the 16S rRNA gene obtained at each corresponding time point to give the normalized gene expression ratio. These normalized ratios were then graphically represented to establish the trends in gene expression.

The explanations given are based on the training which we received on-site for operation of the ABI7000SDS. The certified copy of the certificate of competence is attached (Appendix A) The ABI system generates a standard curve based on quantities that are provided by the user. The parameters of a good standard curve lie in the slope which determines the PCR efficiency and the R^2 values that should be as close to one as possible.

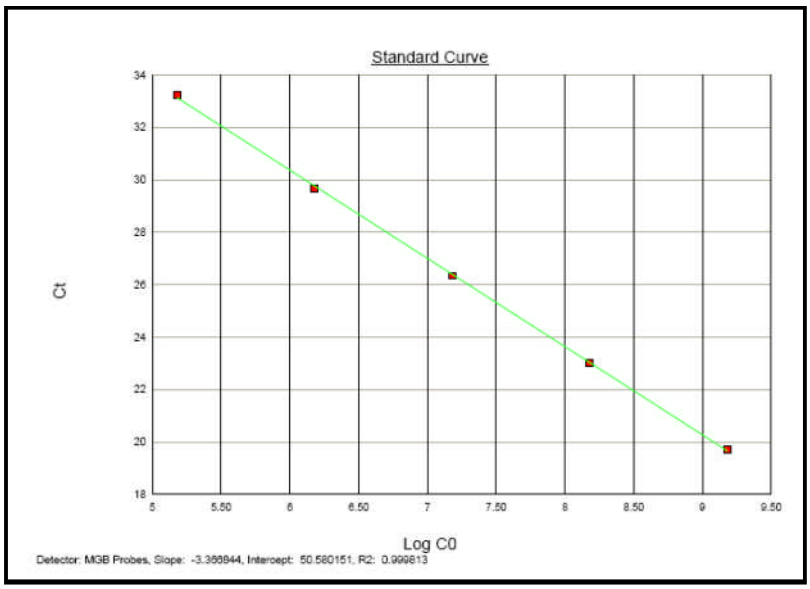


Figure A7.1 System Generated Standard Curve from a typical Run

The standard curve above is system generated and has a slope of -3.37 with an R^2 value of 0.99. This is a high quality standard curve and is more cumbersome to establish then it appears. The Taqman probes are extremely sensitive and will detect the slightest inconsistencies in pippetting technique.

The system also generates spectral data graphically to show the progression of the reaction. The following graphs represent spectral graphs at the beginning and end of the run. The 7000SDS has 4 filters that are able to filter signals at different frequencies. We use MGB probes that are labeled with FAM and a non-fluorescent quencher (NFQ) The signal of FAM is detected by filter A. The figures below indicate an increase in fluorescence over the duration of the PCR. Each line represents a single well. The lines with the highest signal are the wells with the highest template copies. Evaluating these graphs in the software can tell you whether the copy

numbers were too high, as you can see the progression of the signal for each cycle. If the copy numbers are too high the dye will peak early and then decrease towards the end. This type of data can also indicate whether the probes were not functional. Although it is not within the scope of this project, this type of data can be useful for fine examination of multiplex PCR as well.

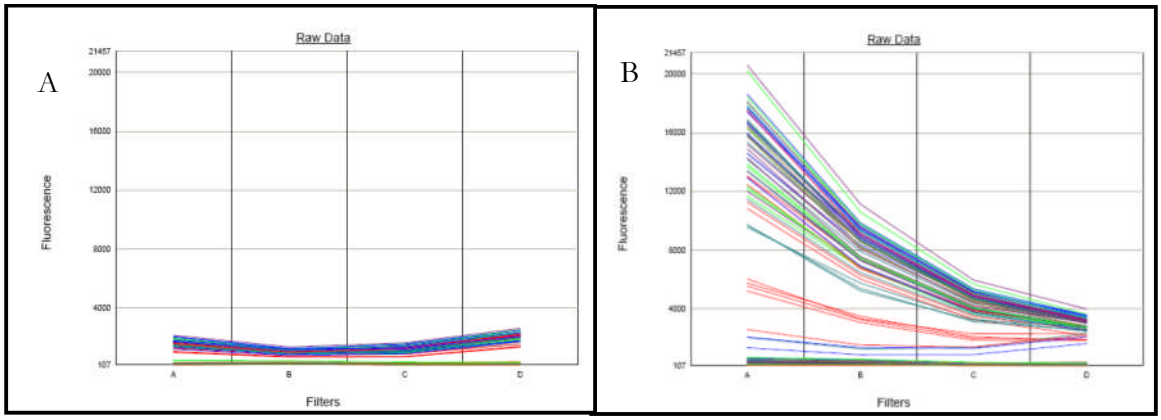


Figure A7.2 a&b: Raw Fluorescence Signal reporting at the start and end of a run

The software also generates scatter plot graphs that show the threshold cycle relative to the well positions in plates. When reactions are conducted in triplicate, one can track how close the readings are based on the pattern formed. It is also useful when looking at the standard curve as you can easily spot outliers if they are present.

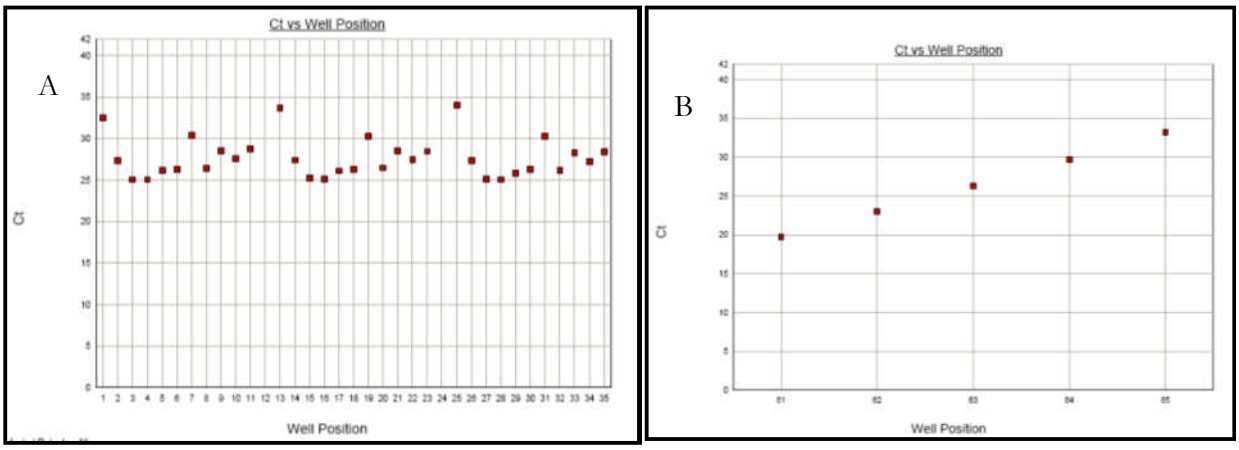


Figure A7.3 a&b: Scatter plot based on threshold cycles for a typical run of samples in triplicate and the corresponding standard curve

here are also component graphs that show the pattern of the dye in each well relative to the background dye that is present in the master mix. These graphs show the threshold cycle per well. The example below shows the first graph of a sample with a much higher target template than the second one. The higher the template the earlier the threshold cycle appears. This type of graph is useful for analysis and is available for each well. It also shows the distinctly different patterns for non-template controls.

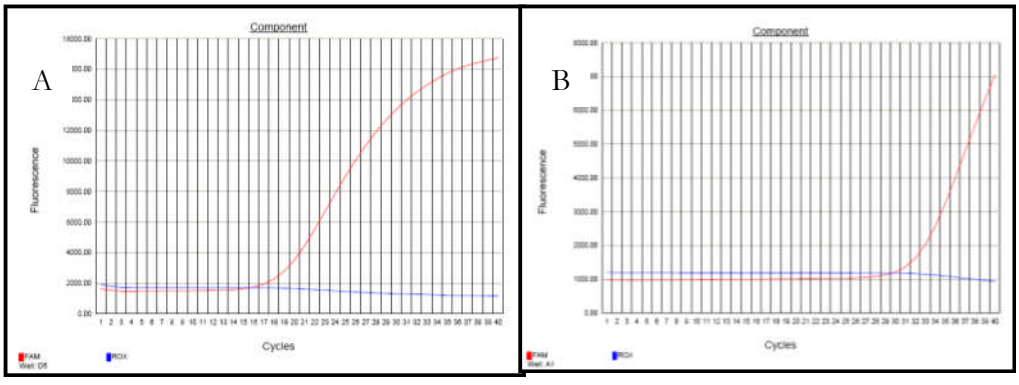


Figure A7.4 a&b: Component fluorescence data for individual wells with a high and low target template respectively

In the case of the negative control, the FAM (indicated as red in the figure) should be below that of the background dye ROX (indicated as blue in the figure) as shown below

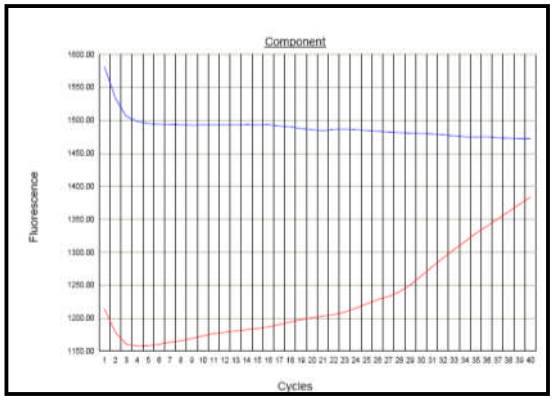


Figure A7.5: Typical fluorescence data for Non Template (Negative) Control

Finally there are the typical sigmoidal amplification curves that show the signal of the reporter dye over the duration of the PCR. These graphs can be used to view multiple wells. In the case of the standard curve, the signals should be approximately equally spaced. Negative signals are normally below the baseline. Signals that just start to increase in strength towards the end of the PCR are also considered negative. The first graph below is the corresponding amplification plot for the standard curve shown above. The other sigmoidal curves represent signals for all the wells in the plate. The negative controls are seen as virtually horizontal just above the X-axis.

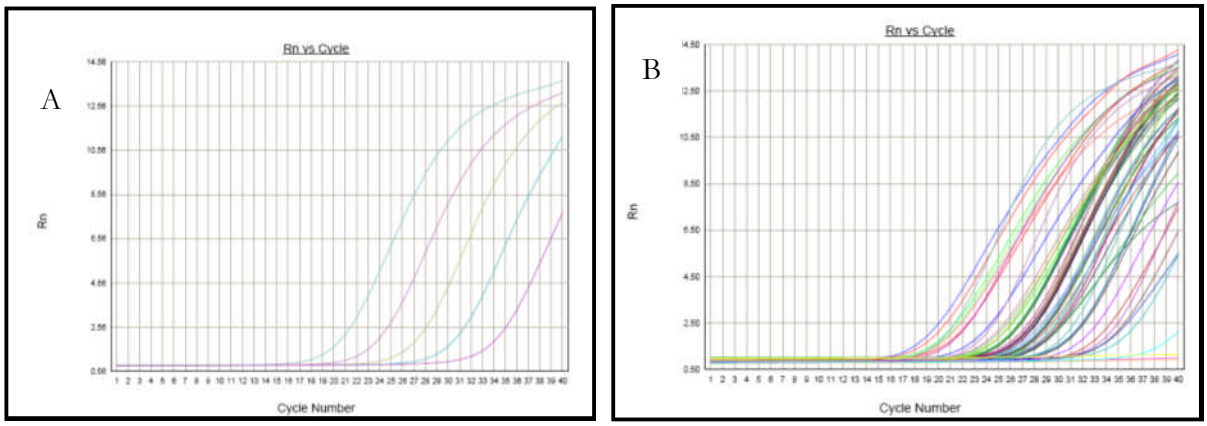


Figure A7.6a&b: amplification data for a standard curve and corresponding run.

8. **Statistical Analysis : Definitions**

Statistical definitions below are quoted directly from the websites indicated

Mann-Whitney U test is the alternative test to the t-test. Mann-Whitney U test is a non-parametric test that is used to compare two population means that come from the same population. Mann-Whitney U test is also used to test whether two population means are equal or not. Mann-Whitney U test was developed by Wilcoxon in 1945. It is used for equal sample sizes, and is used to test the median of two populations

http://www.statisticssolutions.com/methods-chapter/statistical-tests/mann-whitney-u-test/

One way Analysis of Variance (ANOVA) is a statistical technique that is used to compare the means of more than two groups. A detailed explanation is available at this site:

http://www.statisticssolutions.com/methods-chapter/statistical-tests/one-way-anova/

F-test: F-statistics is used to compare the means in one way ANOVA. F-statistics is the ratio of MS between the group variance and MS within the group variance.

http://www.statisticssolutions.com/methods-chapter/statistical-tests/one-way-anova/

The F-test in one-way analysis of variance is used to assess whether the expected values of a quantitative variable within several pre-defined groups differ from each other.

An **F-test** is any statistical test in which the test statistic has an F-distribution under the null hypothesis. It is most often used when comparing statistical models that have been

fit to a data set, in order to identify the model that best fits the population from which the data were sampled. Exact F-tests mainly arise when the models have been fit to the data using least squares. The name was coined by George W. Snedecor, in honor of Sir Ronald A. Fisher. Fisher initially developed the statistic as the variance ratio in the 1920s (**Lomax, Richard G. (2007) "Statistical Concepts: A Second Course", p. 10**

In statistics, the Kruskal–Wallis one-way analysis of variance by ranks (named after William Kruskal and W. Allen Wallis) is a non-parametric method for testing equality of population medians among groups. It is identical to a one-way analysis of variance with the data replaced by their ranks. It is an extension of the Mann–Whitney U test to 3 or more groups. Since it is a non-parametric method, the Kruskal–Wallis test does not assume a normal population, unlike the analogous one-way analysis of variance. However, the test does assume an identically-shaped and scaled distribution for each group, except for any difference in medians. Siegel and Castellan. (1988). "Nonparametric Statistics for the Behavioral Sciences" (second edition). New York: McGraw–Hill

Both the Tukey test (also called Tukey-Kramer test) and the Newman-Keuls (also called Student-Newman-Keuls test) are used to compare all pairs of means following one-way ANOVA. Although these are called post tests, they can be performed regardless of the results of the overall ANOVA results.

The Newman-Keuls test has more power. This means it can find that a difference between two groups is 'statistically significant' in some cases where the Tukey test

would conclude that the difference is 'not statistically significant'. But this extra power comes at a price. Although the whole point of multiple comparison post tests is to keep the chance of a Type I error in any comparison to be 5%, in fact the Newman-Keuls test doesn't do this[1]. In some cases, the chance of a Type I error can be greater than 5%. Another problem is because the Newman-Keuls test works in a sequential fashion, it can not produce 95% confidence intervals for each difference. Because the Newman-Keuls test has two strikes against it (doesn't control error rate, doesn't generate confidence intervals) we recommend that you use the Tukey test instead.

http://www.graphpad.com/faq/viewfaq.cfm?faq=1093

The Tukey–Kramer method is a single-step multiple comparison procedure and statistical test generally used in conjunction with an ANOVA to find which means are significantly different from one another. Named after John Tukey, it compares all possible pairs of means, and is based on a studentized range distribution q (this distribution is similar to the distribution of t from the t-test

The test compares the means of every treatment to the means of every other treatment; that is, it applies simultaneously to the set of all pair wise comparisons

(Linton, L.R., Harder, L.D. (2007) Biology 315 – Quantitative Biology Lecture Notes. University of Calgary, Calgary, AB) The Student Newman–Keuls and related tests are often referred to as post hoc

Post-hoc analysis, in the context of design and analysis of experiments, refers to looking at the data—after the experiment has concluded—for patterns that were not

specified a priori. It is sometimes called by critics data dredging to evoke the sense that the more one looks the more likely something will be found. More subtly, each time a pattern in the data is considered, a statistical test is effectively performed. This greatly inflates the total number of statistical tests and necessitates the use of multiple testing procedures to compensate. However, this is difficult to do precisely and in fact most results of post-hoc analyses are reported as they are with unadjusted p-values. These p-values must be interpreted in light of the fact that they are a small and selected subset of a potentially large group of p-values. Results of post-hoc analysis should be explicitly labeled as such in reports and publications to avoid misleading readers.

Bartlett's test for homogeneity of variance in statistics, a test to ascertain if multiple samples have the same variance (the square of the sample's standard deviation). The test, which is a standard tool in analysis of variance (ANOVA) computer programs, can be used when a single measurable variable is involved, such as when testing the efficacy of a new drug. The test was introduced by the English statistician Maurice Stevenson Bartlett in 1937

http://www.britannica.com/EBchecked/topic/1508940/Bartletts-test

Bartlett's test (Snedecor and Cochran, 1983) is used to test if k samples have equal variances. Equal variances across samples are called homogeneity of variances. Some statistical tests, for example the analysis of variance, assume that variances are equal across groups or samples. The Bartlett test can be used to verify that assumption.

Bartlett's test is sensitive to departures from normality. That is, if your samples come from non-normal distributions, then Bartlett's test may simply be testing for non-normality.

http://www.itl.nist.gov/div898/handbook/eda/section3/eda357.htm

Assumption Test

All statistical procedures have underlying assumptions. In some cases, violation of these assumptions will not change substantive research conclusions. In other cases, violation of assumptions is critical to meaningful research. All forms of statistical analysis assume sound measurement, relatively free of coding errors. It is good practice to run descriptive statistics on one's data so that one is confident that data are generally as expected in terms of means and standard deviations, and there are no out-of-bounds entries beyond the expected range.

http://faculty.chass.ncsu.edu/garson/PA765/assumpt.htm