

The Role of Ergothioneine in the Physiology and Pathogenesis of *Mycobacterium tuberculosis*

by

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## Abstract

*Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), is capable of synthesizing the small sulfur compound ergothioneine (EGT). The physiological role and regulation of EGT biosynthesis in Mtb is unknown. However, mammalian cells deficient in EGT demonstrate augmented oxidative stress and cell death, suggesting that EGT is an antioxidant.

Through this work, we identified the Mtb EGT biosynthesis genes and characterized *rv3701c* (*egtD*) to encode for a histidine methyltransferase that forms the EGT biosynthetic pathway intermediate, hercynine (*N*- $\alpha$ -trimethylhistidine). Construction of a knockout ( $\Delta$ *egtD*) demonstrated the methyltransferase to be essential for EGT biosynthesis in Mtb.

We investigated the role of EGT in protecting Mtb during macrophage infection and observed a small but significant difference in the *ex vivo* growth and survival of the  $\Delta$ *egtD* strain relative to H37Rv wildtype and the complement strains 120 h post-infection of murine macrophages. However, there was no difference in survival of the  $\Delta$ *egtD* mutant in human THP-1 cells over this time period suggesting an alternative role for EGT in Mtb physiology and pathogenesis.

Upon further analysis we found EgtD to be phosphorylated by and interact with the Mtb Serine/Threonine Protein Kinase PknD. Phosphorylation of EgtD both *in vitro* and inside *Escherichia coli* identified phosphorylation at site Thr-213, permitting us to generate an EgtD phosphomimetic and phosphoablative mutant strains with our  $\Delta$ *egtD* mutant. *In vitro* analysis, demonstrated phosphorylated EgtD to produce significantly lower quantities of methylated histidine relative to the non-phosphorylated form. Further quantification of intracellular EGT levels in the Mtb EgtD phosphomutants and Mtb PknD transposon mutant identified PknD to negatively regulate EGT biosynthesis. From these results, we identified that EGT biosynthesis is

up-regulated in response to nutrient starvation. Under these conditions, the Mtb  $\Delta egtD$  mutant was unable to maintain viability compared to its parental wildtype and complement strains. As starvation induces a non-replicative state in Mtb, these findings indicate that EGT plays a role in mediating persistent infection or disease latency. Further metabolic analysis identified Mtb intracellular EGT levels to be directly correlated with carbon source type and availability, suggesting a role for EGT in long-term energy storage.

## Preface

Parts of this thesis have been published in the peer-reviewed journals listed below in chronological order:

1. **Richard-Greenblatt, M.**, Bach, H., Adamson, J., Peña-Diaz, S., Li, W., Steyn, A.J.C., Av-Gay, Y. (2015). Regulation of ergothioneine biosynthesis and its effect on *Mycobacterium tuberculosis* growth and infectivity. *Journal of Biological Chemistry*. 290, 23064-76.

In the aforementioned manuscript, Y. A.-G. and M. R.-G. conceived and coordinated the study and wrote the paper. M. R.-G., H. B., A. J. C. S., and Y. A.-G. designed experiments. M. R.-G. performed experiments. J. A. designed and performed ergothioneine mass spectrometric analysis. S. P.-D. assisted with mouse macrophages infection studies described in Figure 12A. L. W. cloned PknD for co-expression studies in *E. coli* found in Section 5.6. A version of this published work is located in Chapter 2 (Sections 2.1-2.10) and Chapters 3-5.

2. Saini, V., Cumming, B.M., Guidry, L., Lamprecht, D., Adamson, J.H., Reddy, V.P., Chinta, K.C., Mazorodzo, J., Glasgow, J.N., **Richard-Greenblatt, M.**, Gomez-Velasco, A., Bach, H., Av-Gay, Y., Hyungjin, E., Rhee, K., and Steyn, A.J.C. (2016). Ergothioneine maintains redox and bioenergetic homeostasis essential for drug susceptibility and virulence of *Mycobacterium tuberculosis*. *Cell Reports*. 14, 572-585.

In the aforementioned manuscript, I designed and performed the experiments analyzing EGT levels in whib3 mutant the presence of various carbon sources. A modified version of these published results can be found in Figure 19B.

3. **Richard-Greenblatt, M.**, Av-Gay, Y. Epigenetic control of the persistence phenotype by phosphorylation. In W.J. Jacobs, H. McShane, V. Mizrahi, I. Orme (Eds.). *The Signature Problem of TB: Persistence*. Washington, DC: American Society for Microbiology. *In press*.

The following book chapter is a review on our current knowledge of the involvement of *M. tuberculosis* STPKs in establishing a persistent infection in the host. Both Dr. Y. Av-Gay and I participated in writing and editing the manuscript. Modified version of this published work can be found in Section 1.4.

All experiments performed in this thesis were conducted in accordance with the University of British Columbia Research Policies and Procedures, the University Biosafety Committee, the Public Health Agency of Canada guidelines, and with approval from the University of British Columbia Office of Research Services. Biohazard Approval Certificate: B14-0160 (Containment level 2 laboratory) and B14-0157 (Containment level 3 laboratory). Work performed with radioactive materials was approved by the University of British Columbia Advisory Committee on Radioisotopes and Radiation Hazards. Radioisotope License: MEDI-3175-18.

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## List of Abbreviations

ADS	Albumin, Dextrose, Sodium Chloride
Amp	Ampicillin
ART	Antiretroviral Therapy
Asp	Aspartate
ATP	Adenosine Triphosphate
BCG	Bacille Calmette-Guérin
bp	Base pair
cAMP	cyclic-Adenosine Monophosphate
CD4 <sup>+</sup>	Cluster of Differentiation 4
CFP10	10-kDa Culture Filtrate Protein
CFU	Colony Forming Unit
Cpm	Counts per minute
<i>C. purpurea</i>	<i>Claviceps purpurea</i>
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
DR	Drug Resistant
DS	Drug Sensitive
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EGT	Ergothioneine
EgtA	$\gamma$ -Glutamylcysteine Ligase
EgtB	Sulfoxide Synthase

EgtC	Glutamine Aminotransferase
EgtD	Histidine Methyltransferase
EgtE	Pyridoxal 5'-Phosphate Protein
Egt1	Ergothioneine Biosynthesis Protein 1
Egt2	Ergothioneine Biosynthesis Protein 2
ESAT6	Early Secretory Antigenic Target-6
ESSC	Ergothioneine-Cysteine Mixed Disulfide
ESSE	Ergothioneine Disulfide
ESI LC-MS/MS	Electrospray Ionization Liquid Chromatography Tandem Mass Spectrophotometry
FAS	Fatty Acid Synthase
FBS	Fetal Bovine Serum
Fe <sup>2+</sup>	Ferrous Iron (II)
gDNA	Genomic Deoxyribonucleic Acid
GDP	Guanine Diphosphate
GST	Glutathione S-Transferase
GSH	Glutathione (reduced)
GSSG	Glutathione Disulfide (oxidized)
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
His	Histidine
HPLC	High Performance Liquid Chromatography

Hyg	Hygromycin
ICAM-1	Intracellular Adhesion Molecule-1
IFN- $\gamma$	Interferon- $\gamma$
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible Nitric Oxide Synthase
IPTG	1-Thio- $\beta$ -D-galactopyranoside
JNK	C-Jun N-Terminal Kinase
Kan	Kanamycin
kDa	KiloDalton
LB	Luria Bertani
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrophotometry
LmbT	Glycosyltransferase (Lincomycin A Biosynthesis)
LMW	Low Molecular Weight
LTBI	Latent Tuberculosis Infection
<i>M. africanum</i>	<i>Mycobacterium africanum</i>
<i>M. bovis</i>	<i>Mycobacterium bovis</i>
<i>M. leprae</i>	<i>Mycobacterium leprae</i>
mDHFR	Murine Dihydrofoltate Reductase
MDR-TB	Multi-Drug Resistant Tuberculosis
MHC-II	Major Histocompatibility Complex Class II
min	Minute
M-PFC	Mycobacterial Protein Fragment Complementation

MPP <sup>+</sup>	Methyl-4-Phenylpyridinium Ion
MSH	Mycothiol
<i>M. smegmatis</i>	<i>Mycobacterium smegmatis</i>
Mtb	<i>Mycobacterium tuberculosis</i>
MTBC	<i>Mycobacterium tuberculosis</i> Complex
<i>m/z</i>	Mass-to-Charge-Ratio
Na <sup>+</sup>	Sodium
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
<i>N. crassa</i>	<i>Neurospora crassa</i>
NO	Nitric Oxide
NOX2	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NRP	Non-Replicating Persistence
OADC	Oleic Acid, Albumin, Dextrose and Catalase
OCTN1	Organic Cation Transporter 1
OD	Optical Density
ORF	Open Reading Frame
p38	p38 Mitogen-Activated Protein Kinase
pSer	Phosphoserine
pThr	Phosphothreonine
pTyr	Phosphotyrosine
PAS	Para-Aminosalicylic Acid
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PDIM	Phthiocerol Dimycocerosate
Pkn (A,B,D-L)	Protein kinase (A,B,D-L)
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
SAH	<i>S</i> -adenosyl homocysteine
SAM	<i>S</i> -adenosyl methionine
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Ser	Serine
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
STPK	Serine/Threonine Protein Kinase
TAG	Triacylglycerol
TB	Tuberculosis
TCA	Tricarboxylic Acid Cycle
Thr	Threonine
TLC	Thin Layer Chromatography
TMP	Trimethoprim
Tn	Transposon
TNF	Tumour Necrosis Factor
Tyr	Tyrosine
VCAM-1	Vascular Cell Adhesion Molecule-1
WT	Wildtype



XDR-TB

Extensively Drug-Resistant Tuberculosis

## Acknowledgements

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During my studies I was very fortunate to travel to Durban, South Africa to complete a portion of my research as well as gain knowledge on the clinical and societal aspects of TB.

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## Dedication

To all that have worked toward reducing the suffering of those affected by tuberculosis. Your work and compassion has been most courageous and admirable.

*“The Great White Plague, tuberculosis, is never done with its insidious work; for numberless years it has been silently, continuously and unrelentingly pursuing its destructive course, never remitting its deadly work, always reaching out for new victims, and today it is the most universal scourge of the human race.”*

Otis, Edward O. *The Great White Plague: Tuberculosis*. New York: Thomas Y. Crowell & CO, 1909. Print, p. 2.

# **Chapter 1: Introduction**

## **1.1 Tuberculosis**

### **1.1.1 Historical Significance of Tuberculosis**

Often the importance of a disease to mankind is measured by the number of fatalities it causes. Therefore, tuberculosis (TB), responsible for approximately a billion deaths in the last two centuries, must be considered one of the most significant human diseases of all time (1). Even as we attempt to grasp the magnitude of one billion lives, our imagination must extend further over many more centuries and beyond written history to begin to appreciate the extraordinary nature of the disease itself. Nowhere in the ancient communities of Eurasia is there a record of its beginning. Throughout history it remained a familiar fear, written in the ancient texts of Rigveda and Huangdi Neijung (2).

The later stages of TB can sometimes result in disfiguring changes that are easily recognizable in ancient skeletal remains of humans. Particularly, the ulcerative destruction of the vertebrae in TB causes the collapse of the upper region of the spine, generating a characteristic “hunchback” (3). Such pathology has been observed in the spines of Egyptian mummies and in paintings of hunchback figures on the walls of tombs, suggesting TB was endemic to Egypt as early as 5000 BC (2,4). Yet even further in history, morphological and molecular analysis of the remains of a population living in one of the first known villages of agricultural and animal domestication (9000 BC) confirmed the presence of TB (5) suggesting that the disease has been present since the beginning of civilization.

Establishment of the disease in mainland Europe occurred between 2500-1500 BC, but is not predicted to have manifested itself in Britain for a thousand or more years later (6).

Throughout succeeding centuries, the prevalence of the disease increased markedly, and by the

mid-17<sup>th</sup> century it was recorded in the London Bills of Mortality that one in five of deaths in the city was due to TB. At the turn of the 19<sup>th</sup> century, the global death rate was an estimated seven million people a year. Both New York and London, the two most affected cities shared fifty million active cases, and at least half the world's population was latently infected (1).

Up until this point the cause of TB remained entirely unknown. It was not until the French military doctor, Jean-Antoine Villemin (1827-1892) showed that TB could be transmitted from infected human tissue to animals in 1865 (7). Still, no treatment existed and by the end of the nineteenth century there was a growing fear that TB may destroy European civilization (1).

### **1.1.2 Robert Koch and the Tubercle Bacillus**

On the evening of March 24<sup>th</sup>, 1882, Dr. Robert Heinrich Herman Koch (1843-1910), a German medical doctor gave a lecture titled "*Die Tuberculose*" (On Tuberculosis) to the Physiological Society in Berlin (8,9). "The nature of TB has been studied by many, but this has led to no successful results." Acknowledging the work of the previous investigators Villemin, Klebs, Cohnheim and Salomonsen, he reminded the audience that none of the previous investigations led to a bacterial cause for TB. Traditional staining methods (methylene blue) used for the demonstration of pathogenic microorganisms in other diseases were unsuccessful. Every experiment devised to identify and culture the TB infective agent had failed. For his own studies, Koch had begun by using the same staining and culturing methods; however, a number of observations led the brilliant scientist in another direction. Following staining with methylene blue, Koch counterstained his slides with vesuvin, a diazo dye, to remove the deep blue stain which coloured everything. At this point, when looking under a microscope, the structures of the animal tissues were brown, but for those known cases of TB, the presence of beautiful blue

tubercle bacteria could also be observed. When Koch extracted the tuberculous material from human tissues, he could grow the microorganism, although very slowly, on solidified serum slopes. Upon injecting these cultures into test guinea pigs, in all cases, the disease which developed was the same, and the bacteria obtained from the guinea pigs was identical to that of the serum slopes. Through this set of experiments, Koch not only identified the etiological agent of TB, but conceived his famous postulates, known as Koch's postulates (9). Even today, Koch's lecture is generally accepted as the most important in medical history, and was the first step toward a cure for TB.

### **1.1.3 Drug Discovery**

Remaining as a leading cause of death at the turn of the twentieth century, discovering a cure for TB was the most urgent medical problem faced by the world. Despite the discovery of arsphenamine (Salvarsan), penicillin and Prontosil (the first sulfonamide) in the early 1900s, it was soon determined that a cure for TB was not so simple. None of these first antimicrobial agents were active against TB. As a result, the early optimism associated with Koch's discovery was replaced by pessimism. By the 1930s, few individuals believed a cure would ever be possible (1).

Dr. Selman Waksman (1888-1973), one of the most prominent soil microbiologists of his time, recognized that a large number of disease-causing Gram-negative bacteria were resistant to sulfonamides and penicillin. Waksman was already well aware that soil microorganisms had anti-microbial potential due to the discovery of penicillin (10), gramicidin (11), and his own observations (12,13).

In 1943, Waksman hired the graduate student, Albert Schatz, to search for an antibiotic agent possessing broader range activity, especially against Gram-negative bacteria.

Streptothricin, which was previously extracted from *Streptomyces lavendulae* was shown to kill both Gram-positive and –negative bacteria (14,15). These findings led Schatz to focus on *Streptomyces* species for new antibiotic substances for his studies. One morning, only a couple of months after commencing his PhD, Schatz identified a unique greenish grey colony of bacteria grown from a sample of soil that soon became known as *Streptomyces griseus* (16). Like all the other bacteria Schatz isolated from soil samples, he analyzed the growth of various infectious bacteria in the presence of *S. griseus*. To Schatz's amazement, the zones of inhibition were millimetres and sometimes even centrimetres away from the grey-green streaks of *S. griseus*. It was clear that Schatz had discovered his first antibiotic, which he called streptomycin. Upon further testing, streptomycin was also found to kill the previously resistant staphylococcus and Gram-negative bacteria (16). Due to its broad range of antimicrobial activity, Waksman and Schatz decided to test streptomycin's antimicrobial activity as a treatment for TB. Schatz grew the TB bacilli on agar slants to which he had added streptomycin. After three weeks the control slants clearly showed the TB bacillus, meanwhile those containing streptomycin were devoid of growth (16). Despite these seemingly miraculous findings, the two scientists knew based on Waksman's previous work with streptothricin that, once tested in animals, streptomycin could exhibit significant toxicity (17). The anti-tubercular activity was tested in animal trials with streptomycin in guinea pigs, where it was found that Mtb infection was abolished and the guinea pigs appeared to have suffered little side-effects from streptomycin (18). A year after these animal trials, streptomycin treatment in the first human suffers from TB took place (19). Shortly prior to this publication, Waksman received a telegram from Feldman and Hinshaw who conducted the studies stating that: "Our streptomycin studies... were fully confirmed



experimentally and clinically, establishing this as the first effective chemotherapeutic remedy for tuberculosis. Hearty congratulations.” (20)

The celebrations for streptomycin’s success in treating TB were short lived. Within five years of its clinical appearance, streptomycin, which had previously saved the lives of hundreds of thousands, was no longer helpful. In 1948, a publication by the British Medical Research Council compared the outcome of fifty-five patients with pulmonary TB treated with streptomycin to fifty-two patients treated solely with the sanatorium regime (21). Within the first six months, most patients treated with streptomycin showed clearing of TB in their chest X-rays, with only four deaths in this time frame. In contrast, fourteen patients treated with the sanatorium regimen died within the first six months and only 8% showed improvement. As one might expect, these initial findings demonstrated the potency of streptomycin, but a five year follow-up of the same group told a more harrowing tale. Of the fifty-two patients prescribed solely to the sanatorium regimen, seventeen remained, and of those fifty-five treated with streptomycin, twenty-three.

The results were heartbreaking. Streptomycin was not a cure; it reduced, usually only temporarily, the severity of the disease. The American bacteriologist William Steenken Jr. explained these results through drug resistance, as when he monitored the patients treated with streptomycin the majority had germs that were sensitive to the antibiotic. However, after four months of therapy, 90% of patients now had sputum containing streptomycin resistant bacteria (22). Furthermore, in approximately 10% of patients, streptomycin caused ototoxicity, leaving them deaf or permanently dizzy (23).

During the development of streptomycin, the Swedish physician and chemist Jorgen Lehmann was also investigating the use of para-aminosalicylic acid (PAS) in the treatment of

TB. It had been previously shown by Bernheim that salicyclic acid was able to increase the oxygen consumption of the TB bacillus (24), and thus Lehmann deduced that by slightly altering the structure of aspirin he could develop a competitive inhibitor. PAS worked wonderfully against TB in animals (25). The drug not only improved their health in comparison to the control group, it was also free of poisonous side-effects. However, from 1944-1947, the discovery of PAS was overshadowed by streptomycin due to difficulties in its manufacturing. Once mass production was made possible, doctors worldwide could receive large quantities of PAS for clinical trials. PAS was effective in treating patients suffering from severe pulmonary TB that had disseminated to the bowel (26). Later work by Carstensen further showed that PAS could cure other forms of miliary TB (27), and additional studies to follow would confirm these findings (28,29). Despite its observed effectiveness, these trials also ended with a concerning outcome. Although not as dramatically seen with streptomycin, one-third of patients were harbouring bacilli resistant to PAS (30). However, studies performed by the British Medical Council (31) showed that when PAS was given with streptomycin the risk of developing resistance was significantly lower six months following the start of treatment, indicating that the use of combination therapy could be a superior treatment for TB.

Having spent over a decade trying to develop an anti-TB drug derived from sulfonamides, Gerhard Domagk, a German Nobel Prize winner for his work in developing the first commercially available antibiotic, stood in front of his audience at the 12<sup>th</sup> International Congress of Pure and Applied Chemistry. Displaying four drug groups effective against TB, gram for gram (in terms of effectiveness) he demonstrated streptomycin as ten times more effective than PAS for killing the TB bacteria, thiosemicarbazones ten times more than streptomycin, and a new compound that was ten times more potent than thiosemicarbazones.

Domagk's new discovery, called isoniazid (32), had a similar healing effect to that of PAS and streptomycin combined (33). Isoniazid also had a low incidence of side-effects, and was relatively inexpensive to produce. Yet, similar to when PAS and streptomycin were used individually during therapy, the tuberculosis bacteria quickly developed resistance to isoniazid (33). At this point, it became apparent that regardless of the antibiotic used, monotherapy was not a cure for TB.

In 1958, Sir John Crofton, a TB expert at the University of Edinburgh discovered that in patients not harboring a drug resistant strain, adherence to a prolonged combination of all three drugs resulted in cure (34). In theory, if a bacterium develops resistance to one of the drugs during treatment, the remaining two drugs would still be adequate to eradicate the infection, but Crofton knew this was not enough. In his 1960 lecture to the Royal College of Physicians in London, Crofton cautioned that combination therapy alone could not defeat the TB epidemic (35). Necessary action included pasteurization of milk, mass radiography of the disease followed by adequate triple drug therapy for all infected, quarantine of all patients until no longer infectious, and general population living standards to reduce overcrowding. To prove this idea, Crofton and colleagues screened 80% of the adult population for TB in Edinburgh and treated all known infections (36), and within eight years the most common cause of death in Edinburgh had been brought under control and the presence of TB was virtually abolished (1).

In a short time, Crofton's triple therapy would be composed of a more powerful combination. In 1963, the pharmaceutical company Lepetit extracted Rifamycin B from *Streptomyces mediterranei* (known today as *Amiclatopsis rifamycinia*) and found it to be as equally potent as isoniazid (37). Kuschner and colleagues (38) discovered pyrazinamide, and Lederle laboratories ethambutol (39,40). Streptomycin and PAS were replaced by Rifamycin B,

ethambutol and pyrazinamide in modern therapy, meanwhile isoniazid remains. To these first six antibiotics, others with less potency or greater toxicity have been added to the list and remain as second and third-line therapies. With the advent of the new drug combination, and the implementation of the all new embracing approach initiated by Crofton across the developed world, although taking almost two decades to achieve, today it appears that people no longer die from TB. It became a disease of the past.

#### **1.1.4 How the Battle Against TB was Won – and Lost**

Although TB had become greatly reduced in developed countries it has never been eradicated. Despite our current efforts and the determination of all of those before us we have not been successful. In reality, extreme poverty, ignorance, war, corruption and the lack of medical infrastructure observed in the third world has allowed the disease to evolve to appalling proportions. The World Health Organization (WHO) reports that TB remains the primary cause of death due to an infectious agent worldwide. In 2014, 1.5 million people succumbed to the disease, while another 9.6 million were newly infected. It is estimated that one-third of the world's population has the asymptomatic form of the disease (latent TB), to which 13 million individuals have active TB at any point in time. Currently, 22 countries are referred to as “high burden” for TB, constituting 82% of global TB prevalence. Of these locations, India (23%), China (10%) and Indonesia (10%) account for the largest number of cases, yet Africa has the greatest burden relative to its population (41).

The arena of the Third World not only prevents the eradication of TB, but has also led to the emergence of drug-resistant strains. Table 1 outlines our current challenges in the global control of TB. It is estimated that 20% of previously treated cases and 3% of new cases are resistant to both isoniazid and rifampin (derived from the early Rifamycin), which is known as

multidrug-resistant TB or MDR-TB. Of these cases, 10% are considered extensively drug-resistant (XDR-TB) where patients are unresponsive to at least one injectable second-line drug (kanamycin, amikacin and capreomycin) plus a fluoroquinolone. Even more regrettably, we are beginning to see cases of total drug-resistant TB develop in areas such as South Africa, China, Russia and India.

**Table 1. Challenges for the Global Control of TB.** Adapted from (42).

<b>Diagnostics</b>	<ul style="list-style-type: none"> <li>• Sputum microscopy, main diagnostic in endemic areas: Not sensitive (<math>&gt;10^4</math> microorganisms/ml sputum)</li> <li>- Cannot differentiate between DS- from DR-TB</li> <li>- Not effective in diagnosis of pediatric TB, HIV+ patients and extra-pulmonary TB</li> <li>• Culture based methods are the most accurate, but require biosecure laboratories and trained personnel which are costly</li> <li>• Genotypic resistance offers rapid turnaround and fair accuracy but high probability of false-positive diagnosis</li> <li>• GeneXpert Mtb/RIF assay only identifies rifampicin resistance</li> </ul>
<b>Infection control</b>	<ul style="list-style-type: none"> <li>• Prevention of aerosol transmission in health care facilities and hot-spots in the community is difficult due to high burden</li> <li>• Early diagnosis and treatment are essential to decrease transmission, difficult due to slow treatment response</li> <li>• Lack of infrastructure and funds for prolonged isolation of patients</li> </ul>
<b>HIV co-infection</b>	<ul style="list-style-type: none"> <li>• HIV epidemic has resulted in poorer treatment outcomes, and higher rates of recurrent disease</li> <li>• Shared toxicities for second-line TB and antiretrovirals</li> <li>• HIV patients susceptible to TB immune reconstitution disease</li> </ul>
<b>Complicated treatment</b>	<ul style="list-style-type: none"> <li>• Long treatment duration</li> <li>- 6-9 months DS, &gt; 2 years DR</li> <li>• Second-line treatment expensive, more toxic, less potent</li> <li>• Optimal drug regimens for DR cases poorly characterized</li> </ul>
<b>Inadequate vaccine</b>	<ul style="list-style-type: none"> <li>• BCG vaccine ranges from 0-80% effectiveness in protecting against adult pulmonary TB (43)</li> </ul>
<b>Limited funding</b>	<ul style="list-style-type: none"> <li>• Cost of treatment is high</li> <li>• Most endemic countries cannot afford to maintain infrastructure and services to optimally manage patients</li> </ul>
<b>Lack of political commitment</b>	<ul style="list-style-type: none"> <li>• Lack of TB awareness in general</li> <li>• Poor quantification of true disease burden</li> <li>• No immediate threat perceived (Western countries)</li> <li>• No international political pressure</li> </ul>

Abbreviations: TB, tuberculosis; HIV, human immunodeficiency virus; DS, drug sensitive; DR, drug resistant; BCG, Bacille Calmette-Guérin.

The emergence of the human immunodeficiency virus (HIV) epidemic has also added another unexpected twist to the TB dilemma due to co-infection. The progression of HIV results in the body being in a state of chronic immune inactivation, enhancing one's susceptibility to infection. During latent TB, the bacteria is contained within a granuloma (collection of immune cells), but in the case of TB-HIV co-infection, the immune system breaks down resulting in reactivation and systemic dissemination of the infection. Consequentially, for individuals with HIV, TB is the number one cause of death. Of further concern are the complications arising out of therapy for TB-HIV co-infection. Restoration of immune competence by antiretroviral therapy (ART), results in a hyper-immune response to the TB bacilli, known as immune reconstitution inflammatory syndrome. Co-administration of TB chemotherapy and ART can also result in severe side-effects such as hepatotoxicity and peripheral neuropathy. Furthermore, rifamycins are strong inducers of the liver metabolic enzyme cytochrome P450, causing enhanced antiretroviral clearance and suboptimal HIV suppression (44). As a result, the challenges associated with treating TB-HIV co-infection increases TB mortality rates, transmission, and risk of disease reactivation.

Our neglect of TB as a global health priority has not only been morally indefensible, but reckless. Perhaps the Western World's fortune in the lack of epidemiological importance of TB has made us ignorant to this global health threat, but in the era of globalization it was only a matter of time that TB would come knocking at our backdoor. Since the advent of TB chemotherapy, the annual incidence of TB has dropped to 4.7 per 100,000 people in Canada and

has remained consistent for the last decade (45). However, foreign-born individuals continue to be the main source of our cases (71%) (45). The same holds true for the United States (46) and United Kingdom (47) where migrants from TB endemic countries account for 63% and 73% of cases, respectively, further demonstrating the global spread of the disease. The influx of migrants from TB endemic locations is also a major contributor to the incline of drug-resistant cases in low incidence countries (48). Our measures to detect and treat latent infection of individuals can only be deemed futile as our present prophylactic treatments with isoniazid or rifampicin are likely ineffective for cases of MDR. Consequentially, we continue to observe countless MDR outbreaks throughout the developed world, with direct costs for treatment of each case approximating \$134,000 US (49). Thus, if we do not take on the challenge of controlling TB in the undeveloped world now, the work and dedication of all those before us to eradicate this disease will have been for nothing.

## **1.2 Biology of *Mycobacterium tuberculosis***

In 1882, Prof. Robert Koch identified the bacterium *Mycobacterium tuberculosis* (Mtb) to be the causative agent of TB in humans (9). When Koch peered down his microscope he observed his stained bacteria in brilliant blue take the long slender shape of a bacilli that barely reaches 3 millionths of a meter in length. At the same time, he noticed many of Mtb's characteristics were similar to another bacterium, *M. leprae*, which causes leprosy. Indeed, both of these microorganisms are members of the genus *Mycobacterium* that belong to the suborder Corynebacterineae and order of Actinomycetales.

Mycobacteria are predominantly environmental organisms, living in soil and aquatic environments; however, several transitions from the environment to pathogenicity have occurred in this genus. Mtb is a member of a group of mycobacteria known as the *M. tuberculosis*

complex (MTBC), which is believed to have risen from the clonal expansion of a smooth tubercle bacillus progenitor population some 2.5 million years ago (50). In addition to Mtb, the MTBC also includes the human pathogen *Mycobacterium africanum* and a clade of animal-infecting mycobacteria (such as *Mycobacterium bovis*), all of which exhibit low genetic diversity (99.9% nucleotide similarity and identical 16S rRNA) (51,52). Genomic data of smooth tubercle bacilli indicate that the origin of MTBC is from a previous human-infecting mycobacterial species derived from East Africa, and human migration out of Africa led to the emergence of seven separate Mtb lineages. During this period, a strain of Mtb evolved to infect non-human mammals, generating the animal-adapted *M. bovis* ecotypes approximately 70,000 years ago. These genomic studies indicate that human TB did not arise from zoonosis, and mycobacterial diseases likely predates *Homo sapiens* (50).

Mycobacterial pathogens can also be divided into two groups based on growth rate: slow-growing and fast-growing species. Rapid growers, such as *Mycobacterium fortuitum*, generally show visible growth from dilute inocula within 3-4 days. In contrast, those slow-growing species, including Mtb, have a doubling time at barely a thirtieth the rate (doubling time ~ 20 hour) of well-studied bacteria and are visible after three weeks of growth. Despite growing slowly, the metabolic rates of mycobacteria are comparable to fast-growing bacteria. This characteristic confers mycobacteria with a major survival advantage over their counterparts. Slow growth with normal rates of metabolism permits the induction of cellular adaptation before reaching determinants of cell survival. For fast-growing bacteria, the inhibition of a cell process coupled with rapid growth results in imbalances in metabolism and ultimately cell death (53). There are several examples of the success of mycobacterial adaptation to environmental conditions, most notable being anaerobiosis (54,55), low pH (56) and starvation (57-59). These



conditions induce a state of quiescence where the bacterial population slows or arrests its growth and can persist in a viable non-replicating state for months or even years.

Another unique aspect of mycobacteria is their metabolic requirements. These bacteria are neither fastidious nor auxotrophic (53). Despite metabolic activity being influenced by oxygen tension, mycobacteria are facultative aerobes, and can generate fumarate and use it as an electron sink through the reductive tricarboxylic acid (TCA) cycle for the synthesis of ATP during anaerobiosis (60). Furthermore, mycobacteria lack catabolite repression systems that enable many bacteria to selectively use carbon sources (61). Rather, mycobacteria co-catabolize multiple carbon sources simultaneously, permitting the exploitation of nutrients available and ease the adaptation to nutrient deficiencies. Consequentially, this unusual metabolic plasticity further enables mycobacteria to adapt to a wide range of environments.

Perhaps the most unique characteristic of mycobacteria is its cell wall. The Mtb cell wall is a complex structure that differs significantly from the canonical cell envelope of Gram-positive and Gram-negative microorganisms (62). One of its key aspects is its high lipid content, accounting for up to 60% of the dry weight of the bacterium (63,64). This unique architecture confers the cell envelope with an unusually high hydrophobicity and low permeability. Consequentially, the low permeability of the cell wall prevents accurate Gram staining, and thus requires mycobacteria to be diagnostically recognized using acid-fast dyes, such as Ziehl-Neelsen stain (65).

The cell envelope itself is composed of three main components: a plasma membrane, a covalently linked complex of peptidoglycan, arabinogalactan and mycolic acids, and a polysaccharide-rich capsule-like layer (62). The most interior portion of the cell envelope, the plasma membrane, is similar to that of other Gram-positive bacteria with the exception that it is

rich in phosphatidylinositol mannosides, a feature similar to all members of Actinomycetales (66). Surrounding the plasma membrane is a thin peptidoglycan layer consisting of an unusually high degree of cross-linking. Traditionally, peptidoglycan precursors inserted into the cell wall are linked by transpeptidases to produce 3-4 crosslinks; however, in the case of Mtb, up to 80% of peptidoglycan contains 3-3 peptide crosslinks (67,68). This heavy cross-linking not only provides added structural stability to the cell, but also plays an important role in resisting immune clearance (69-72). Attached to the *N*-acetyl glucosamine polymers of peptidoglycan are molecules of arabinogalactan, which are in turn esterified at their distal ends to a variety of 60- to 90- carbon long, branched  $\alpha$ -alkyl,  $\beta$ -hydroxy mycolic acids. Mycolic acids are a large family of related lipids present in mycobacteria, and are the hallmark of this genus, as they are responsible for the observed thick waxy coat and a major contributor to their cell wall impermeability. This mycolyl-arabinogalactan-peptidoglycan complex layer is further intercalated with an assortment of “free lipids”, such as phthiocerol dimycoserates (PDIMs), phenolic glycolipids, trehalose-containing glycolipids, and sulfolipids that are anchored to the plasma membrane and extend to the exterior of the cell wall. Lastly, a loosely bound extracellular material, in the form of the capsule, acts as the interface between the mycobacterial environment and cell wall. This matrix is primarily composed of proteins and polysaccharides (73), with only a small proportion (2-3%) containing lipid (74). Variation in the composition and structure of the mycobacterial membrane exist and is a major determinant of growth, physiology and virulence between these species.

Although only briefly described here, the biology of mycobacteria is complex and differs significantly from other bacteria. It might be expected that the slow growth of mycobacteria would result in being outcompeted by other microorganisms in their environment. However, these bacteria have evolved compensating factors such as a hydrophobic, lipid-rich impermeable

cell wall envelope, the ability to transition into a state of quiescence during environmental stress, and metabolic flexibility, that have permitted their survival, growth and persistence in a wide range of environments. Furthermore, this slow growth allows time for mycobacterial cells to adapt to changing conditions before loss in viability. Taken together, it is evident that the adaptive strategies employed by Mtb are essential in its pathogenesis, yet we understand so little about it. Thus, if we want to manage TB, we must prioritize our understanding of Mtb biology and its response to changes in the environment.

### **1.3 Pathogenesis of Mtb**

Mtb has evolved into an extraordinarily successful pathogen that has enabled it to intimately sense the host's environment, and adapt its physiology to prevent unrestrained replication or perfect symbiosis that would threaten its existence as a species. Thus, to avoid killing of the host or itself, the lifecycle of Mtb consists of a prolonged period of replicative quiescence tolerant to host immunity and conventional antibiotics, which can at times last decades or even the lifetime of the host, followed by an obligatory episode of replication, required for transmission to a new host.

#### **1.3.1 Gaining Access to the Phagosome**

Following aerosol transmission to a new host, Mtb first encounters the alveolus of the lung where it is phagocytosed by tissue resident alveolar macrophages, neutrophils, or dendritic cells. Although macrophages are the primary reservoir for Mtb during infection, the bacillus has also been reported to infect non-phagocytic cell types such as adipocytes and epithelial cells (75,76). Entry of Mtb into cells can occur through macropinocytosis (77) or through a variety of receptors. Receptor mediated phagocytosis is believed to be primarily non-opsonic (C-type lectin) and triggered by the surface components of Mtb (78). However, entry into the

macrophage through these receptor mediated pathways is thought to block its microbicidal mechanisms (79), assisting the bacterium to acquire safe passage to its intracellular niche, the phagosome.

### **1.3.2 Evasion of the Innate Immune Response**

Following internalization of a particle or pathogen, phagosomes normally undergo a series of rapid and extensive modifications that alter their biochemical composition and function in a process known as phagosome maturation. The process of maturation includes subsequent fusion of the phagosome with endosomes and lysosomes, leading to progressive acidification, alteration in ionic composition, and accumulation of lytic enzymes, oxygenated lipids, and reactive oxygen and nitrogen species (ROS and RNS, respectively) that can further be intensified with immune activation by cytokines, such as interferon- $\gamma$  (IFN- $\gamma$ ). The acidic pH of the maturing phagosome activates enzymes that degrade bacterial proteins and lipids, and simultaneously suppresses bacterial metabolism. ROS and RNS act to damage the cell components of microorganisms by modifying their DNA, lipids, thiol groups, tyrosine side chains and active centres of metalloproteins. As maturation progresses, the invading microbe experiences further insult by cationic antimicrobial peptides that cause permeabilization of their membrane (80).

As an intracellular pathogen, Mtb is able to circumvent the killing mechanisms of the macrophage permitting the bacterium to reside and replicate in the phagosome. During the process of phagocytosis, Mtb encounters ROS generated by NADPH oxidase (NOX2), known as the phagocyte oxidative burst. The transfer of electrons from NADPH to phagosomal oxygen by NOX2 creates superoxide, which can further be converted into other microbicidal molecules such as hydrogen peroxide and hydroxyl radicals. *In vitro* experiments performed by Chan *et al.*

(81) showed that intracellular growth of NOX2-deficient macrophages had no effect on intracellular bacterial growth, suggesting that Mtb is resistant to the inhibitory effects of the oxidative burst. However, in murine models of infection conflicting evidence exists (82-84). Further studies investigating the response of Mtb to ROS identified the bacilli to be resistant to high levels of oxidative stress, where exposure to 10mM of hydrogen peroxide had no effect on cell viability (85). From these results, it appears that ROS play a minor role in controlling Mtb growth during infection and likely depends on a variety of factors including mode of entry into phagocytes (79), degree of ROS production (86), and the induction of Mtb's response to oxidative stress (85,87).

In contrast, the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) has been shown to play a crucial role in the immune control of Mtb (87,88). NO is a highly diffusible free radical, soluble in both lipids and water, and is capable of reacting with oxygen and ROS, to form a variety of RNS, including the highly mycobactericidal peroxynitrite. Mice deficient in iNOS are highly susceptible to TB infection (83,88,89), and the killing of Mtb by human alveolar macrophages is dependent on the activity of iNOS (87). Furthermore, the production of NO has been identified to play a crucial role in maintaining disease latency (89), indicating that NO also exhibits a bacteriostatic effect. Since the products of iNOS are highly reactive, it is important that iNOS is localized in close proximity to the phagosomal membrane to effectively kill the invading microbe.

To enhance its survival within the phagosome, Mtb employs a number of mechanisms to defend itself against host derived ROS and RNS. One approach utilized by Mtb is to prevent its exposure to these toxic compounds, by either directly inhibiting the assembly of the NOX2 complex (90) and/or interfering with the delivery of iNOS to the proximity of the phagosomal

membrane (91). Although significantly contributing to the virulence of Mtb, these two mechanisms are not fool proof. To compensate, Mtb has a number of detoxification processes comprising of (a) scavenging enzymes such as catalase, superoxide dismutase, alkyl hydroperoxide reductases, (b) redox buffering systems (thioredoxins and mycothiol), and (c) truncated haemoglobins (92). Furthermore, Mtb's resistance to ROS is further augmented by the cell wall components, lipoarabinomannan, cyclopropanated mycolic acids, as well as phenolic glycolipid I, which act as potent scavengers of oxygen radicals (93).

Despite its well evolved defense system against ROS and RNS, the intracellular pathogenesis of Mtb is primarily achieved by (a) prevention of phagosome acidification (94) and (b) blocking the fusion of the mycobacteria-containing phagosomes with lysosomes (95). In addition to non-opsonic phagocytosis, Mtb is also known to actively block phagosomal acidification and maturation through the secretion of protein phosphatases (96-98). Preventing the fusion of phagosomes with lysosomal compartments fails to expose Mtb to hydrolytic enzymes, not only enabling bacterial survival, but also halting antigen processing and presentation by the major histocompatibility complex (MHC) class II. In parallel, a number of Mtb lipoproteins act as Toll-like receptor 2 (TLR2) agonists and thus induce signalling pathways responsible for the inhibition of MHC-II expression (79). Attenuation of macrophage antigen presentation impedes T-cell activation and successive secretion of various cytokines required to initiate an adaptive immune response. Through the modulation of MHC-II activity, Mtb is able to escape host immune surveillance and persist long-term within the phagosome.

### **1.3.3 Disease Latency and *Mycobacterium tuberculosis* Persistence**

Innate immune response has little immediate antibacterial effect on Mtb which undergoes "rapid" division until the onset of adaptive immunity. Yet, internalization of Mtb by alveolar

macrophages in the airway triggers a pro-inflammatory response that results in the macrophage invading the subtending epithelium. Infected macrophages also secrete a number of cytokines and chemokines that initiate the recruitment of mononuclear cells from nearby blood vessels (99). This process generates a primary lesion of infection that consists of a core of infected macrophages surrounded by foamy macrophages that are further enclosed by lymphocytes. The eventual accumulation of infected antigen presenting cells in the regional lymph nodes of the lungs leads to the development of the adaptive immune response against Mtb (99). This process stimulates the activation of phagocyte antimicrobial activities and leads to the recruitment of additional mononuclear leukocytes into the site of infection. In time, the focus of infection matures and becomes a well-organized, dynamic structure of immune cells at various stages of differentiation known as a granuloma (99). Despite this extensive cell-mediated response, adaptive immunity is unable to eradicate or provide sterile immunity against Mtb (100).

The onset of adaptive immunity in conjunction with the tissue response described above act to contain the infection and arrest the period of rapid replication of Mtb (99). At this stage the majority of individuals become asymptomatic, do not shed bacteria, and are considered to have latent TB infection (LTBI). Clinically, LTBI is defined by a reactive tuberculin skin test, indicating a delayed-type hypersensitivity response to intradermal injection of Mtb-derived purified proteins, in the absence of radiographic findings or symptoms of active TB. During this period of latency, the infection is sustained mainly by a population of non-replicating bacilli.

During LTBI, the granuloma exhibits a solid architecture comprised of mononuclear phagocytes at different developmental stages, dendritic cells and fibroblasts at its core. A fibrous cuff as well as T and B lymphocytes encases these phagocytic cells, separating them from the surrounding lung tissue. However, in a small number of individuals (2-23%), the immune system

cannot effectively contain the infection. The centre of the granuloma can become necrotic and collapse, releasing live bacilli into the airways. This stage of infection is known as reactivation, and leads to active, symptomatic disease which generally results in shedding of Mtb in respiratory secretions, completing the life-cycle of the bacillus (99). While some individuals are never able to contain the primary infection, the majority of adult TB cases are the result of reactivation (with the exception of geographical regions with an extremely high prevalence of TB) (101), and can even occur decades after the initial infection (102). The inability to progress to or maintain LTBI is widely attributed to defects in immunity. Although CD4<sup>+</sup> T cells and tumour necrosis factor (TNF) have been described as two major elements in the control of TB and prevent its reactivation, these mechanisms can only explain the minority of situations (101).

As previously mentioned, the maximal growth rate of Mtb is approximately 20 h, a finding that is more so related to optimal growth conditions *in vitro*. In contrast, bacterial populations inside chronically infected animals are found to replicate every 100 h or more (103,104). It is suspected that the unfavourable growth conditions of the host trigger a metabolic adaptation in the bacilli, where a subset of bacteria undergoes complete growth cessation for significant periods of time. Nutrient limitation (58,59), acidic pH (105), low oxygen tension (106), and other host gases (107) associated with the granuloma are suspected to play a major role in slowing the growth of Mtb and causing a drastic drop in its respiration. This physiological adaptation, known as cellular quiescence, is considered a “norm” in the microbial world. When organisms are exposed to growth-limiting stress, the bulk of the bacterial population will slow or arrest its growth and can persist in a viable non-replicating state for months or even years (108). It is important to note that Mtb does not enter a state of true microbial dormancy (sporulation) during its life cycle. Rather, Mtb displays nominal metabolic capacity, maintains its membrane



potential, and does not undergo morphological differentiation (106,109). Consequentially, this strategy permits Mtb to maintain its population size throughout a period of stress, relieving the necessity for rapid growth when environmental conditions become favourable which may hinder the bacterium from adapting to its environment. Therefore, the ability of Mtb to adapt its physiology to a variety of conditions, a crucial factor in its pathogenic success, depends on the bacilli's ability to sense its environment and orchestrate an appropriate response.

## **1.4 Mtb Pathogenesis is Dependent on Signal Transduction**

### **1.4.1 Protein Phosphorylation in Bacteria**

Phosphorylation is the most common type of post-translational modification that occurs in the cell, and it is the principle mechanism by which extracellular signals are translated into cellular responses (110). Protein kinases are responsible for catalyzing the transfer of phosphate (phosphorylation) to a specific residue on its target protein. The addition of a phosphoryl group provides a double negative charge, a characteristic that is not carried by naturally occurring amino acids, and the ability to form hydrogen bonds through the phosphoryl oxygens. This modification converts a hydrophobic portion of the protein into a polar or hydrophilic region that usually induces a conformational change in the protein's structure. For example, phosphorylation of a protein can alter its biological activity, stabilize or mark it for destruction, facilitate or inhibit movement between subcellular compartments, or promote conformational changes that can initiate or disrupt protein-protein interactions. With regards to signal transduction, phosphorylation can activate the substrate to transfer the accepted phosphate group to a downstream effector, initiating a cascade of signal-response reaction.

Phosphorylation reactions can be reversed through the action of protein phosphatases. The removal of phosphate from the substrate, a process known as dephosphorylation, can be

thought of as restoring activators and effectors to their original or an alternative functional state. Therefore, kinases and phosphatases together represent intracellular on/off control mechanisms of many physiological processes.

Today, protein phosphorylation is known to occur across all three domains of life; however, the study of post-translational modification in bacteria was neglected for considerable time. Early attempts to detect its presence were unsuccessful, generating the dogma that protein phosphorylation was a regulatory mechanism that emerged late in evolution to meet the needs of organisms composed of multiple and differentiated cells. The pioneering work of several groups in the 1970s identified protein kinase activity in both *Escherichia coli* and *Salmonella typhimurium* (111-113), which soon led to the discovery of the histidine/aspartate (His/Asp) kinases of the two component systems (114,115). The first aspect of this system involves the stimulation of a His kinase by a particular environmental or intracellular signal, resulting in auto-phosphorylation on a key His residue. The phospho-His can then be used as a substrate by the cognate response regulator for its own auto-phosphorylation on an Asp residue. The majority of response regulators are DNA-binding proteins that trigger expression from target promoters. Usually, two component systems work in isolation where a given pair of His kinase and response regulator is highly selective for each other via protein-protein interaction.

During the initial phase of His/Asp – dependent phosphorylation discovery, these systems were regarded as the major signal transduction pathway in bacteria, and led to the hypothesis that Ser/Thr/Tyr phosphorylation was a eukaryotic trait, whereas His/Asp phosphorylation was exclusive to prokaryotes. Since this time, our knowledge of protein phosphorylation has been revised. Hundreds of two-component systems have been discovered in eukaryotic cells (116), and recent genomic data indicates that “eukaryotic-like” Serine/Threonine protein kinases

(STPKs) are as prevalent in prokaryotes as their His kinase counterparts (117). However, two component systems remain the main signalling mechanism in all phyla of bacteria, with STPKs being most abundant among Acidobacteria, Actinobacteria, various groups of Cyanobacteria as well as bacteria belonging to the order of Myxococcales.

#### **1.4.2 Signal Transduction in Mtb**

The pathogenic success of Mtb is largely dependent on its ability to sense and adapt to the dynamic environment of the host. As a result, Mtb has evolved an extensive intracellular signalling network consisting of 12 paired two-component regulatory systems (also including four orphan regulators), 11 STPKs, a single protein-tyrosine kinase and three phosphatases, which have been extensively reviewed in the past two decades (97,118,119). The presence of Serine (Ser), Threonine (Thr) and Tyrosine (Tyr) protein kinases enables the cell to generate phosphorylated residues with far greater stability. Generally, the hydrolytic half-time of phosphoryl-Asp is only a couple of hours whereas Ser/Thr/Tyr phosphor-esters, or *O*-phosphorylation, can produce signals that are stable and require a phosphatase to be reversed (120). Consequently, our current hypothesis is that Mtb uses phosphoryl-Asp for rapid, short-term signal transduction and Ser/Thr/Tyr phosphorylation for long-term global responses giving cells the advantage to adapt and survive in complex environments.

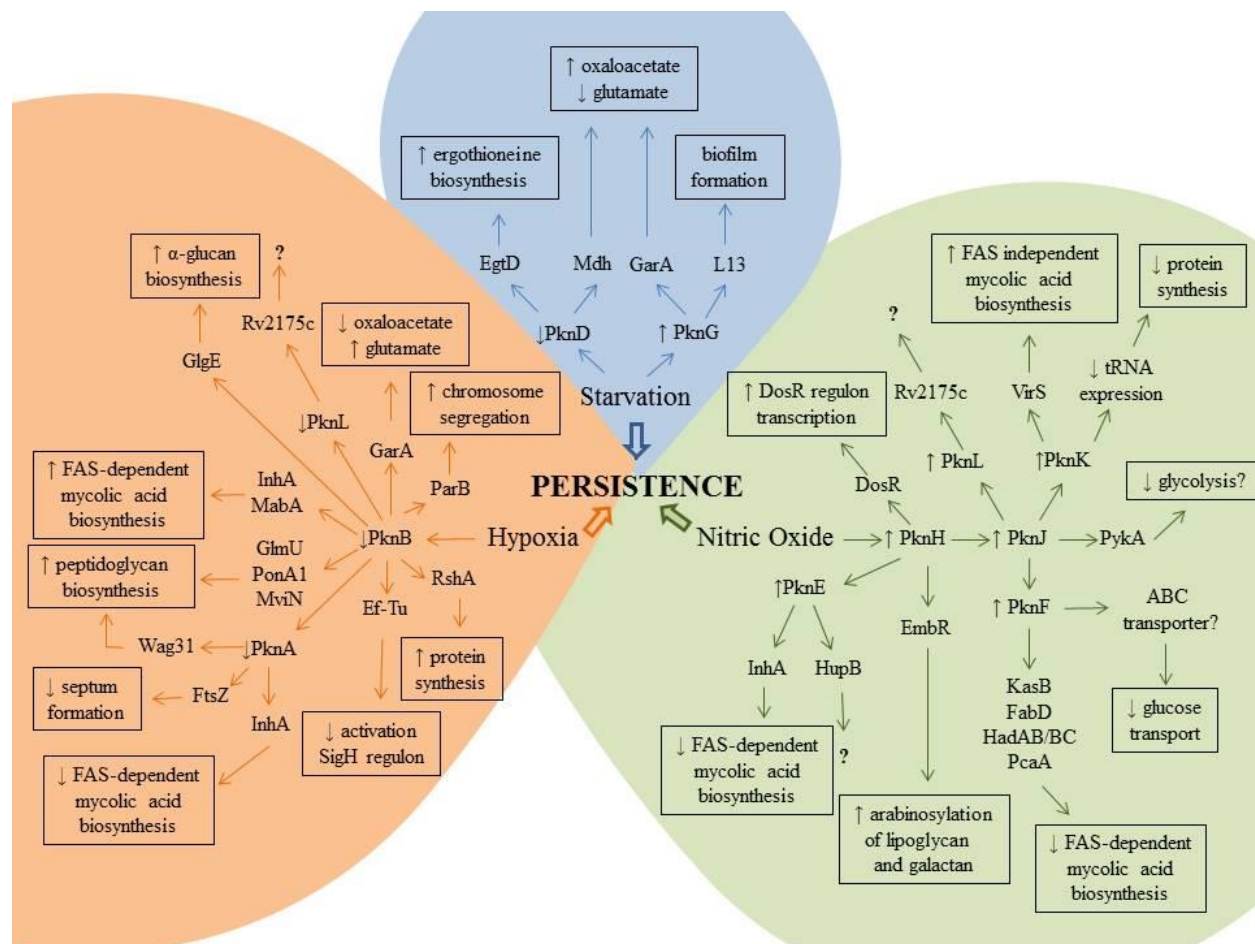
The discovery of Mtb STPKs originated from the identification of 11 genes encoding for the subdomains of the Hank's superfamily of kinases, resulting in their annotation as "eukaryotic-like" STPKs (121). Of the 11 STPKs (PknA, B, D-L), the sequences of nine of these kinases contain a transmembrane region that connects the intracellular N-terminal kinase domain to a C-terminal sensory component that is located extracellularly. Current structural data indicates that these transmembrane receptor kinases are activated by dimerization of their kinase

domains resulting in the phosphorylation of the activation loop and ultimately leading to kinase activation (122). The remaining two kinases, PknK and PknG, lack a transmembrane domain. Yet, subcellular fractions of Mtb lysates showed PknK to be present in the cell wall/membrane fraction rather than the cytosol through an unknown anchoring mechanism (123). Therefore, PknG is described as the sole soluble STPK in Mtb.

Similar to other prokaryotes, the overall extent of *O*-phosphorylation in Mtb is limited, amounting to 7.5% of all proteins being phosphorylated (124) in comparison to the 40-45% of eukaryotes (125). As expected, the number of phosphoserine (pSer) and phosphothreonine (pThr) sites in Mtb is significantly greater than phosphotyrosine (pTyr) sites, with over 500 sites identified (124). These findings indicate that each STPK can act on multiple substrates. However, little is known regarding STPK signalling cascades and kinase hierarchy in Mtb. In contrast to His-kinases, which typically phosphorylate a single response regulator, the cross-reactivity observed with Ser/Thr/Tyr protein kinases results in complex signalling cascades, and rarely does the direct output of Mtb Ser/Thr protein phosphorylation involve the direct regulation of expression of target genes. However, some evidence exists of STPK signalling organization typical of two-component systems (123,126-128).

Our understanding of bacterial signal transduction has become of great significance due to its role in pathogenicity. During the course of infection, the growth and/or survival of a pathogen relies largely upon its successful adaptation to a continuously changing environment. Our laboratory has argued that STPKs play a crucial role in Mtb's ability to establish a persistent infection in its host (129). As seen in Figure 1, these intracellular signalling cascades can culminate in alterations in gene transcription, enzymatic activity, cellular localization, or protein-

protein interactions that result in the rapid adaptation of the bacterium to changes in its environment resulting in a persistent infection.



**Figure 1. Mtb STPK Signalling Network Associated with Persistence.** STPKs sense specific environmental cues (starvation, hypoxia, and nitric oxide) and coordinate a physiological response that trigger Mtb to enter a state of non-replicating persistence. FAS, fatty acid synthase.

Image from: Richard-Greenblatt and Av-Gay. Epigenetic control of the persistence phenotype by phosphorylation. In W.J. Jacobs, H. McShane, V. Mizrahi, I. Orme (Eds.). *The Signature Problem of TB: Persistence*. Washington, DC: American Society for Microbiology. *In press*.

## 1.5 Ergothioneine

### 1.5.1 Introduction

In the middle ages, Europe was haunted by epidemics of gangrenous and convulsive symptoms, a disease known today as ergotism (130). The cause of the disease remained

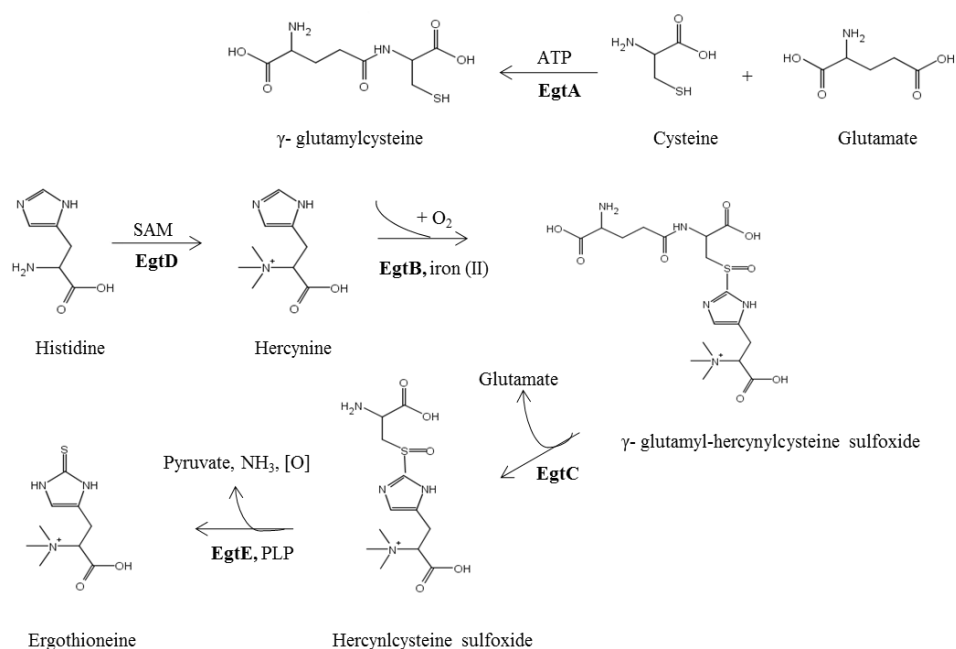
unknown for many centuries until a French physician, Dr. Thuillier, linked these symptoms with the consumption of rye grain infected with ergot in 1670 (131). Ergot, a group of fungi classified by the genus *Claviceps*, parasitize a number monocotyledonous plants, including corn, wheat, barley, and rye. These fungi produce an assortment of alkaloids that are responsible for the disease that occurs in humans and animals that consume infected grains. The devastating effects from ergot prompted a number of scientists to search for a method to protect crops from infection, and in 1909 this is precisely what French pharmacist and ethnobotanist, Charles Tanret, was undertaking. However, rather than finding a solution he isolated an unusual compound from ergot, to which he named *ergothionéine* (132).

### **1.5.2 Ergothioneine Biosynthesis**

Although ergothioneine (EGT) was originally identified in the fungi *Claviceps*, its presence has since been found to be distributed across all domains of life. However, not all organisms are able to synthesize EGT. As early as 1911, Barger and Ewins (133) isolated EGT from the blood of pigs and since this discovery all studied animals are found to accumulate EGT in specific tissues. Despite extensive investigation, no evidence exists for the biosynthesis of EGT in mammalian cells (134,135), but the discovery of its occurrence in plant material clarified the question as to its origin in animals (136). EGT is not present in the majority of commonly consumed foods, but is found at high concentrations in specialty mushrooms (*Boletus edulis* and *Pleurotus ostreatus*), meat products (liver and kidney) and certain plant products (black beans, red beans, oat bran and garlic) (137). A number of investigations have shown that tissue EGT levels are directly dependent on diet (135,138,139); however, high variability exists in EGT concentration between food groups and within food groups as a result of different cultural practices, geographical location, and timing of harvest (137,140,141).

Very little work has been performed regarding its origin in plant material but it is suspected that plants also acquire EGT as a nutrient from their environment (136). To date, EGT biosynthesis is found to be exclusive to a subset of fungi and bacteria. A number of studies have demonstrated that EGT is synthesized by Ascomycetes (142-144) and bacterial species belonging to the Actinobacterial (145-147) and Cyanobacterial (148) phyla. Furthermore, a genetic survey of prokaryotic genomes revealed that EGT biosynthesis also occurs in specific Bacteroidetes, Proteobacteria, Acidobacteria, and Archaeal species (149).

Between microorganisms, EGT biosynthesis follows a similar path, that is, the conversion of His to EGT through the intermediate hercynine. Initial studies in the fungi *Neurospora crassa* identified the amino acids His, methionine and cysteine to serve as precursors of EGT (150). Further investigation established that intact histidine is incorporated into EGT (150,151), followed by a triple transmethylation process at the  $\alpha$ -amino carbon to form the intermediate hercynine (152,153). Next, hercynine undergoes a sulfurization reaction catalyzed by  $\text{Fe}^{2+}$  and  $\text{O}_2$  to form a second intermediate compound, *S*-( $\beta$ -amino- $\beta$ -carboxyethyl) EGT sulfoxide, which is subsequently converted by a pyridoxal-requiring enzyme to EGT and pyruvate (154). The genes involved in the synthesis of EGT remained entirely unknown until Seebeck (155) identified the mycobacterial EGT gene cluster in *M. smegmatis* that codes for: a  $\gamma$ -glutamyl cysteine synthase (EgtA), a formylglycine-generating enzyme-like protein (EgtB), a glutamine amidotransferase (EgtC), a methyltransferase (EgtD), and lastly, a pyridoxal 5-phosphate binding protein (EgtE) (Figure 2).



**Figure 2. EGT Biosynthetic Pathway in *M. smegmatis* According to Seebeck (155).** EGT biosynthesis occurs through five enzymatic steps, and encodes for a  $\gamma$ -glutamyl-cysteine synthase (EgtA), a formylglycine-generating enzyme-like protein (EgtB), a glutamine amidotransferase (EgtC), a methyltransferase (EgtD), and a pyridoxal 5-phosphate protein (EgtE). The pathway proceeds from L-histidine through the intermediary precursor hercynine, hercynyl  $\gamma$ -glutamyl-cysteine sulfoxide, and hercynylcysteine sulfoxide. EGT acquires its sulfur from  $\gamma$ -glutamylcysteine. Modified from (155). SAM, *S*-adenosyl methionine; ATP, adenine triphosphate; PLP, pyridoxal 5'-phosphate.

Studies in mycobacteria have revealed that EGT biosynthesis follows a similar pathway as in fungi; however, subtle differences have been noted. Interestingly, cysteine and other alternative thiols were unable to act as substrates in the conversion of hercynine, with the exception of  $\gamma$ -glutamylcysteine, a dipeptide formed by EgtA (155). Thus, hercynylglutamylcysteine sulfoxide is a necessary intermediate in the mycobacterial biosynthesis of EGT and the pathway appears to differ in the thiolation process of the imidazole ring of hercynine from fungi (154). Since  $\gamma$ -glutamylcysteine is a precursor for reduced glutathione (GSH; Figure 3A), the major redox buffer in fungi and essential to cellular survival, competition for EGT biosynthesis is not desirable. This idea is supported by the finding that EGT

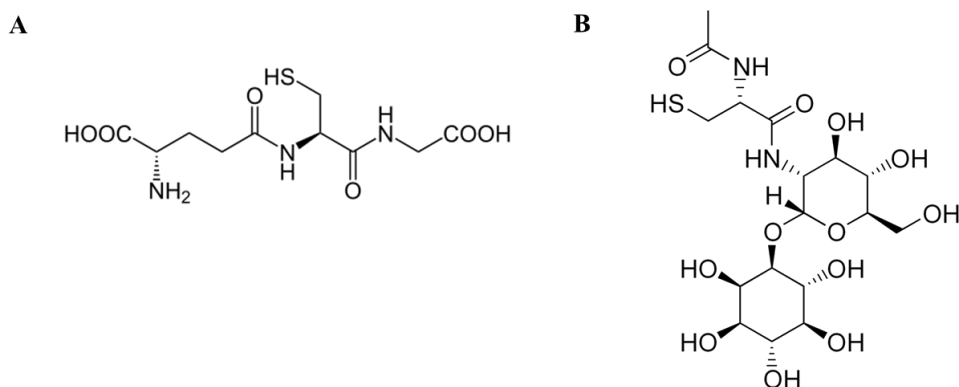


biosynthesis has a 62-fold greater specificity for cysteine than  $\gamma$ -glutamylcysteine by *N. crassa* (156). However, this does not rationalize the utilization of  $\gamma$ -glutamylcysteine in mycobacterial EGT biosynthesis. Perhaps these findings represent the evolution of GSH biosynthesis in microorganisms. Interesting to note is that when GSH, but not  $\gamma$ -glutamylcysteine, is depleted from the cyanobacterium *Synechocystis*, EGT is no longer produced, indicating that GSH or a similar mechanism is required for the biosynthesis of EGT (157); however, the role of GSH in EGT biosynthesis remains to be investigated.

A second variation exists between the fungal and mycobacterial pathways, as fungi contain a bifunctional enzyme (Egt-1) that catalyzes the first two steps of EGT biosynthesis from His to hercynine to hercynlcysteine sulfoxide. The enzyme contains an N-terminal methyltransferase, and the DinB\_2 and FGE-sulfatase domains in the C-terminal region (143). Since EGT biosynthesis in fungi relies on cysteine and does not require an additional enzymatic step for the removal of glutamate, the resulting pathway consists of only two enzymes (Egt-1 and Egt-2).

There is increasing evidence that EGT biosynthesis in fungi is linked to sporulation since there is a significantly higher concentration of EGT in the conidia, and the *Schizosaccharomyces pombe* Egt-1 homologue is up-regulated ~10-fold during ascospore development (143,158). However, certain bacteria belonging to the order Actinomycetales, such as mycobacteria, do not sporulate, and consequently EGT biosynthesis cannot be directly attributed to spore formation. As mentioned earlier, mycobacteria do enter a state of quiescence which can be induced in *in vitro* cultures through prolonged stationary phase. Interestingly, EGT was found to accumulate in stationary phase cultures of *M. smegmatis* (159) as well as during quiescence induced by starvation of *S. pombe* (160,161). It was also demonstrated that EGT biosynthesis increases in

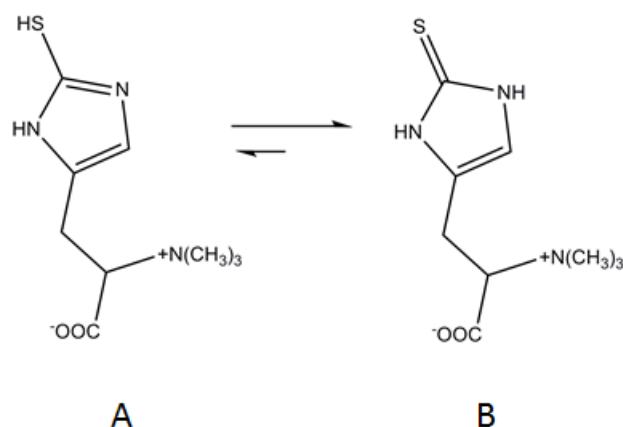
response to the depletion of the main redox buffer, mycothiol (MSH; Figure 3B), in *M. smegmatis* during exponential growth (159). Despite the enhanced production of EGT under these conditions, its quantity always remains in the micromolar range and does not appear to be metabolized by microorganisms known to synthesize it (162-164). Intracellular EGT content is also dependent on the microbial species, their culturing conditions, and phase of growth (146).



**Figure 3. Structural Representation of GSH (A) and MSH (B).** GSH is derived from the amino acids cysteine, glutamic acid and glycine meanwhile MSH is the product of cysteine, glucosamine and inositol.

### 1.5.3 Chemistry

Two years following its discovery, Barger and Ewins (133) predicted the structure of EGT to be a novel amino acid, a betaine of 2-mercaptohistidine, which was later confirmed by Akabori (165). The location of the sulfur atom on the amidine carbon of the imidazole ring enables EGT to exist in three different tautomeric forms (Figure 4) (166). Although generally misclassified as a thiol, EGT is found predominantly in its thione form under physiological conditions (167), and it is only at a higher pH that EGT ionizes to its thiolate (168).



**Figure 4. Structure of the Thione-Thiol Tautomers of EGT.** Although characterized as a thiol (A), EGT exists primarily as a thione (B) under physiological conditions.

The thiol group of an amino acid is a versatile site for many chemical processes due to its  $pK_a$ , nucleophilicity, redox properties, metal ion affinity, and bonding characteristics (169).

Thiols are generally easily oxidized due to the low dissociation energy of the sulfhydryl (-SH) group. This feature of thiol chemistry became of particular importance following the evolution of aerobic respiration as the amino acid cysteine is highly sensitive to auto-oxidation. Therefore, for a cell to be able to utilize and/or produce oxygen it must cope with thiol oxidation. The most common mechanism employed by cells to prevent cysteine auto-oxidation is by keeping the concentration of free cysteine low and maintaining the bulk of the cysteine supply in a form that is more resistant to auto-oxidation than cysteine itself. These cysteine-containing compounds are generally referred to as low-molecular-weight (LMW) thiols. GSH is the most abundant LMW thiol in mammalian cells and Gram-negative bacteria, and is generally produced at millimolar levels (169). In contrast, mycobacteria do not produce GSH, rather they synthesize MSH as their major LMW thiol. These cysteine based compounds can undergo autooxidation without serious consequence as the cell contains disulfide reductases that act to restore the compound to its

reduced state. Therefore, compounds such as GSH and MSH behave as redox buffers, protecting the cell from endogenous and exogenous oxidative stress.

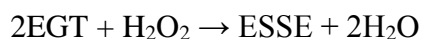
However, the thiol/thione tautomerism confers enhanced stability of ESH. The standard redox potentials ( $E_o'$ ) for thiol/disulfide couples generally ranges between -0.20 to -0.40 V (170). In contrast, EGT has an exceptionally high reduction potential of  $E_o' = -0.06$  V (170), which is almost 200 mV more positive than the GSH/GSSG or MSH/MSSM couple (171). The implication of this distinct redox behaviour results in the resistance of EGT to autoxidation under physiological conditions, a situation where LMW thiols would be easily oxidized (172). Furthermore, this insensitivity to autoxidation enables EGT to participate in heavy metal binding (173), and thereby prevents metal ions from partaking in Fenton-type radical-generating reactions (174).

Perhaps the most significant difference between EGT and LMW thiols is the instability of the disulfide (ESSE) under physiological conditions. As mentioned, LMW thiols require reductase enzymes to maintain them in their reduced form (175). To date, there is no evidence of the existence of enzymes catalyzing the reduction of the EGT disulfide back to its thione form. The calculated net free energy of the reduction of ESSE by Hand and Honek (176) indicate that this reaction is thermodynamically favorable in the presence of millimolar concentrations of GSH and EGT. In comparison to other aromatic thiols, the EGT disulfide is less thermodynamically stable due to the tautomerization step of the thiol form of EGT to its thione. Conversion of the thiol to the lower energy thione serves to drive the overall thermodynamics of the ESSE reduction reaction (176). As a result of the inherent instability of the EGT disulfide at physiological pH, EGT is found entirely in its reduced state in biological systems. Thus, it is

only possible to observe EGT and ESSE simultaneously under strongly acidic conditions, although it has been observed as a transient species in biological systems (177).

The original premise that EGT existed in its sulfhydryl form led many authors to assume its physiological role to be similar to that of GSH. This generalization still appears to dominate literature even today, although early on Woodward and Fry (178) described distinct differences in redox behaviour between these two compounds. Unlike GSH, EGT oxidation in the presence of iodine was almost negligible under extremely acidic conditions. Interestingly, in the presence of GSH in acidic blood filtrates, EGT oxidation can be induced, and is completely oxidized when increasing pH to 5.9 (178). It was later shown that in the presence of excess reduced GSH, EGT does not undergo oxidation. It is only in the presence of oxidized GSH (GSSG) that oxidized EGT can be detected. Thus, the oxidation of EGT in this situation does not result in the restoration of GSH ( $\text{GSSG} + \text{ESH} \rightarrow \text{GSSE} + \text{GSH}$ ), indicating that GSH is involved in the immediate reduction of EGT (172).

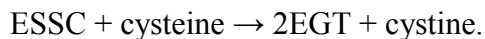
A more in depth investigation of the oxidation conditions for EGT was performed by Heath and Tonnie (172), where it was discovered that EGT can be slowly oxidized (one week) by oxygen in the presence of copper and in strongly acidic solutions, but not under neutral or alkaline conditions. In the same study, EGT was also found to be oxidized immediately after the addition of hydrogen peroxide, but only in acidic solutions. The original spectrum of EGT from these reactions could be restored by the addition of cysteine, indicating that EGT can be reversibly oxidized. The process of reduction by cysteine is described to involve the formation of a mixed disulfide with EGT. In contrast to cysteine, EGT immediately oxidizes to its disulfide (ESSE) in strongly acidic solutions by hydrogen peroxide,



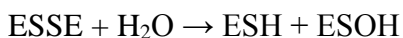
The formation of ESSE results in its rapid reaction with cysteine enabling the formation of a mixed disulfide (ESSC),



In the presence of excess cysteine, the mixed disulfide is immediately converted to EGT and cystine,



The reduction of EGT by GSH was shown to occur in the same manner as described above for cysteine. In the absence of cysteine, the ESSE disulfide slowly reverts back to its reduced form, with decomposition of millimolar solutions taking 3-5 days in water. This slow decomposition can be accelerated if the solution is made alkaline. Once decomposition is complete, only 60-70% of the theoretical amount of EGT can be detected, even following the addition of cysteine. This finding indicates that the decomposition of the disulfide is not solely due to a reduction reaction, and it has been hypothesized to additionally proceed through hydrolytic fission of the disulfide bond to sulfenic acid (has not been experimentally confirmed),



which then decomposes further



Therefore, it appears that there are two mechanisms that work in biological systems to maintain EGT in its reduced form: the thermodynamics of the ESSE and GSH. The fact that there are multiple paths to maintain EGT in its reduced form in addition to its resistance to autoxidation indicates the importance of the thione structure for its biological function. Furthermore, the above reactions indicate that ESSE is a highly oxidizing agent, and would cause significant

damage to thiol groups in the cell. Perhaps this is one of the reasons that it is maintained at low quantities in the cell.

#### **1.5.4 Biological Function**

##### **1.5.4.1 EGT as a Scavenger of ROS and RNS *in vitro***

Based on the presence of its sulfur atom, the majority of research surrounding the biological function of EGT has focused on its role as an antioxidant in cells. The control of ROS and RNS generated by the cell, or from exogenous sources, is a major challenge for aerobic organisms. The hydroxyl radical is one of the most aggressive species as it is capable of reacting with virtually any molecule it encounters in the cell at a diffusion controlled rate ( $10^9$  to  $10^{10} \text{ M}^{-1} \text{ s}^{-1}$ ) (179). EGT has been found to scavenge hydroxyl radicals near diffusion controlled rates and was 60% more active than the reference antioxidant, uric acid (180). However, it is expected that EGT does not function as a scavenger of hydroxyl radicals *in vivo* due to the presence of LMW thiols existing at higher intracellular concentrations that are also equally as reactive with the radical. Ozone, a major constituent of photochemical smog and air pollution, is also highly toxic to cells as it can easily react with water to form hydroxyl radicals or extract hydrogen from molecular oxygen to initiate chain reactions. As the skin employs an antioxidant-based system to protect from environmental reactive oxygen species, EGT was analyzed for its ability to neutralize ozone-induced oxidation of  $\beta$ -phycoerythrin. Of the known antioxidants of the skin, EGT demonstrated to have the greatest activity against ozone *in vitro* (181).

Another biologically relevant ROS is hypochlorous acid, a compound generated by myeloperoxidase from neutrophils at sites of inflammation. One of the major targets of hypochlorous acid *in vivo* is  $\alpha_1$ -antiproteinase, the main inhibitor of serine proteinases, such as elastase (182). At concentrations present *in vivo*, EGT is found to almost completely protect  $\alpha_1$ -

antiproteinase from hypochlorous acid (180). Therefore, it has been concluded that this molecule may assist in protecting tissue against the toxic effects of hypochlorous acid.

Peroxynitrite is a RNS that is formed through the reaction of NO and superoxide. This anion is both a nitrating agent and powerful oxidant of many biologically important compounds such as lipids and DNA (183). Based on a total oxyradical scavenging assay EGT is shown to directly scavenge peroxynitrite 10%, 80%, and 2950% more efficiently compared to uric acid, trolox (an analog of vitamin E), and GSH (184). Peroxynitrite induced DNA damage (185), tyrosine nitration, and  $\alpha_1$ -antiproteinase oxidation (186) was also prevented in the presence of EGT, indicating scavenging occurs at a biologically significant rate. Moreover, EGT displays activity against other peroxy species to a greater extent than other antioxidants (177,184).

Although EGT scavenges a number of ROS and RNS *in vitro*, it has not been found to be an efficient scavenger of superoxide (180,187), singlet oxygen (187,188), nitrite or hydrogen peroxide (173) *in vitro*. The quenching of singlet oxygen by thiols occurs predominately in their thiolate form, and thus its chemical reaction with EGT is highly pH dependent and cannot be observed under physiological conditions (188). Despite these findings, EGT was found to quench singlet oxygen formation by Rose Bengal and phenosafranin, indicating that EGT may function in protecting cells against photochemical damage caused by photosensitizers (189,190). Furthermore, EGT is thought to be unable to scavenge NO due to its inability to protect against the cytotoxic effects of sodium nitroprusside (191).

Enzymatic degradation by catalase and several peroxidases, such as glutathione peroxidase, are the major contributors to controlling levels of hydrogen peroxide in biological systems. Although a weak oxidizing agent, hydrogen peroxide does react with thiols. However, due to the primary occurrence of the thione, oxidation of EGT by hydrogen peroxide is not



observed under physiological conditions (172,192,193). Still, several groups have reported EGT to act as a cytoprotectant against hydrogen peroxide mediated toxicity in cell cultures, indicating that EGT may play a role in the regulation of oxidants (185,194,195) or take part in a cell process that contributes to this effect.

#### **1.5.4.2 Ergothioneine in Mammals and Disease**

The distribution of EGT throughout the mammalian body is non-uniform as a consequence of the plasma membrane being intrinsically impermeable to this hydrophilic compound. Thus, to be able to enter the cell, EGT requires active transport. More recently, the multi-specific, bidirectional novel organic cation transporter (OCTN1) was identified as having EGT as its optimal substrate, but is also known to transport cationic compounds, such as tetraethylammonium, pyrilamine, quinidine, verapamil, donepezil, betonicine and stachydrine (196,197). Two important factors associated with EGT uptake is the influence of pH and Na<sup>+</sup> ions. On the one hand, transport of EGT is optimal under alkaline conditions, and the activity was found to be significantly decreased by 55% when the medium was acidic (198). EGT is also co-transported with Na<sup>+</sup>, and thus the Na<sup>+</sup> gradient across the plasma membrane is a strong driving force for its uptake. On the other hand, the result of the low intracellular Na<sup>+</sup> concentration limits the efflux of EGT from the cell, which ultimately results in the accumulation observed in cells containing OCTN1 (196). The subcellular location of the OCTN1 has been confirmed in the plasma membrane compartments (199,200); however, its presence in the mitochondrial membrane remains controversial (201,202).

A direct relationship between EGT tissue concentration and OCTN1 expression is described in literature (173). Cells expressing OCTN1 were found to accumulate EGT to higher levels than *octn1* (-/-) gene knockout mice and resulted in higher baseline intestinal inflammation

(203). However, an attempt to characterize EGT deficiency in *Caenorhabditis elegans* OCTN1 mutants demonstrated an absence of impaired EGT uptake (204). These findings indicate that EGT may accumulate in the extracellular matrix of cells or have alternative transport mechanisms in addition to OCTN1. Therefore the relevance of the OCTN1 transporter in EGT uptake varies between organisms (173) and thus studies analyzing the effect of EGT deficiencies based off of OCTN1 mutants should be interpreted with caution. Perhaps most interesting from this study was the finding that despite the lack of impaired uptake of EGT in OCTN1 mutant nematodes, these organisms still exhibited higher levels of baseline oxidative stress and a reduced lifespan. Furthermore, the provision of excess EGT was unable to rescue the debilitating phenotype, indicating that EGT transport does not explain the deleterious effects of OCTN1 (204). Therefore, it is questionable as to whether EGT deficiency is directly responsible for the oxidative stress phenotype observed with OCTN1 mutations.

EGT has been implicated in inflammatory processes, although its role remains unclear. Several polymorphisms in the *octn1* gene have been associated with rheumatoid arthritis (205,206) and Crohn's disease (207,208) where these mutations result in elevated intracellular EGT levels. In these chronic inflammatory conditions, increased EGT was hypothesized to stimulate inflammation by promoting the survival of immune cells (209,210) as erythroid cells impaired in EGT uptake were observed to be pro-apoptotic (211). Other studies have indicated that elevated levels of EGT is a secondary effect due to cytokine-mediated up-regulation of OCTN1 by TNF- $\alpha$  (209) and interleukin (IL)-1 $\beta$  (212). In contrast, OCTN1 knockout mice were found to be predisposed to intestinal inflammation, and the authors suggest that EGT may play a role in counteracting inflammation (203). In support of this anti-inflammatory effect, EGT has been shown to inhibit TNF- $\alpha$  mediated secretion of IL-8 in epithelial cell cultures (213) as well

as IL-6 expression induced by p38 and c-jun N-terminal kinase (JNK) signalling pathways (214). The immunomodulatory effects of EGT are also believed to extend to cardiovascular diseases as EGT has been observed to inhibit the induction of the adhesion molecules VCAM-1, ICAM-1, and E-selectin and prevent binding of monocytes to the endothelium (215).

Although its presence in brain tissue is significantly lower (0.3-1 mg per 100 g tissue) relative to other organs, several groups have analyzed the cytoprotective effects of EGT in neurobiology. EGT was observed to protect against  $\beta$ -amyloid induced apoptosis of PC12 cells (191) and prevent the anti-proliferative effects of cisplatin in rat cortical neurons (216). The neurotoxic effects of both  $\beta$ -amyloid and cisplatin are believed to result from ROS generation. Mice treated with cisplatin exhibited decreased brain EGT levels and body weight and showed increased brain lipid peroxidation, all of which could be restored by oral supplementation with EGT (216). Overstimulation of glutamate receptors by *N*-methyl-D-Asp also leads to the production of free radicals and causes neuronal cell death. However, intraperitoneal injection of EGT was found to significantly reduce *N*-methyl-D-aspartate excitotoxicity of neurons (217). EGT has also been shown to protect rat cardiac and liver tissue (192,218,219), but not rabbit hearts from ischemia/reperfusion induced damage (220). These findings may represent differences in experimental design or dissimilar expression of OCTN1 by different animals and tissues. Injury from ischemia/reperfusion occurs as a consequence of oxidative stress upon reperfusion; however, the role EGT plays in protecting tissue remains unknown.

Lastly, elevated blood EGT levels have been associated with other clinical conditions, such as diabetes and TB. In the case of diabetic patients, EGT was believed to bind zinc, and thereby inhibiting the storage of glucagon and insulin (221-223). However, it has also been suggested that EGT prevents insulin resistance and type 2 diabetes by inhibiting palmitic acid-

induction of IL-6 (173,214) and reduces embryo malformations in diabetic pregnant rats (224). A link, although not well established, was originally identified in 1951 between EGT and TB (225). Aboriginal patients with pulmonary TB demonstrated elevated blood EGT levels in comparison to healthy controls. In contrast, Caucasian patients with TB showed no difference in blood EGT levels. This correlative study has been criticized for these observed differences resulting from diet (226), and therefore a well-controlled follow up study is warranted. However, a more recent study observed an association between single nucleotide polymorphisms in OCTN1 and TB susceptibility in the Thai population (227).

Although implicated in a wide range of immune-related diseases, it remains unknown as to whether EGT contributes to the pathogenesis or is a cellular defense mechanism. Perhaps understanding the role EGT plays in simpler life forms can help elucidate its function in higher organisms.

## **1.6 Aims of the Study**

Prior to initiating these studies, there was no known published work on the role of EGT in microorganisms. Based on *in vitro* findings that EGT can scavenge specific oxidants, and its proposed antioxidant function in mammalian cells, we hypothesized that Mtb synthesizes EGT to protect against macrophage-generated ROS and RNS during the early stages of infection. To explore this hypothesis, we constructed an Mtb H37Rv strain devoid of EGT ( $\Delta egtD$ ) and investigated the role of EGT in Mtb physiology and pathogenesis.

To gain insight into the function of EGT in Mtb, we were interested in examining potential regulators of EGT biosynthesis. We screened the proteins involved in Mtb EGT biosynthesis for their ability to act as substrates of Mtb STPKs and identified multiple STPKs that could phosphorylate multiple enzymes involved in EGT biosynthesis. In our work, we

focused specifically on the effect of EgtD phosphorylation by PknD. Here we found that PknD negatively regulates EGT biosynthesis in Mtb. As *pknD* transcripts are down-regulated during nutrient starvation (228) we predicted that intracellular EGT levels may be increased under these conditions. In addition to finding that the depletion of carbon sources increases the intracellular EGT content, we also observed that EGT is required to maintain viability of Mtb under these conditions. Results from this thesis indicate that EGT plays a role in disease latency, possibly through its effects on Mtb's central metabolism.

## **Chapter 2: Materials and Methods**

### **2.1 Bacterial Strains and Growth Conditions**

Mtb H37Rv cultures were grown aerobically at 37°C on Middlebrook 7H10 agar plates with 10% (v/v) OADC (oleic acid/albumin/dextrose/catalase) enrichment (BD) or in Middlebrook 7H9 broth (BD) supplemented with 0.05% (v/v) Tween-80, 0.2% (v/v) glycerol, and 10% (v/v) OADC. Hygromycin (Hyg; 50 µg/ml) and kanamycin (Kan; 25 µg/ml) were added for the selection of the appropriate Mtb strains. *E. coli* DH5α and BL21 (DE3) were grown at 37°C in Luria Bertani (LB) broth or on LB agar and were supplemented with Kan (50 µg/ml), or ampicillin (Amp; 100 µg/ml) when required.

### **2.2 Cloning and Protein Expression and Purification**

Tables 2 and 3 summarize the primers, vectors, and restriction enzymes utilized for cloning and site-directed mutagenesis in this study. DNA was amplified by polymerase chain reaction (PCR) from H37Rv genomic DNA using standard methods for cloning. After sequencing, recombinant plasmids were further transformed into *E. coli* BL21 (DE3) cells for protein expression. Strains harboring the genes to produce recombinant protein were used to inoculate LB broth from an overnight culture (1:100) and the cells were induced with 1-thio-β-D-galactopyranoside (IPTG) once an OD<sub>600</sub> = 0.6-0.8 was reached (see Table 4 for over-expression conditions). Protein purification was carried out on either nickel-nitrilotriacetic acid (Qiagen) or GSH agarose (Sigma-Aldrich) resins, according to the manufacturers' instructions. Site-directed mutagenesis was performed following established protocol (Stratagene Quick Change). Parental pMV261-*egtD* or pET28-*egtD* was used as a template to generate the EgtD phosphomutants in Mtb or *E. coli*, respectively.

**Table 2. Plasmids Used for the Construction of Strains and Recombinant Proteins.**

Plasmids	Characteristics	Source/Ref.
<i>Methylation Assay</i>		
pET-28a	Produces N-term His6-tagged proteins, Kan <sup>R</sup>	Novagen
pET-28- <i>egtD</i>	pET-28a carrying <i>egtD</i> cloned between NdeI and XhoI, Kan <sup>R</sup>	This study
<i>egtD</i> knockout mutation		
p0004S	Cloning vector containing two Van91I sites, Hyg <sup>R</sup>	(229)
phAE159	Temperature-sensitive mycobacteriophage phasmid, Amp <sup>R</sup>	(229)
pMRG	p0004S carrying 999 bp fragments up- and downstream of <i>rv3701c</i> , Hyg <sup>R</sup>	This study
pGEX-RF	pGEX-4T3 carrying 999 bp downstream region of <i>rv3701c</i> , Amp <sup>R</sup>	This study
<i>Kinase Assays</i>		
pET-22b	Produces C-term His6-tagged proteins, Amp <sup>R</sup>	Novagen
pET-22b- <i>pknG</i>	pET-22b carrying <i>pknG</i> cloned between NdeI and HindIII, Amp <sup>R</sup>	(194)
pET-22b- <i>pknH</i>	pET-22b carrying <i>pknH</i> <sub>(1-401)</sub> cloned between XhoI and NdeI, Amp <sup>R</sup>	(230)
pET-28- <i>egtA</i>	pET28a carrying <i>egtA</i> cloned between NdeI and XhoI, Kan <sup>R</sup>	This study
pET28- <i>egtD</i> <sub>T213A</sub>	pET-28a carrying full-length <i>egtD</i> <sub>T213A</sub> cloned between NdeI and XhoI, Kan <sup>R</sup>	This study
pET28- <i>egtD</i> <sub>S70A</sub>	pET-28a carrying full-length <i>egtD</i> <sub>S70A</sub> cloned between NdeI and XhoI, Kan <sup>R</sup>	This study
pGEX-4T3	Produces N-term GST-tagged proteins, Amp <sup>R</sup>	GE Health Care
pGEX- <i>pknA</i>	pGEX-4T3 carrying <i>pknA</i> <sub>(1-337)</sub> cloned between BamHI and SalI, Amp <sup>R</sup>	(231)
pGEX- <i>pknB</i>	pGEX-4T3 carrying <i>pknB</i> <sub>(1-332)</sub> cloned between BamHI and SalI, Amp <sup>R</sup>	This study
pGEX- <i>pknD</i>	pGEX-4T3 carrying <i>pknD</i> <sub>(1-378)</sub> cloned between BamHI and NotI, Amp <sup>R</sup>	(231)
pGEX- <i>pknE</i>	pGEX-4T3 carrying <i>pknE</i> <sub>(1-336)</sub> cloned between BamHI and SalI, Amp <sup>R</sup>	(231)
pGEX- <i>pknF</i>	pGEX-4T3 carrying <i>pknF</i> <sub>(1-292)</sub> cloned between EcoRI and NotI, Amp <sup>R</sup>	(231)
pGEX- <i>pknK</i>	pGEX-4T3 carrying <i>pknK</i> <sub>(1-299)</sub> cloned between BamHI and Not I, Amp <sup>R</sup>	(231)

Plasmids	Characteristics	Source/Ref.
<i>M-PFC Assay</i>		
pUAB100	<i>Phps60-BamHI</i> -GCN4- <i>ClaI</i> -Gly-mDHFR-[F1,2], Hyg <sup>R</sup>	(232)
pUAB100- <i>egtD</i>	pUAB100 carrying full-length <i>egtD</i> between BamHI and XhoI, Hyg <sup>R</sup>	This study
pUAB200	<i>Phps60-MfeII</i> -GCN4- <i>ClaI</i> -Gly-mDHFR-[F3], Kan <sup>R</sup>	(232)
pUAB200- <i>pknA</i>	pUAB200 carrying <i>pknA</i> <sub>(1-337)</sub> between AflII and ClaI, Hyg <sup>R</sup>	(231)
pUAB200- <i>pknB</i>	pUAB200 carrying <i>pknB</i> <sub>(1-332)</sub> between AflII and ClaI, Hyg <sup>R</sup>	(231)
pUAB200- <i>pknD</i>	pUAB200 carrying <i>pknD</i> <sub>(1-378)</sub> between AflII and ClaI, Hyg <sup>R</sup>	(231)
pUAB200- <i>pknK</i>	pUAB200 carrying <i>pknK</i> <sub>(1-299)</sub> between AflII and ClaI, Hyg <sup>R</sup>	(231)
<i>Co-expression Analysis</i>		
pGEX- <i>egtD</i>	pGEX-4T3 carrying full-length <i>egtD</i> between BamHI and XhoI, Amp <sup>R</sup>	This study
pET-28a- <i>pknD</i>	pET-28a carrying <i>pknD</i> <sub>(1-378)</sub> between BamHI and XhoI, Kan <sup>R</sup>	This study
<i>Intracellular EGT Levels</i>		
pMV261	Mycobacterial extrachromosomal expression vector with a <i>Phsp60</i> promoter, Kan <sup>R</sup>	(233)
pMV261- <i>egtD</i>	pMV261 carrying full-length <i>egtD</i> between BamHI and ClaI, Kan <sup>R</sup>	This study
pMV261- <i>egtD</i> <sub>T213A</sub>	pMV261 carrying full-length <i>egtD</i> <sub>T213A</sub> between BamHI and ClaI, Kan <sup>R</sup>	This study
pMV261- <i>egtD</i> <sub>T213E</sub>	pMV261 carrying full-length <i>egtD</i> <sub>T213E</sub> between BamHI and ClaI, Kan <sup>R</sup>	This study

**Table 3. Primers and Restriction Enzymes used for the Construction of Strains and Recombinant Proteins.**

Cloning Primers	Sequence	Restriction Site
<i>Methylation Assay</i>		
pET-28a- <i>egtD</i> F	Tttttcatatggtgacttcacccgagcag	NdeI
pET-28a- <i>egtD</i> R	Tttctcgagtcagatgtcccacgcca	XhoI
<i>egtD</i> Knockout Mutation		
pMRG Upstream forward	Tttttttgcataaattgcgcggtcaaacgctgcgcccc	BstAPI
pMRG Upstream reverse	Tttttttgcatttcttgcgcgaggtccttcgcgcggt	BstAPI
pMRG Downstream forward	ttttgcatagattgcgcgcgcgcggcgacgatgca	BstAPI



Cloning Primers	Sequence	Restriction Site
pMRG Downstream reverse	Tttttgcatcttttgcgtcgatcggcgccagggtgg	BstAPI
pGEX-UP F	tttttgatccgcgggtcaaacgtgcgcccc	BamHI
pGEX-UP R	Tttttcgggccgcgaggtcctttcgcgcggt	NotI
pMV261- <i>egtD</i> F	Ttttttgatccatgagagtgtcggttgcc	BamHI
pMV261- <i>egtD</i> R	Tttttatcgattcacttggcggccagcg	ClaI
<i>Kinase Assays</i>		
pET-28a- <i>egtA</i> F	Tttttcatatgacgcttgccgccatg	NdeI
pET-28a- <i>egtA</i> R	Ttctcgagtcacgacgccccgtgc	XhoI
pGEX- <i>pknB</i> F	Ctattggatccatgaccacccctccac	BamHI
pGEX- <i>pknB</i> R	Tatctgtcgactcaccaacggcccaccgaaccgat	Sall
<i>M-PFC Assay</i>		
pUAB100- <i>egtD</i> F	Ttttttgatccatgagagtgtcggttgcc	BamHI
pUAB100- <i>egtD</i> R	Tttttatcgattcacttggcggccagcg	ClaI
<i>Co-expression Analysis</i>		
pGEX- <i>egtD</i> F	Ttttttgatccatgagagtgtcggttgcc	BamHI
pGEX- <i>egtD</i> R	Tttttctcgagtcacttggcggccag	XhoI
pET-28a- <i>pknD</i> F	Ttttttgatccgtgagcgatgccgttccgag	BamHI
pET-28a- <i>pknD</i> R	Tttttctcgagctagccgcggctgtccgc	XhoI
<i>Site-Directed Mutagenesis</i>		
UP P22P F	Gctggacccgcccaggttctgc	-
UP P22P R	Gcagaacctggggcggtccagc	-
UP G107G F	Gttcaccgatggacactggttgc	-
UP G107G R	Gcaaccagtgtccatcggtgaac	-
pMV261- <i>egtD</i> <sub>T213A</sub> F	Cctggcgggggtggcggtcagttcaatcgc	-
pMV261- <i>egtD</i> <sub>T213A</sub> R	Gcgattgaactgagccgccaccccgccagg	-
pMV261- <i>egtD</i> <sub>T213E</sub> F	Cctggcgggggtggaggtcagttcaatcgc	-

**Table 4: *E. coli* BL21 (DE3) Expression Conditions for the His and GST-tagged Recombinant Proteins.**

Plasmid	Temperature (°C)	Incubation (h)	IPTG (mM)
pET-28a- <i>egtA</i>	25	16	0.001
pET-28a- <i>egtD</i>	25	16	0.0001
pGEX- <i>egtD</i>	25	16	1
pGEX- <i>pknA</i> <sub>(1-337)</sub>	25	16	0.5
pGEX- <i>pknB</i> <sub>(1-332)</sub>	25	16	0.4
pGEX- <i>pknD</i> <sub>(1-336)</sub>	16	16	1
pGEX- <i>pknF</i> <sub>(1-292)</sub>	25	16	0.5
pET-22b- <i>pknG</i> <sub>(full length)</sub>	25	16	0.1
pET-22b- <i>pknH</i> <sub>(1-401)</sub>	37	4	1
pGEX- <i>pknK</i> <sub>(1-299)</sub>	25	16	0.5

Plasmid	Temperature (°C)	Incubation (h)	IPTG (mM)
pET-28a_ <i>pknD</i> <sub>(1-336)</sub>	25	16	1

## 2.3 Construction and Complementation of the Mtb H37Rv *ΔegtD* Mutant

### 2.3.1 Allelic Exchange

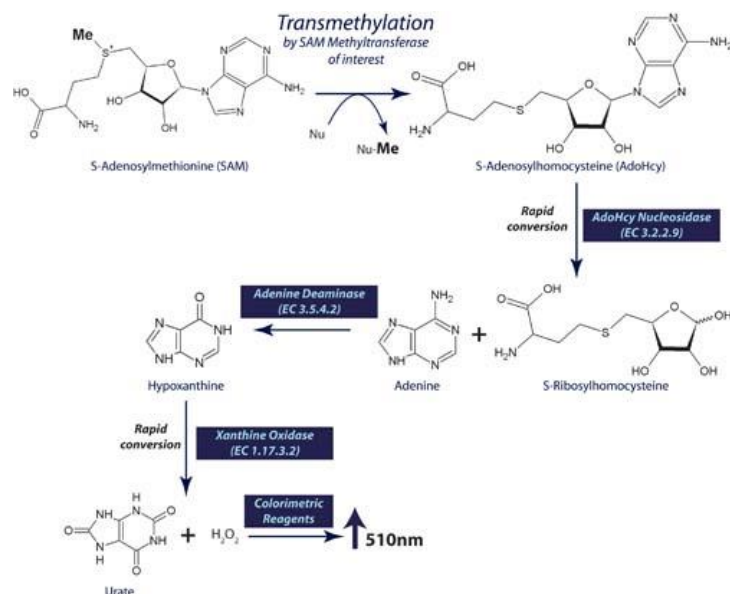
The *ΔegtD* mutant strain was constructed via allelic exchange using the conditionally replicating mycobacteriophage, phAE159, as previously described (229). phAE159 is a temperature-sensitive shuttle plasmid that replicates at 30°C, but not at 37°C. To construct the *egtD* knockout phage, flanking regions comprising of 1000 bp upstream and downstream regions of the *egtD* (*rv3701c*) gene were amplified from H37Rv genomic DNA using the primers for pMRG described in Table 3. Flanking sequences for upstream and downstream regions of *egtD* were amplified by PCR and each fragment contained *BstAPI* sites for directional cloning into the p0004S cosmid. The up and downstream regions flanking *egtD* were cloned on either side of the Hyg-resistance/sacB cassette, which was confirmed by sequence analysis and restriction digest using a combination of EcoRI, NcoI, HindIII and NdeI. The recombinant cosmid was digested with *PacI* and ligated into the *PacI* site of phAE159 to generate a phasmid capable of replicating as a cosmid in *E. coli* and a temperature sensitive phage in mycobacteria. The ligated DNA was packaged into phage λ with Gigapack III Gold packaging extract (Stratagene), and *E. coli* HB101 cells that were previously grown in MgSO<sub>4</sub> and maltose overnight. Colonies were selected for growth on LB plates containing 150 µg/ml of Hyg, and phage DNA was extracted and electroporated into *M. smegmatis* mc<sup>2</sup>155. Transformation plates were incubated at 30°C for 3 days. Several plaques were picked, and used to prepare a high-titer phage stock (10<sup>9</sup> PFU/ml) with *M. smegmatis*. Phages were transduced into Mtb H37Rv and plated on Middlebrook 7H10 supplemented with OADC and Hyg (50 µg/ml). After 4 weeks, Hyg-resistant colonies appeared and were cultured for analysis by PCR and Southern hybridization to identify clones in which allelic exchange had occurred within the *rv3701c* gene.

### 2.3.2 Southern Blot Hybridization

Southern blotting was performed using the digoxigenin (DIG) hybridization system (Roche Applied Sciences). Chromosomal DNA (12 µg) from both the H37Rv wildtype and *rv3701c* null mutant strain was digested with AflIII. Digested DNA was resolved on a 1% agarose gel prior to its transfer to a nylon membrane via capillary method overnight. Hybridization was performed at 68°C overnight with a DIG-11-dUTP labelled probe. Anti-DIG antibodies were used to detect the probe hybridized to its DNA target.

### 2.4 EgtD *in vitro* Methylation Activity

A reaction containing 10 mM histidine, 4 mM *S*-adenosyl methionine (SAM), 1 mM Mg(OAc)<sub>2</sub>, 5 mM NaCl, 20 µg of EgtD and 5 µg *S*-adenosyl homocysteine (SAH) nucleosidase was prepared as described (155) and incubated overnight at 37°C. Detection of methylated His products in the reaction was determined by Electrospray Ionization Mass Spectrometry (ESI-MS) at the University of Victoria Genome BC Proteomics Centre. EgtD substrate specificity was analyzed using a continuous enzyme coupled SAM510™: SAM Methyltransferase Assay (Figure 5; G-Biosciences) according to the manufacture's protocol. Reactions were initiated immediately following the addition of 1 mM of each tested substrate (all proteinogenic amino acids (exception of cysteine), histamine, imidazole,  $\alpha$ -methyl-DL-His, 1-methyl-L-His, 3-methyl-L-His) and monitored at 510 nm for 30 min at room temperature.



**Figure 5. Schematic of the SAM510™ Methyltransferase Assay.** The transfer of a methyl group from SAM to the newly methylated substrate results in the formation of SAH. SAH nucleosidase present in the reaction mix converts SAH to *S*-ribosylhomocysteine and adenine, which is converted into hypoxanthine. Xanthine oxidase further converts hypoxanthine to urate and hydrogen peroxide. The assay is designed to monitor the rate of formation of hydrogen peroxide at 510nm using a colourimetric agent. Image was taken from the G-Biosciences website for the SAM510™:SAM Methyltransferase Assay.

## 2.5 Intracellular Quantification of EGT

### 2.5.1 EGT Extraction from Mtb

Four millilitres wildtype Mtb was harvested by centrifugation. Cells were washed twice in the same volume of ddH<sub>2</sub>O. Following washing, the cells were resuspended in 2 ml of 70% acetonitrile with 25 ng/ml of the internal standard 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>). Cells were disrupted with the MagNAlyser (Roche) and 0.1 mm silica beads (BioSpec) at a speed of 7000 rpm for 60 s intervals followed by 2 min of rest at -20°C (repeated three times). The extract was then filter sterilized using 0.22 µm nylon polypropylene Spin-X® Centrifuge Tubes prior to exiting the Biosafety Containment Level 3 lab for further analysis by ESI LC-MS/MS.

### 2.5.2 ESI LC-MS/MS Analysis of EGT

EGT was quantified using an Agilent 1200 binary HPLC system coupled to an AB Sciex 5500 Q-Trap, triple quadrupole mass spectrometer (Massachusetts, USA). Separation was performed on a Zorbax HILIC Plus column (Agilent Technologies, California, USA; 100 x 2.1 mm, 3.5  $\mu$ m particle size). Acetonitrile (76%), and water (24%), both containing 0.1% formic acid were used as mobile phase at a flow rate of 200  $\mu$ l/min. The peak area of EGT was measured using Analyst 1.5.2 (AB Sciex, Massachusetts, USA) and normalized by the weighted contribution of the peak areas of the MPP<sup>+</sup> internal standard. Identification of EGT was based on its theoretical  $m/z$  value, MS/MS fragmentation data, and its retention time, which was verified by analyzing a pure standard (Oxis International Inc., California, USA). Calibration curves were generated through a series of EGT standard additions to the sample. Regression coefficients of each calibration curve were all greater than 0.99.

### 2.6 *In vitro* Kinase Assays

*In vitro* phosphorylation screen was performed as previously described (230) using 1  $\mu$ g of EgtD or EgtA in 20  $\mu$ l of the assay buffer (20 mM Tris-HCl pH 7.4, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 1 mM DTT) and varying concentrations of kinase (0.1-1  $\mu$ g) to obtain optimal autophosphorylation activity. The kinase domains of the following Mtb STPKs used for screening in this assay were PknA, PknB, PknD, PknE, PknF, PknG, PknH, and PknK (see Table 3 for region cloned). Reactions were commenced by the addition of 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P] ATP (PerkinElmer Life Sciences, 3000 Ci/mmol) and were incubated at room temperature (23°C) for 30 min. Following the incubation period, reactions were arrested using SDS-sample loading buffer, heated at 95°C and resolved by SDS-PAGE. Protein was visualized using Coomassie Blue. EgtD dose-dependent phosphorylation kinetics were performed as described above with

minor changes. First, the kinase was left to autophosphorylate for 20 min prior to the addition of EgtD. Cold ATP (100  $\mu$ M) was spiked into 10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P] ATP prior to the addition of ATP to the reactions. The reaction kinetics were monitored by excising the bands corresponding to EgtD and subjecting them to scintillation counting (Beckman Coulter LS 6500). Kinetic parameters were calculated using Prism Software (GraphPad 6.04). EgtD phosphorylation sites were analyzed using LC-MS/MS (phosphopeptide analysis) as described (126) with 1 mM non-radiolabelled ATP by the Genome British Columbia Proteomics Centre at the University of Victoria.

## **2.7 Protein-Protein Interaction Assay**

Protein-protein interactions were investigated using the mycobacterial protein fragment complementation (M-PFC) assay as previously described (232). Mtb EgtD and STPKs (PknA, PknB, PknD, and PknK) were amplified by PCR and cloned into pUAB100 (expressing murine dihydrofolate enzyme fragments F1, F2) and pUAB200 (expressing murine dihydrofolate fragment F3), respectively. EgtD was co-transformed with each of the four kinases into *M. smegmatis* mc<sup>2</sup>155 and co-transformants were selected on 7H11/ Kan/Hyg plates. Co-transformants were re-plated on 7H11/Kan/Hyg plates supplemented with 0 and 10  $\mu$ g/ml trimethoprim (TMP) and analyzed for growth over 4-5 days.

## **2.8 Cell-based Phosphorylation**

*EgtD* was cloned into pGEX-4T3 and *pknD* into pET-28a. Both plasmids were co-transformed into *E. coli* BL21 (see Table 2). Co-transformants were selected on LB plates containing Amp and Kan. Cultures were induced with 1 mM IPTG and further grown for 16 h at 25°C. Both proteins were purified as described above and resolved by SDS-PAGE and stained using Coomassie Blue to ensure adequate expression of both proteins in the culture.

Approximately 20 µg of recombinant protein were then subjected to phosphopeptide analysis by LC-MS/MS to determine *egtD* phosphorylation sites in a cell-based system.

## **2.9 TLC Analysis of Phosphorylated EgtD Activity**

Phosphorylated EgtD was obtained through the *in vitro* kinase assay outlined above. The reaction varied slightly in that 8 µM EgtD, 10 µM non-radiolabelled ATP and 0.4 µM of kinase were used. Reactions were incubated at room temperature for 1.5 h to obtain the maximum yield of phosphorylated EgtD (stoichiometry not determined). Next, 15 mM histidine, 2.5 mM NaCl, 500 µM Mg(OAc)<sub>2</sub>, and 10 µM SAH nucleosidase were added to the 8 µM of phosphorylated EgtD. The methylation reaction was initiated upon the addition of 10 µmol of S-[methyl-<sup>14</sup>C]-Adenosyl-L-Methionine (PerkinElmer Life Sciences, 60mCi/mmol). Samples (10 µl) were taken from the reaction at various time points, which were stopped with 1 µl of 1% trifluoroacetic acid, and stored at -20°C prior to analysis. Samples were subjected to TLC, using PEI Cellulose F plates (EMD Millipore, Darmstadt, Germany) developed in butanol/water/acetic acid (60:25:15; v/v). The separation of radiolabelled SAM and methylated His was visualized using the PhosphorImager SI (Molecular Dynamics) following 7 days of exposure. Ninhydrin (0.03% butanol solution) was used to detect His on the plate. The spots corresponding to His were cut from the plate and subjected to scintillation counting (Beckman Coulter LS 6500) to quantify the formation of methylated His over time.

## **2.10 Murine J774A.1 Macrophage and THP-1 Monocyte Infection**

### **2.10.1 Tissue Culture**

Both the human monocytic leukemia cell line THP-1 (TIB-202; American Type Culture Collection, Manassas, VA), and murine J774A.1 macrophages (American Type Culture Collection; ATCC catalogue number TIB-67) were grown in 125 cm<sup>2</sup> tissue culture flasks

(Corning Inc., Corning, NY). J774A.1 cells were cultured in Dulbecco's modified Eagle medium, high glucose supplemented with 10% fetal bovine serum (FBS), and with 1% of each HEPES, streptomycin, penicillin and non-essential amino acids. In contrast, THP-1 cells were grown in RPMI 1640 medium supplemented with 10% FBS, and 1% of each: L-glutamine, penicillin and streptomycin. Both strains were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> for 18 h.

### **2.10.2 J774A.1 and THP-1 Differentiation**

Both J774A.1 and THP-1 cells were washed with their respective incomplete culture media (media described above without antibiotics) and seeded in 24-well plates (Corning Inc., Corning, NY) at a density of  $2.5 \times 10^5$  cells/well. THP-1 cells were differentiated into a macrophage-like cell line with 20 ng/ml phorbol myristate acetate. Strains were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 18 h.

### **2.10.3 Mtb Infection of J774A.1 and THP-1 Cells**

Following differentiation, either cell line was infected with exponentially growing Mtb (OD<sub>600</sub> = 0.5) at an MOI of 5:1. However, infection of THP-1 cells was used with human serum-opsonized Mtb. Macrophages were incubated with Mtb for 3 h at 37°C, 5% CO<sub>2</sub>. Wells were washed three times and resuspended in culture medium containing 100 µg/ml gentamicin to kill any remaining extracellular Mtb. For colony forming unit (CFU) counting, cells were further washed with incomplete culture medium and the macrophages were lysed using 0.025% SDS at the selected time points post-infection. Serial dilutions of the lysate were plated onto Middlebrook 7H10 agar medium supplemented with OADC and the appropriate antibiotics. Colonies were counted after incubation for 3 weeks at 37°C.



## **2.11 Mtb Starvation Studies**

Mycobacterial cultures were grown by shaking in Middlebrook 7H9, 0.2% glycerol (v/v), 10% OADC and 0.05% tyloxapol to an  $OD_{600} = 0.8$ . Cells were washed twice with phosphate buffered saline (PBS) and then resuspended in PBS prior to leaving the cultures to stand at 37°C in sealed 125 ml Erlenmeyer culture flasks (Corning Inc., Corning, NY). Mtb viability during starvation was determined by counting CFUs from triplicate cultures over a four-week period. Serial dilutions of the cells were performed and followed by plating onto Middlebrook 7H10 agar medium supplemented with OADC and the appropriate antibiotics. Colonies were counted after incubation for three weeks at 37°C. The extraction and quantification of intracellular EGT levels was performed at various time points throughout starvation as described in Methods Section 2.5.

## **2.12 Quantification of Mtb Intracellular EGT in the Presence of Carbon Sources**

H37Rv wildtype was grown to an  $OD_{600} = \sim 1.0$  in 7H9 supplemented with 10% OADC, 0.2% glycerol and 0.05% tyloxapol. Cells were washed three times in 7H9 containing 0.05% tyloxapol and these cells were used to inoculate new cultures at a starting  $OD_{600} = 0.2$ . Each culture was grown in the presence 0.05 mM and/or 25 mM of the respective carbon source (glucose, acetate, propionate, myristate, stearate, behenate, palmitate, cholesterol). Lipid and cholesterol stocks (100 mM) were prepared fresh in tyloxapol/ethanol (1:1 v/v) and dissolved at 80°C immediately prior to use. Carbon sources were added to previously filter sterilized 7H9 and were filtered a second time to prevent contamination. Glucose, acetate and propionate were dissolved in water; however, an equal volume of the tyloxapol:ethanol mixture was added to the media prior to filter sterilization (tyloxapol:ethanol concentration in all carbon source media was

a final concentration of 0.5%). Following four days of growth, intracellular EGT was extracted and quantified from the three strains grown in each carbon source.

## Chapter 3: Rv3701c Encodes for a Histidine Methyltransferase Required for EGT Biosynthesis in Mtb

### 3.1 Introduction

To investigate the role of EGT in the physiology and pathogenesis of Mtb we needed to generate a strain devoid of EGT and to identify a candidate gene in EGT biosynthesis for the knock-out procedure. In the majority of mycobacteria, EGT biosynthesis is thought to occur through five enzymatic steps, and the genes encode for a  $\gamma$ -glutamylcysteine ligase (EgtA), a sulfoxide synthase (EgtB), a glutamine aminotransferase (EgtC), a histidine methyltransferase (EgtD) and pyridoxal 5-phosphate protein (EgtE) (155). Of these genes, we were interested in selecting one to construct a knockout strain. Rather than immediately constructing an EGT deficient strain, we wanted to verify that the selected gene was in fact involved and is potentially essential for EGT biosynthesis.

### 3.2 Identification of EGT Biosynthesis Pathway in Mtb

Using the NCBI Basic Local Alignment Search Tool, we identified a five gene cluster in the open reading frames *rv3700c-rv3704c* to putatively encode for EGT biosynthesis in Mtb (Table 5).

Table 5. Sequence Identity of Mtb's EGT Biosynthetic Gene Cluster (*Rv3700c-Rv3704c*) with the *M. smegmatis* (*msmeg6246-6250*).

Enzyme	Accession Number	% Identity	% Similarity	E-value <sup>a</sup>
EgtA	<u>NP_218221.1</u>	66	77	0.0
EgtB	<u>NP_218220.1</u>	77	83	0.0
EgtC	<u>NP_218219.1</u>	74	82	4e <sup>-116</sup>
EgtD	<u>NP_218218.1</u>	74	81	6e <sup>-173</sup>
EgtE	<u>NP_338356.1</u>	66	79	9e <sup>-158</sup>

<sup>a</sup>Obtained from BLAST analysis

The five enzymes described in Mtb are similar to that of *M. smegmatis* (77-83%), and are the reciprocal best hits which provided considerable confidence that this gene cluster is responsible for EGT biosynthesis in Mtb. Furthermore, we did not identify any other gene clusters encoding for these five enzymes. Therefore, based on our *in silico* analysis we concluded that EGT biosynthesis occurs in Mtb as described in Figure 6.

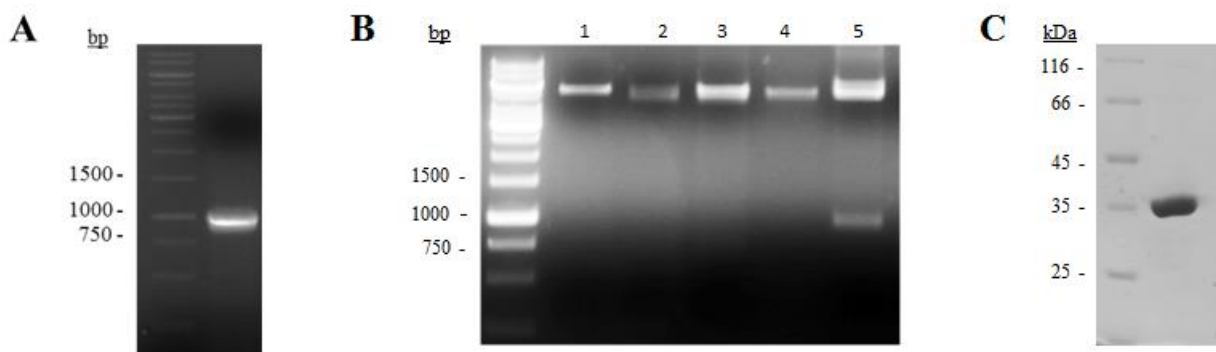


**Figure 6. *In silico* Analysis Identified the Gene Cluster *rv3701c-rv3704c* to Encode for EGT Biosynthesis in Mtb.**

### 3.3 *Rv3701c* Encodes for EgtD in EGT Biosynthesis

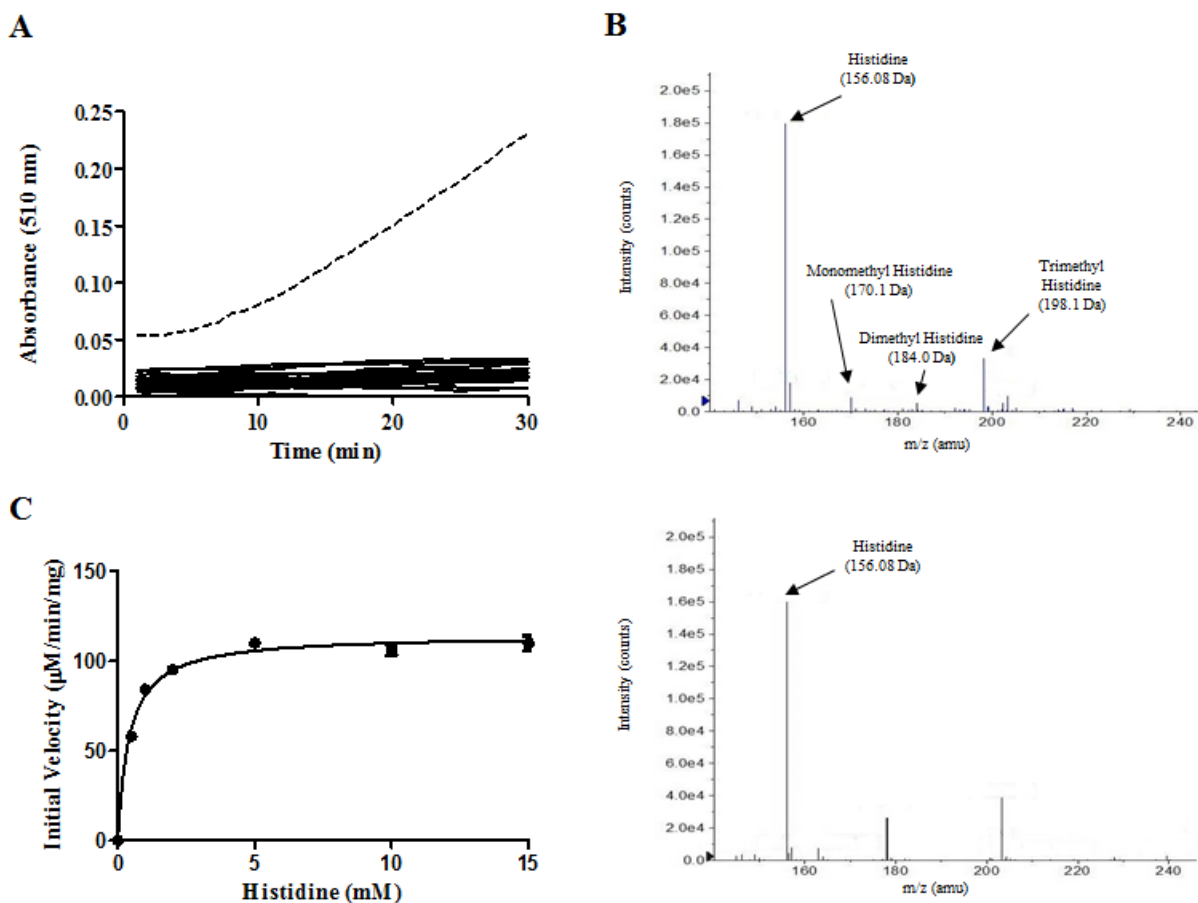
As the main tool needed for the identification of the EGT biosynthetic pathway in Mtb was to generate a single gene knockout mutant, we next determined which gene we were interested in removing. As seen in Figure 4, we predicted that *rv3701c* encodes for EgtD, a histidine methyltransferase that catalyzes the first reaction step involved in EGT biosynthesis (Figure 2). We believed that knocking out *rv3701c* would be best suited for our goal of understanding the role of EGT in Mtb physiology. Deleting the first enzyme in the pathway would prevent the accumulation of any intermediates, generating a phenotype specific for the loss of EGT. In addition, His methylation is a very rare reaction in nature and thus we deemed it unlikely that Mtb would contain a methyltransferase homologue which can compensate for EgtD. The synthesis of  $\gamma$ -glutamylcysteine by EgtA represents a second entrance to the pathway. However, we deemed the removal of EgtA would generate a phenotype in Mtb representative of a deficiency in  $\gamma$ -glutamylcysteine, which is a known antioxidant in bacteria (169).

We next wanted to confirm that *rv3701c* was indeed a histidine methyltransferase that is involved in EGT biosynthesis. To test this hypothesis, we cloned *rv3701c* from *Mtb* H37Rv for expression in *E. coli* (Figure 7) and assayed the purified recombinant protein (Figure 8).



**Figure 7. Production of the EgtD Recombinant Protein.** **A**, PCR amplification of *egtD* (966 bp) from *Mtb* H37Rv genomic DNA; 1% agarose gel. **B**, *egtD* positive clone in pET-28a (lane 5), double digested (NdeI-XhoI); 1% agarose gel. **C**, Purification of the over-expressed EgtD recombinant protein. EgtD was prepared following the induction of *E. coli* BL21 (DE3) harbouring the pET-28a-*egtD* plasmid. Proteins were purified using Ni-NTA resin. The elution fraction (containing 250 mM imidazole) was resolved by SDS-12% PAGE and stained with Coomassie blue. The DNA and molecular mass markers are indicated on the left in bp and kDa, respectively.

Using the SAM510 assay, the methylation activity of EgtD was assessed in the presence of each of the proteinogenic amino acids with the exception of cysteine due to its interference with the assay (234). The consumption of SAM in the reaction was continuously monitored at 1-min intervals over a 30-min time period. No change in absorbance was observed, with the exception of His, indicating that only this amino acid could undergo methylation by EgtD (Figure 8A). We further explored substrate specificity using the same methods, and did not detect methylation activity using imidazole and other His-derived substrates such as histamine and the 1-methyl-L-His, 3-methyl-L-His and  $\alpha$ -methyl-DL-His. These findings established that EgtD is a methyltransferase with high specificity for the amino acid His.



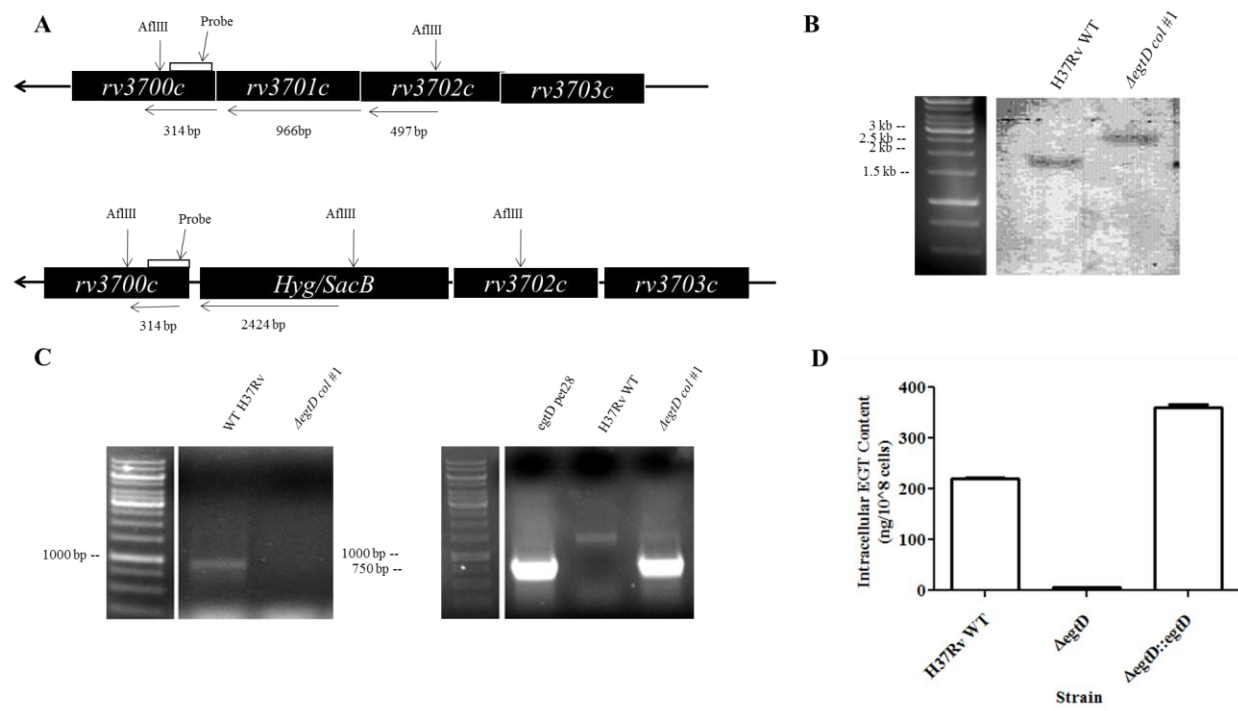
**Figure 8. EgtD Methylation Activity and Kinetics.** **A**, Screen of Rv3701c in the presence of each of the proteinogenic amino acids and additional compounds was performed to confirm Rv3701c as a His methyltransferase. *Dash line*: His; *solid line*: all proteinogenic amino acids (exception of cysteine), histamine, imidazole,  $\alpha$ -methyl-DL-His, 1-methyl-L-His, 3-methyl-L-His. Methylation activity was analyzed by continuously monitoring the consumption of SAM at 510 nm. **B**, ESI-MS analysis of the methylation activity of Rv3701c in the presence of SAM and His. *Upper*, Rv3701c catalyzes the methylation of the  $\alpha$ -amino nitrogen atom of His to form mono-, di-, and trimethylated His; *Lower*, Reaction in the absence of Rv3701c. No methylated His products were observed. **C**, EgtD activity was assessed using the enzyme-coupled SAM510 assay at various concentrations of His. Methylation kinetics indicate that EgtD follows Michaelis-Menten reaction ( $R^2=0.99$ ).

The methyltransferase assay we used for EgtD characterization does not identify the number of methyl groups added to a substrate, but solely describes if methylation is in fact occurring. Thus, we performed ESI-MS analysis on a methylation reaction containing EgtD and His with the attempt to isolate hercynine ( $\alpha$ -N,N,N-trimethyl His) variants. As observed in the

upper panel of Figure 8B, three methylation products, mono-, di-, and trimethylated His were produced in the presence of EgtD. However, when the same reaction was prepared in the absence of the methyltransferase (Figure 8B lower), His was not transformed into any of the expected methylated products. Lastly, the dependence of reaction velocity on His concentration was measured in the presence of 5.3  $\mu\text{M}$  EgtD (Figure 8C) using the SAM510 assay at various concentrations of His (0-15 mM). Experimental data was best fitted using the Michaelis-Menten equation ( $R^2=0.99$ ), and  $V_{\max}$  ( $115 \mu\text{M}/\text{min}\cdot\text{mg} \pm 2$ ) and  $K_m$  ( $422 \mu\text{M} \pm 4$ ) were calculated based on the averages of multiple measurements with standard error less than 15%. Based on these values, we further calculated the enzyme's  $k_{\text{cat}}$  ( $2 \times 10^{-2} \text{ s}^{-1} \pm 0.0003$ ) and  $k_{\text{cat}}/K_m$  ( $5 \times 10^3 \pm 89 \text{ M}^{-1}\cdot\text{s}^{-1}$ ).

### 3.4 EgtD is Essential for EGT Biosynthesis in Mtb

To further confirm the role and determine the necessity of EgtD in EGT biosynthesis, we constructed an Mtb  $\Delta\text{egtD}$  mutant in the H37Rv strain. We replaced the genomic *Rv3701c* open reading frame by specialized transduction with a Hyg-resistant cassette (Figure 9A) and verified the knock-out strain formation by Southern blot hybridization and PCR (Figure 9B and C, respectively). Intracellular EGT levels were quantified in the parental wildtype Mtb, the *Rv3701c* null mutant, and a complemented strain whereby the *rv3701c* mutant was transformed with a plasmid containing the *rv3701c* gene (pMV261: $\Delta\text{egtD}$ ). Negligible traces of EGT could be detected in extracts prepared from the  $\Delta\text{egtD}$  mutant, while both the wildtype and complemented strains synthesized considerable amounts of EGT (Figure 9D).



**Figure 9. Construction and *in vitro* Characterization of  $\Delta egtD$  in Mtb.** **A**, Schematic diagram of the *Rv3701c* region of the chromosome of Mtb. Genomic DNA was digested with AflIII, and the blot was probed with a DIG-11-dUTP labelled DNA fragment containing 314 bp of the *egtD* 3' flanking sequence. **B**, Confirmation of the  $\Delta egtD$  mutant through Southern blot. AflIII digested gDNA giving rise to an expected 1.77 kbp fragment in wildtype Mtb (lane 1) and 2.74 kbp fragment in the Hyg-resistant transductant in which *egtD* was disrupted with the *hyg* marker (lane 2). **C**, PCR analysis of the Hyg-resistant transductant gDNA for the  $\Delta egtD$ . *Left panel*, PCR amplification of the *egtD* (966 bp). *Right panel*, PCR amplification of the Hyg-resistant cassette (~700 bp), which replaces *egtD* in the mutants. **D**, Intracellular EGT levels from wildtype Mtb,  $\Delta egtD$  Mtb and  $\Delta egtD$  complemented with pMV261:*egtD* quantified by ESI LC-MS/MS. Error bars indicate the means  $\pm$  S.D. of three independent experiments.

### 3.5 Chapter Summary

Based on its close sequence identity to the previously characterized pathway in *M. smegmatis* and from the findings presented in this chapter, we found that ORF *rv3700c-rv3704c* encodes for EGT biosynthesis genes in Mtb. Through a number of biochemical assays, we further characterized the gene product of *rv3701c* to be a methyltransferase that catalyzes the conversion of His to hercynine in the first reaction step of EGT biosynthesis. Therefore, for the



remainder of our work we will refer to *rv3701c* as *egtD*. Lastly, the absence of EGT production in the  $\Delta egtD$  mutant indicates that EgtD is essential for the synthesis of EGT in Mtb.

## Chapter 4: Role of EGT in the *in vitro* Growth and Intracellular Survival of Mtb

### 4.1 Introduction

To ensure survival throughout infection, Mtb must overcome oxidative and nitrosative challenges associated with xenobiotics, including free radicals produced by the host to cope with infection, antibiotics, and general bacterial respiration. This stress is countered by an intricate detoxification system employed by Mtb that is composed of (a) enzymes such as catalase, superoxide dismutase, and alkyl hydroperoxidase, (b) truncated haemoglobins (c) oxidoreductases, and (d) redox coupling systems (92). The most common molecules involved in defense mechanism against cellular damage induced by ROS are LMW thiols. These small molecules (<500 Da) act as buffers against the hazards of ROS toxicity, scavenging oxidants through their sulfur group. GSH tends to be the dominant thiol in eukaryotes; however, considerable diversity exists in this protective system in prokaryotes (235). Mycobacteria, like most Gram-positive bacteria, do not produce GSH. Rather, they synthesize MSH at millimolar levels designating it as the most abundant LMW thiol in these species (236). Similar to GSH, MSH also serves as an antioxidant that is important in maintaining a highly reducing environment inside the cell (237). Several studies have demonstrated the role of MSH in detoxifying reactive species by either (i) donating a reducing equivalent (238), or (ii) forming an S-conjugate composed of MSH and the respective agent (239). Consistent with these findings, mycobacterial species deficient in MSH are found to have varying yet increased sensitivity to hydrogen peroxide (237,238,240), NO (241), and other redox cycling agents (237,238,242,243). Despite the importance of MSH in protecting mycobacteria against oxidative and nitrosative

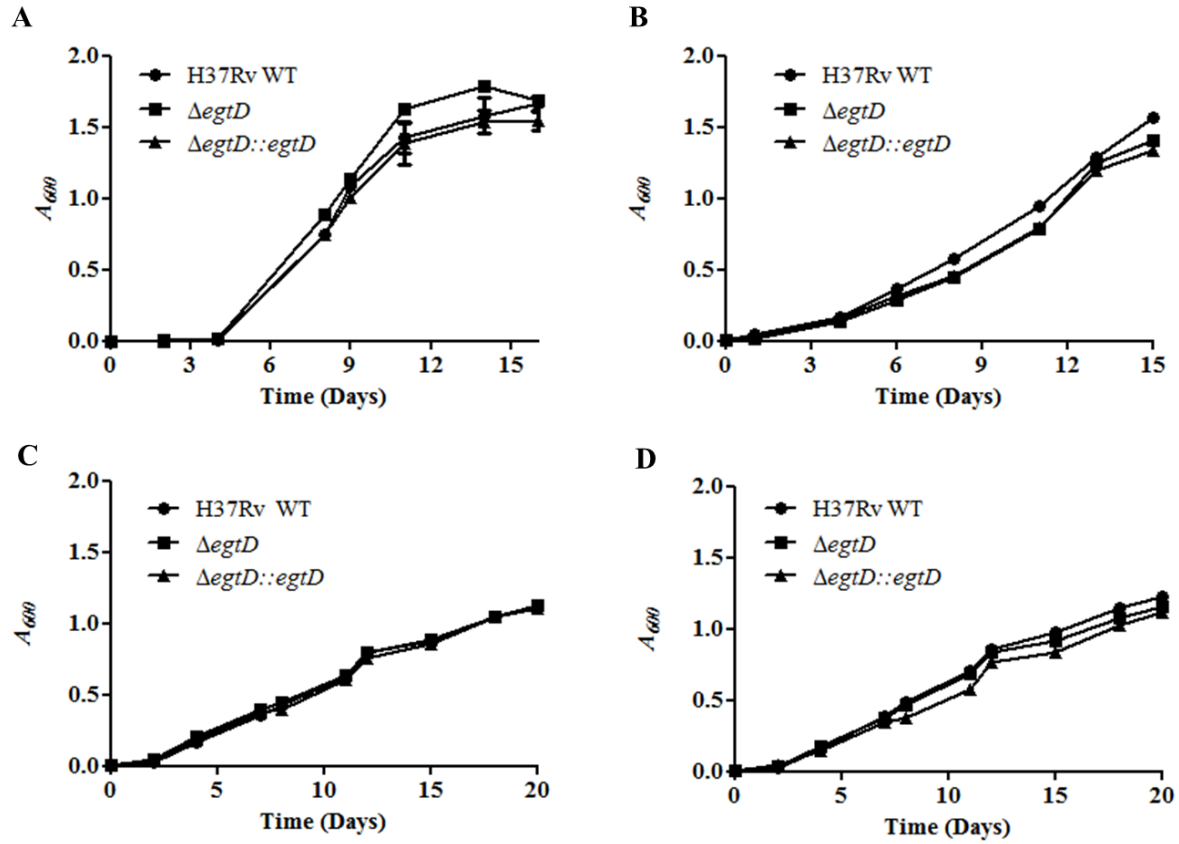
stressors, Mtb strains lacking MSH remain viable *in vivo*, which indicates a compensatory mechanism (244).

Fahey's group (159) showed that *M. smegmatis* mutants devoid of MSH displayed a marked elevation in EGT levels. As mentioned earlier, EGT exists primarily as a thione under physiological conditions, and possesses a lower redox couple value ( $E_0'$  -0.06V) (245). Although a poorer reductant, EGT is described as an effective antioxidant through quenching of singlet oxygen, scavenging of hydroxyl radicals, and inhibition of heavy metal-catalyzed reactions, as reviewed in (173), and its depletion in mammalian cells leads to augmented oxidative damage and cell death in the presence of exogenous stressors (195). Furthermore, we show that a transposon mutant that disrupts *egtD* (hereafter referred to as *egtD:Tn*) in Mtb CDC1551 exhibits increased sensitivity to hydrogen peroxide, paraquat, menadione, and cumene hydroperoxide (246). Therefore, we were interested in examining the role of EGT in protecting Mtb against ROS toxicity that results from growth *in vitro* and its influence on the survival of Mtb *ex vivo*.

#### **4.2 Role of Intracellular EGT during *in vitro* Growth**

We first explored a potential role for EGT in Mtb growth *in vitro*. Previously it was shown that MSH is essential for Mtb growth in the absence of catalase (244). The function of catalase supplementation is to remove toxic peroxides in the media (242). As EGT was previously suggested to have overlapping functions with MSH in mycobacteria and protect against peroxides (247), we tested whether the  $\Delta egtD$  mutant possesses similar growth defects to the MSH deficient mutant under *in vitro* growth conditions. No differences in growth were observed in rolling or standing cultures containing Middlebrook 7H9 medium supplemented with 10% OADC, 0.2% glycerol and 0.05% Tween-80 (Figure 10A and C). The same results were also observed when these strains were grown in ADS (albumin, dextrose and NaCl) for the

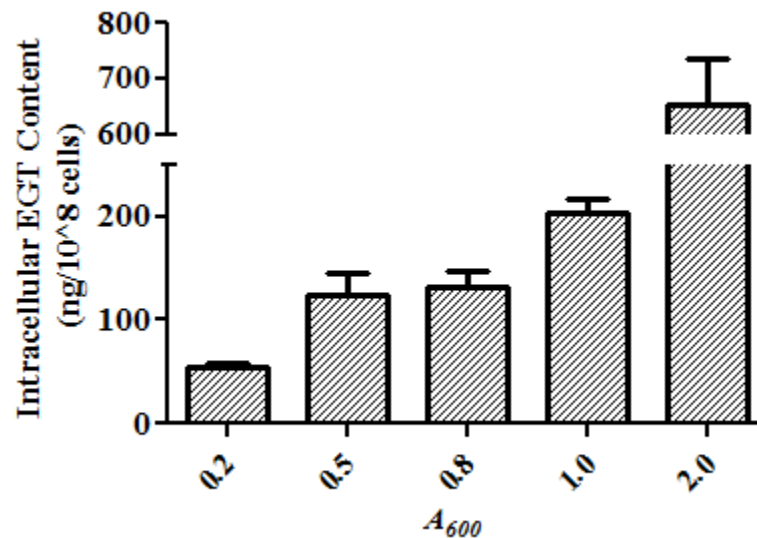
wildtype,  $\Delta egtD$ , and  $\Delta egtD$  complemented strains (Figure 10B and D). These findings indicate that Mtb does not require EGT for *in vitro* growth, and the  $\Delta egtD$  mutant does not require catalase for protection against ROS.



**Figure 10. *In vitro* Growth of Mtb in Nutrient Rich Media.** Growth of H37Rv wildtype,  $\Delta egtD$ , and the corresponding complemented strain ( $\Delta egtD::egtD$ ) grown in Middlebrook 7H9 medium supplemented with 10% OADC (A and C) or ADS (B and D), 0.2% glycerol, and 0.05% Tween-80. Strains were further grown in rolling bottle (A and B) or stationary cultures (C and D). Results are representative of two independent experiments  $\pm$  S.E. (error bars).

Despite not having observed a growth defect in the  $\Delta egtD$  mutant, we quantified intracellular EGT levels in wildtype at varying culture densities. Interestingly, we observed EGT to accumulate in Mtb during late-logarithmic phase (Figure 11). These findings indicate that Mtb

may utilize EGT under conditions involving growth limiting factors such as nutrient limitation, endogenous waste production, or low pH, rather than the growth process itself.

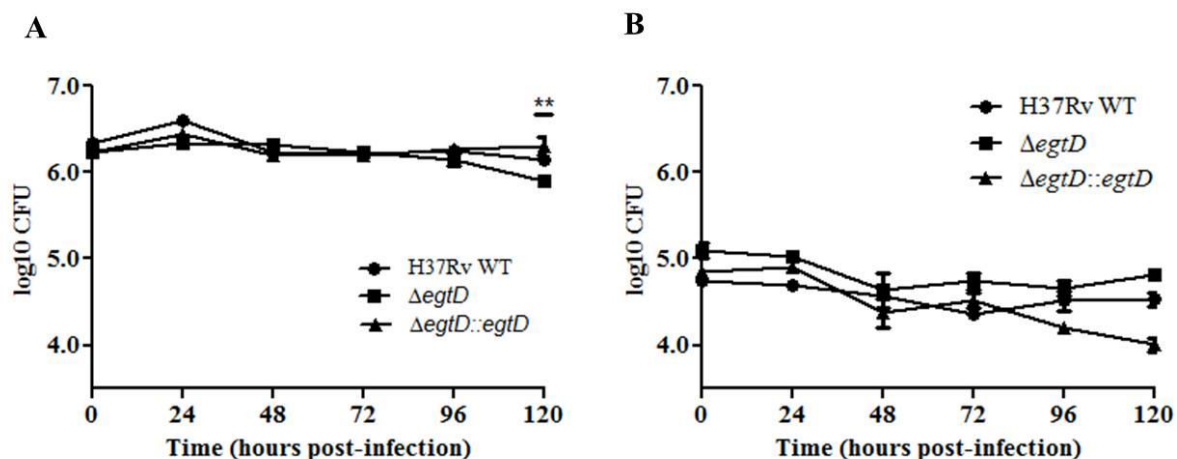


**Figure 11. Intracellular EGT Levels During Growth of H37Rv Wildtype Mtb.** Mtb was grown in nutrient rich broth (Middlebrook 7H9 supplemented with 10% OADC, 0.2% glycerol and 0.05% tyloxapol).

#### 4.3 Growth and Survival of Mtb $\Delta egtD$ in J774A.1 and THP-1 Cells

Since the macrophage generates ROS and RNS as a first line of defence against invading microorganisms, we hypothesized that EGT enhances the survival of Mtb during intracellular infection. Controversy still exists as to whether human macrophages produce adequate levels of NO. Since the expression of functional iNOS in mouse macrophages has been clearly demonstrated (248), we decided to study the role of mycobacterial EGT in both human monocytes and mouse macrophages. We assessed the growth and survival of the H37Rv wildtype,  $\Delta egtD$ ,  $\Delta egtD::egtD$  strains in human THP-1 cells and J774A.1 macrophages. Independent infection of the J774A.1 cells showed an approximate half-log reduction in CFUs at 120 h in the  $\Delta egtD$  mutant (Figure 12A). In contrast, no difference in the survival of the  $\Delta egtD$

mutant was observed relative to the wildtype in THP-1 cells (Figure 12B). These findings indicate that EGT provides Mtb with greater protection from the environment of murine macrophages rather than human monocytes five days post-infection.



**Figure 12. Replication and Survival of H37Rv Wildtype (WT),  $\Delta egtD$ , and the Corresponding Complement Strain ( $\Delta egtD::egtD$ ) in Macrophages.** J774A.1 macrophages (A), and THP-1 monocytes (B) were infected at a multiplicity of infection of 5:1. CFUs were calculated at the specified time points post-infection. Results are representative of two independent experiments that were each performed in triplicates  $\pm$  S.E. (error bars), \*\*  $P$  value  $<0.01$ .

#### 4.4 Chapter Summary

We examined the role of EGT in the growth of Mtb both *in vitro* and *ex vivo*. Through these studies we found that growth of  $\Delta egtD$  mutant is similar to that of wildtype parental strain when grown in rolling bottle or stationary cultures and the media is supplemented with ADS or OADC. From these findings, it appears that EGT is not required for the *in vitro* growth of Mtb and plays little to any role in protecting against oxidative stress generated under these conditions. When we monitored intracellular EGT levels during Mtb growth in oxygenated nutrient rich cultures, we found EGT to accumulate in late-stationary phase indicating a role for EGT under growth limiting conditions. Despite the observed accumulation of EGT, there was no apparent

defect in the  $\Delta egtD$  mutant entering stationary phase. Our *ex vivo* experiments showed reduced viability of the  $\Delta egtD$  mutant in murine macrophages. Interestingly, a similar phenotype for the  $\Delta egtD$  mutant was not observed in the THP-1 cells, which indicated that the protective effect of EGT is species or cell-line dependent. Furthermore, as the protective effect of EGT does not coincide with the oxidative burst of the macrophage and had no effect on the survival of Mtb infecting THP-1 cells, we suspect that EGT is needed to a greater extent at later stages of the disease.

## Chapter 5: Regulation of EGT Biosynthesis by Mtb STPKs

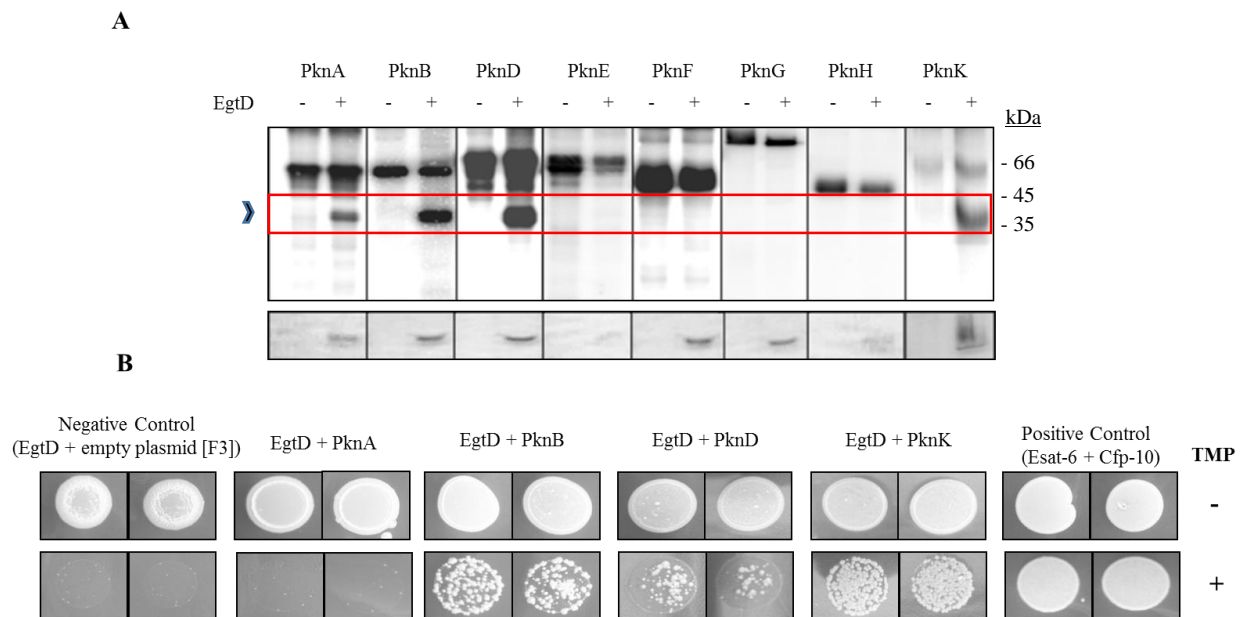
### 5.1 Introduction

The limited impact EGT has on the survival of Mtb inside of the macrophage led us to hypothesize that EGT has an alternative function in the bacilli's physiology and pathogenesis. Upon further analysis of the EGT biosynthetic pathway we realized that the synthesis of this molecule is quite an expensive process, consuming the amino acids His and cysteine as well as the co-factors ATP, SAM, and PLP (Figure 2). Therefore, we rationalized that its synthesis must be regulated. We further stipulated that if we could identify the environmental conditions under which EGT is regulated we could gain insight into its role. To start, we surveyed the literature to determine if any of the genes (*rv3700c-rv3704c*) in the cluster were regulated at a transcriptional level; however, we did not find any evidence for it. Consequently, we postulated that the Egt enzymes might be regulated post-translationally. Since phosphorylation is the most common type of regulatory post-translational modification, we became interested in exploring if the EGT biosynthetic enzymes are a substrate of one of Mtb's protein kinases. In Mtb, protein phosphorylation occurs through either two-component systems or STPKs. Generally, two component systems work in isolation, where a given pair of a His kinase and response regulator is highly selective for each other via protein-protein interaction (121), and thus we deemed it unlikely that EGT biosynthesis would be regulated in this manner. In contrast, Mtb STPKs have multiple substrates and have previously been identified in regulating several metabolic pathways, including mycolic acid (249-252), glutamine (194), PDIM (253) and glucan (254) biosynthesis. Therefore, we hypothesized that EGT biosynthesis may be regulated through Mtb's STPK.



## 5.2 EgtD is a Substrate of Mtb STPKs

Since EgtD is essential for EGT biosynthesis in Mtb (Figure 9D), the enzyme is a logical candidate for regulation in the pathway. As a result, we screened a set of Mtb STPKs for their ability to phosphorylate purified EgtD using *in vitro* kinase assays. From this screen, we identified PknA, PknB, PknD and PknK as kinases capable of phosphorylating EgtD (Figure 13A), as no radioactive bands were observed in the presence of the other STPKs or when EgtD was incubated in the absence of kinase. These findings indicate that EgtD is an *in vitro* substrate of several of Mtb's STPKs.



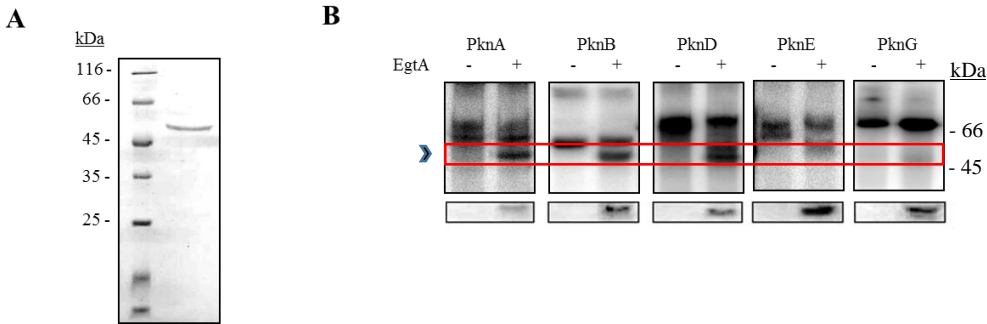
**Figure 13. EgtD is a Substrate of Multiple Mtb STPKs.** **A**, *In vitro* phosphorylation of EgtD by multiple kinases. Mtb STPKs purified as GST or His fusions were incubated with His-tagged EgtD and [ $\gamma$ - $^{32}$ P] ATP. Samples were separated by SDS-PAGE, stained with Coomassie blue followed by visualization by autoradiography. Upper bands represent autophosphorylation activity of each kinase (Pkn); lower bands reflect phosphorylated EgtD. **B**, Interaction between EgtD and Mtb's STPKs facilitates the reassembly of complementary fragments F1, F2, and F3 of murine dihydrofolate reductase and thus confers *M. smegmatis* resistance to trimethoprim (TMP). Growth was monitored over 4 days on Kan/Hyg plates supplemented with 0 or 10  $\mu$ g/ml TMP. Control plates without TMP revealed growth of all strains. *Positive control*, Mtb ESAT-6

(F1, F2), CFP-10 (F3); *Negative control*, EgtD (F1, F2) with F3 alone. Experiments are shown in duplicates.

To further explore the possibility that EgtD may be regulated post-translationally, we investigated whether EgtD interacts with the four identified kinases in a cell-based interaction assay (232). Performed in *M. smegmatis*, this assay involves the reassembly of complementary fragments F1, F2 (expressed by pUAB100) and F3 (expressed by pUAB200) of murine dihydrofolate reductase (mDHFR) enzyme, conferring resistance to trimethoprim (TMP). As illustrated in Figure 13B, growth of *M. smegmatis* co-transformed with pUAB100-*egtD* and pUAB200 containing *pknB*, *pknD* or *pknK* was present. No growth was observed in the strain co-expressing EgtD and PknA, indicating that these two proteins do not interact under *in vivo* growth conditions. The interaction between EgtD and the kinases is weaker than the positive control, CFP-10 and ESAT-6, which is expected due to the transient nature of kinase-substrate interactions. Therefore, the M-PFC assay provides evidence that EgtD is potentially a substrate for Mtb STPKs, PknB, PknD and PknK in mycobacteria.

### **5.3 EgtA is an *in vitro* Substrate of Mtb STPKs**

As EgtA represents the second entrance to the pathway and commits glutamate and cysteine to EGT biosynthesis, we believed that this could be a second site of post-translational regulation. To test this hypothesis, we cloned *egtA* into pET-28a, overexpressed and purified the corresponding protein to determine if EgtA was a substrate of Mtb's STPKs (Figure 14). In this screen we also found that PknA, PknB, and PknD were able to phosphorylate EgtA.



**Figure 14. EgtA is Phosphorylated by Multiple STPKs *in vitro*.** **A**, Purification of the overexpressed EgtA recombinant protein. EgtA was prepared following the induction of the *E. coli* BL21 (DE3) harbouring the pET-28a-*egtA* plasmid. The EgtA protein was purified using Ni-NTA resin and the elution fraction was resolved by SDS-12% PAGE and stained with Coomassie Blue. The molecular mass markers are indicated on the left in kDa. **B**, *In vitro* phosphorylation of EgtA by multiple kinases. Mtb STPKs purified as GST or His fusions were incubated with His-tagged EgtA and [ $\gamma$ - $^{32}$ P]-ATP. Samples were separated by SDS-12% PAGE, stained with Coomassie Blue followed by visualization by autoradiography. Upper bands represent autophosphorylation activity of each kinase (Pkn); lower bands reflect phosphorylated EgtA. This assay was performed with the assistance of Elenore Divry, an intern from the École Supérieure de Biologie-Biochimie-Biotechnologies (Lyon, France).

#### 5.4 PknD Preferentially Phosphorylates EgtD *in vitro*

Although it is apparent from Figure 13A that PknD possesses the greatest phosphorylation capacity towards EgtD, we wanted to further verify this notion by analyzing EgtD's phosphorylation kinetics. As observed from the results in Table 6, PknD possesses the greatest  $k_{cat}/K_M$  and lowest  $K_M$  for the methyltransferase, indicating that the interaction between these two proteins has the greatest relevance. Therefore, for the remainder of our work, we focused on the effect of PknD phosphorylating EgtD.

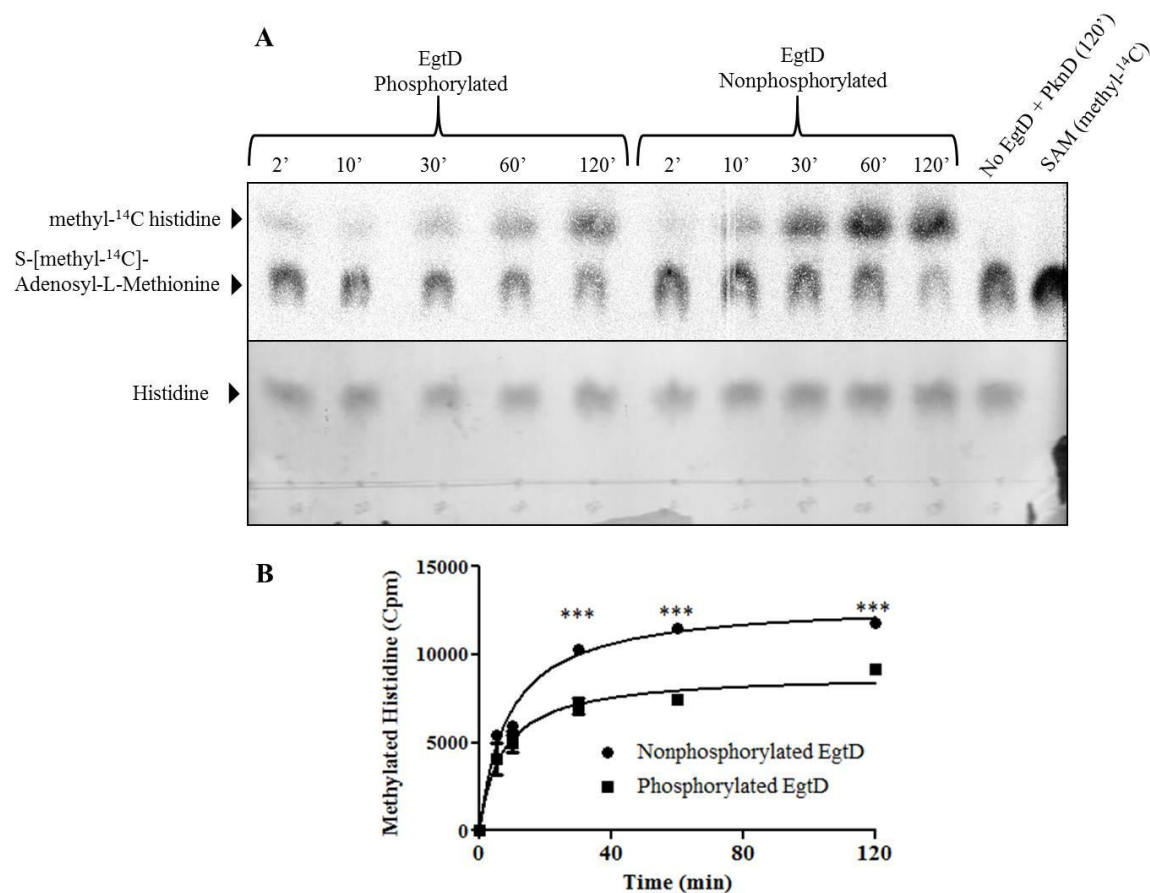
**Table 6. EgtD Phosphorylation Kinetics.** Various concentrations of EgtD (1-12  $\mu\text{M}$ ) were phosphorylated by 0.7-1 nM of kinase. The transfer of  $\gamma\text{-}^{32}\text{P}$  was measured via scintillation counting to determine phosphorylation kinetics. Data is representative of three independent experiments and presented as average values  $\pm$  S.E.

<b>Kinase</b>	<b><math>V_{max}</math> (nmol/min/mg)</b>	<b><math>K_m</math> (<math>\mu\text{M}</math>)</b>	<b><math>k_{cat}</math> (<math>\text{s}^{-1}</math>)</b>	<b><math>k_{cat}/K_m</math> (<math>\text{M}^{-1}\cdot\text{s}^{-1}</math>)</b>
<b>PknB</b>	$27 \pm 2$	$2 \pm 0.6$	$0.005 \pm 0.0003$	$3 \times 10^3 \pm 918$
<b>PknD</b>	$15 \pm 0.8$	$0.2 \pm 0.09$	$0.002 \pm 0.0001$	$1 \times 10^4 \pm 4528$
<b>PknK</b>	$10 \pm 1$	$0.6 \pm 0.2$	$0.001 \pm 0.0002$	$2 \times 10^3 \pm 777$

### 5.5 Phosphorylation of EgtD Reduces the Formation of Methylated His

Phosphorylation of a protein introduces a negative charge on the targeted amino acid(s) which may ultimately affect protein activity. To investigate the effect of phosphorylation on EgtD's methylation activity, we developed an assay designed to monitor the transfer of methyl- $^{14}\text{C}$  from SAM to His. Phosphorylated and non-phosphorylated EgtD were prepared using the *in vitro* kinase assay in the presence or absence of PknD. Following 2 h of incubation at room temperature, the kinase assays containing EgtD were added to the methylation assays containing S-[methyl- $^{14}\text{C}$ ]-Adenosyl-L-Methionine and His. Samples were taken at various time points from the reaction and separated by TLC (Figure 15A). The formation of methyl- $^{14}\text{C}$  His was visualized by autoradiography (*upper*), while total His was observed on TLC plates developed with ninhydrin (*lower*). As expected, the combined kinase and methylation assay absent of EgtD did not form methyl- $^{14}\text{C}$  His following 2 h. However, the production of methylated His by phosphorylated EgtD was visibly less than that of the non-phosphorylated enzyme.  $^{14}\text{C}$  His spots were then scraped from the TLC plate and the cellulose stationary phase was added to scintillation fluid for quantification. Counts per minute (Cpm) of phosphorylated and non-phosphorylated EgtD methylated His were plotted as a function of time for both enzyme sets

(Figure 15B). An approximate 20% reduction in product formation in the phosphorylated EgtD reactions was observed compared to the non-phosphorylated EgtD reactions following 30 min of incubation. However, there was no significant difference in the initial rate of methylated product formed during the first 10 min of these reactions suggesting phosphorylation may decrease EgtD's affinity for the methylated His intermediates, rather than decrease the catalytic efficiency of the enzyme itself. Nonetheless, the formation of methyl- $^{14}\text{C}$ -His was significantly lower in the reaction containing phosphorylated EgtD, suggesting that PknD may negatively regulate EgtD in Mtb.

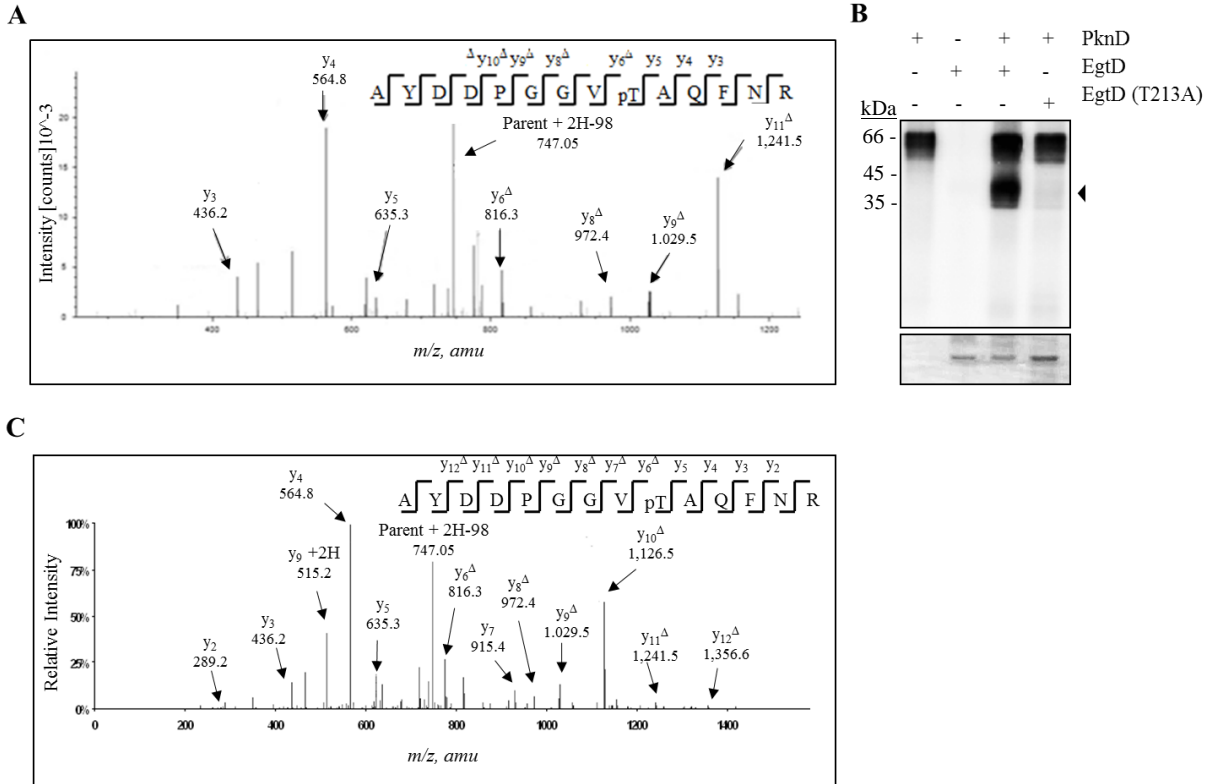


**Figure 15. Phosphorylation of EgtD Reduces the Formation of Methylated His. A,** Phosphorylated and non-phosphorylated EgtD was obtained from an *in vitro* kinase assay and

was added to a methylation assay containing S-[methyl- $^{14}\text{C}$ ]-Adenosyl-*L*-Methionine (2  $\mu\text{Ci/ml}$ ). The transfer of methyl- $^{14}\text{C}$  to His was monitored over a two-hour period and analyzed by one-dimensional TLC using butanol/acetic acid/water (60:15:25, v/v). *Upper*, Detection of methyl- $^{14}\text{C}$ -His was performed by autoradiography, exposing the TLC plate to X-ray cassettes for one week. *Lower*, TLC plate developed with ninhydrin to visualize His. **B**, Graphical representation of the effect of phosphorylation on methylated His formation from the EgtD methylation reactions. \*\* $P < 0.005$ , \*\*\* $P < 0.0005$  for comparison of phosphorylated vs. non-phosphorylated EgtD. Error bars indicate the means  $\pm$  SEM of three independent experiments. Cpm, counts per minute; SAM, S-adenosylmethionine.

## 5.6 PknD Negatively Regulates EGT Biosynthesis in Mtb

Introduction of a negative charge, through the substitution of acidic residues such as Asp or Glu, has been previously shown to mimic phosphorylation of a protein with regards to functional activity (249,251,252,254). We were interested in further determining the effect of phosphorylation on EGT levels in Mtb, and thus constructing EgtD variant strains with constitutively altered activities. To do this, we were first required to identify EgtD phosphorylation site(s) by PknD. Mass spectrometry was used to identify the nature and location of phosphorylation on Mtb EgtD as performed previously (126). MS/MS analysis of purified recombinant EgtD incubated in the presence of PknD identified phosphorylation on the trypsin-digested  $^{205}\text{AYDDPGGVTAQFNR}^{218}$  peptide which was located on Thr-213 in the C-terminus of the protein (Figure 16A). No autophosphorylation was observed in the negative control containing EgtD incubated with ATP in the absence of PknD. The phosphopeptide identified by mass spectrometry was validated by substituting EgtD Thr-213 to alanine (EgtD T213A) by site-directed mutagenesis in order to prevent phosphorylation. The autoradiogram in Figure 16B shows that T213A site-directed mutagenesis resulted in abrogation of EgtD phosphorylation compared with wildtype EgtD. The above findings further supported EgtD Thr-213 as the major phosphorylation site for PknD *in vitro*.



**Figure 16. Identification of EgtD Phosphorylation by PknD.** **A**, MS/MS spectra at (+2) representing peptide positions 205-218 with a monoisotopic mass of 1,510.69 Da from EgtD phosphorylated by PknD *in vitro*. Phosphorylation at Thr-213 was shown by the “y” C-terminal daughter ion series, where all y ions identified lose phosphoric acid (-98 Da) after the phosphorylated residue. *pT*, phosphothreonine; *amu*, atomic mass units. **B**, *In vitro* kinase assay confirmed Thr-213 as the major phosphorylation site of EgtD by PknD. EgtD T213A is defective in phosphorylation. *Upper*, phosphorimage; *lower*, Coomassie Blue stain. *Arrowhead* points to EgtD. **C**, MS/MS spectra *m/z* 795.83 (+2) representing peptide positions 205-218 from EgtD phosphorylated in a cell-based system with PknD showing phosphorylation of Thr-213. Phosphorylation at Thr-213 is shown by the “y” C-terminal daughter ion series, where all y ions after Thr-213 lose phosphoric acid.

We wanted to further validate EgtD’s phosphorylation site in an “*in vivo*” cell-based system. Due to potential interference of other mycobacterial STPKs, we decided to study the interaction between EgtD and PknD using *E. coli* as a heterologous host, which lacks STPKs. The active kinase domain of PknD and full-length recombinant wildtype EgtD were sub-cloned into pET-28a and pGEX-4T3, respectively; to achieve compatible expression conditions.

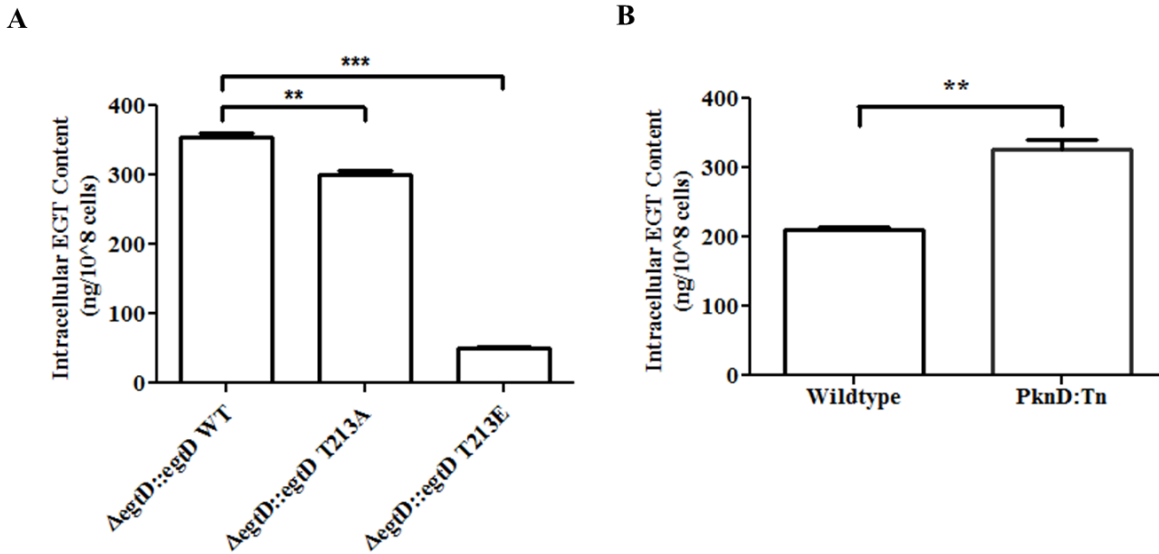
Purified EgtD expressed in the presence and absence of PknD inside *E. coli* underwent LC-MS/MS analysis to identify the phosphorylation sites. As seen previously *in vitro*, EgtD co-expressed with PknD was monophosphorylated on peptide <sup>205</sup>AYDDPGGVTAQFNR<sup>218</sup> (Figure 16C), but no phosphorylation was observed in the absence of the kinase in *E. coli*. From these results, we concluded that Thr-213 is the major phosphorylation site of EgtD both *in vitro* and in an *in vivo* cell-based system.

Following the identification of Thr-213 as the major phosphorylation site, we constructed and then transformed the  $\Delta$ *egtD* mutant with pMV261 derivatives allowing constitutive expression of different *egtD* alleles under the control of the *hsp60* promoter: *egtD*\_WT, phosphomimetic *egtD*\_T213E and phosphoablative EgtD\_T213A. As shown in Figure 17A, EGT levels in the EgtD\_T213A over-expression strain were comparable to EgtD\_WT, with a minor reduction likely due to the nature of the amino acid substitution. In contrast, over-expression of EgtD\_T213E was accompanied by a significant decrease in EGT levels in comparison to EgtD\_T213A and EgtD\_WT, providing evidence that EgtD phosphorylation negatively regulates EGT biosynthesis in Mtb.

To further confirm the effect of EgtD phosphorylation on EGT biosynthesis we investigated EGT levels in the CDC1551 *pknD*:Tn mutant generously provided by John Hopkins School of Medicine (Figure 17B). Since PknD is expressed during mid- log phase (255), and we observed phosphorylation to negatively regulate EgtD methylation activity and EGT biosynthesis, we hypothesized that the *pknD* mutant would exhibit higher levels of EGT than wildtype Mtb in culture. Intracellular EGT was extracted from CDC1551 wildtype and *pknD*:Tn mutant at an OD<sub>600</sub>=0.5. As expected, the *pknD*:Tn mutant had significantly higher levels of EGT than



wildtype, indicating that in the absence of PknD regulation EgtD's enzymatic activity is enhanced.



**Figure 17. Phosphorylation of EgtD Reduces EGT Levels in Mtb.** **A**, Electrocompetent H37Rv Mtb cells were transformed with pMV261\_egtD\_WT, pMV261\_egtD\_T213A, pMV261\_egtD\_T213E to allow for the constitutive expression of the *egtD* alleles under the control of the *hsp60* promoter. Bacteria were harvested at mid log phase, washed with purified water, and lysed in 70% acetonitrile using MagNAlyser (Roche). Bacterial lysates were collected and analyzed for EGT by ESI LC-MS/MS. EGT intracellular levels were normalized to number of cells. **B**, CDC1551 wildtype and a PknD:Tn (point of insertion at bp 1166) were generously provided by the John Hopkins Mutant Library (Baltimore, MD) and were grown to mid log phase prior to extraction and EGT was quantified by ESI LC-MS/MS. The results presented for both A and B are expressed as the mean of three independent experiments  $\pm$  S.E. \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ .

## 5.7 Chapter Summary

In this chapter, we show that EgtD is a substrate of multiple Mtb STPKs both *in vitro* and intracellularly as shown in *M. smegmatis* and *E. coli*. Studies describing phosphorylation kinetics further demonstrated that PknD preferentially phosphorylates EgtD, and that phosphorylation by this kinase negatively regulates EgtD's methylation activity *in vitro*. To understand the effect of PknD on EGT biosynthesis in Mtb we identified the major phosphorylation site for EgtD both *in*

*vitro* and in a cell-based system. Complementation of the  $\Delta egtD$  mutant with a phosphomimicking (T213E), but not the phosphoablative (T213A) or wildtype EgtD protein led to significantly reduced intracellular EGT levels. Further confirming these results, we showed that a Mtb mutant harbouring a defective PknD protein showed elevated levels of intracellular EGT in comparison to the wildtype strain. Together, these findings indicate that through the phosphorylation of EgtD, PknD negatively regulates EGT biosynthesis in Mtb.

## Chapter 6: Role of EGT in Mtb Starvation and Central Metabolism

### 6.1 Introduction

Given our new information on the regulation of EGT biosynthesis in Mtb, we sought out conditions where intracellular EGT levels may be up-regulated. Microarray studies analyzing the global adaptation of Mtb described *pknD* to be down-regulated 4 h following nutrient starvation (228). As we described PknD to be a negative regulator of Mtb EgtD, we therefore hypothesized that EGT levels will increase during starvation.

Although still unclear, it is generally believed that the phagosome is a site of nutrient deprivation for pathogens (256). Genome-wide expression profiling of Mtb RNA isolated from chronically infected mouse lungs or sputum of tuberculosis patients revealed transcriptional signatures reflective of nutrient limitation (257-260). Further evidence indicates Mtb secretes a siderophore to acquire iron within the phagosome (261), as well as upregulating its glyoxylate cycle to limit the loss of carbon through the production of CO<sub>2</sub> from the TCA cycle (262-265). In addition, Mtb isolated from lung lesions demonstrated an altered morphology and staining properties that were similar to cultures starved in distilled water for two years (266).

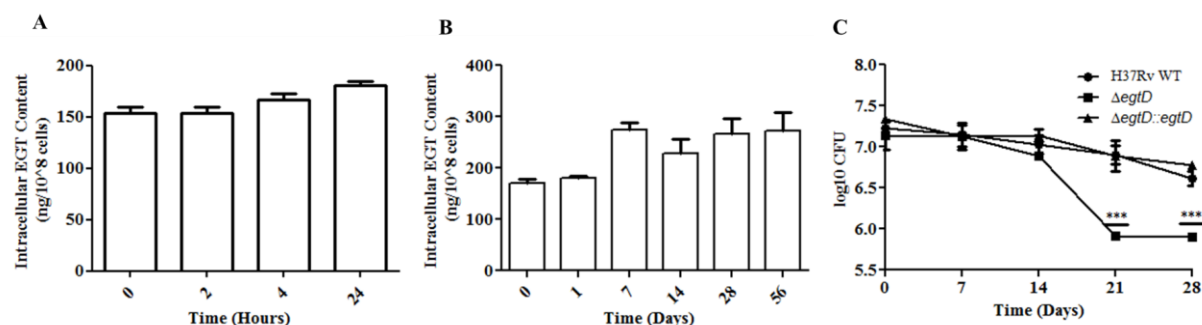
Upon further analysis, Loebel *et al.* (58) and Betts *et al.* (228) identified that starvation of Mtb in PBS resulted in the gradual shutdown of respiration to minimal levels, and the bacteria remained viable but non-replicating, a physiological state similar to that observed in during latent infection. NRP confers Mtb with phenotypic resistance against major first- and second-line anti-TB drugs (267-269), which not only makes latent disease difficult to eradicate, but the presence of these sub-populations in active TB cases are responsible for prolonged treatment times. The WHO estimates that one-third of the world's population has latent TB, further placing these individuals at risk of disease reactivation during their lifetime. Despite the apparent importance

of understanding bacterial persistence in TB to improve disease management, the physiology of Mtb quiescence is poorly understood. Therefore, it was of interest to verify and gain insight into the role of EGT in Mtb persistence.

## **6.2 Mtb Intracellular EGT Levels Increase During Starvation**

To identify if EGT plays a role in starvation we grew wildtype H37Rv in PBS containing 0.05% tyloxapol and extracted intracellular EGT from cells over a 24 h time period.

Corresponding to a decrease in *pknD* transcripts described by Betts *et al.* (228) 4 h following starvation, we observed a slight increase in EGT at 4 and 24 h (Figure 18A). From these results, we speculated that EGT levels likely continue to increase following a 24 h time period, and thus we decided to monitor its synthesis during long-term starvation. At one week following starvation, EGT levels increased and were maintained over the six-week experimental time frame (Figure 18B). Despite being elevated during long-term starvation, we wanted to determine if EGT was indeed necessary for the survival of Mtb under these conditions. Again, we grew our cultures in PBS containing 0.05% tyloxapol and monitored bacterial survival by counting CFUs for up to four weeks. The H37Rv wildtype and the  $\Delta egtD$  complement strains did not show a significant loss in viability over this time period; however, the  $\Delta egtD$  mutant CFUs were reduced by a magnitude of approximately 1.5 log after three weeks (Figure 18C). From these findings, we conclude that Mtb requires EGT for its survival during long-term starvation.



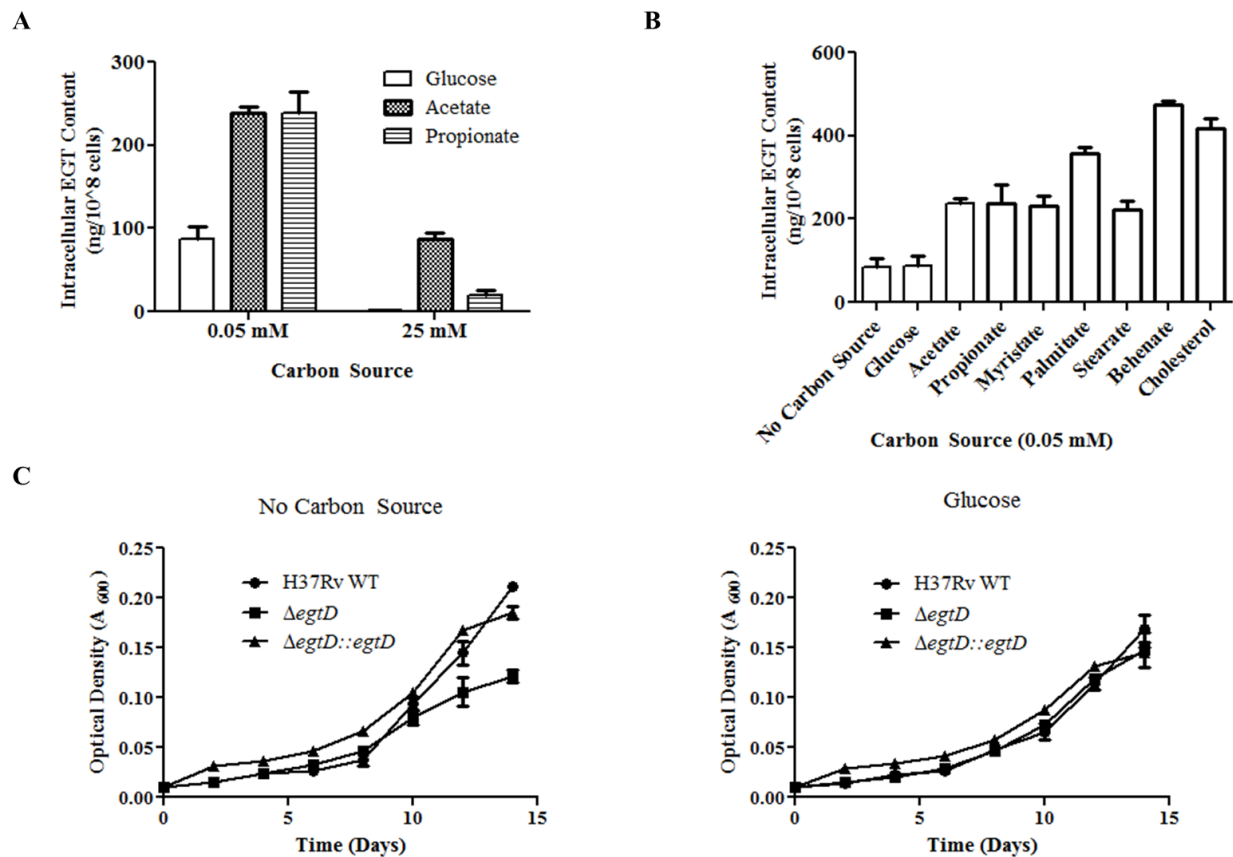
**Figure 18. EGT Biosynthesis is Up-Regulated and Necessary for Mtb Survival During Long-Term Starvation.** Wildtype Mtb intracellular EGT levels were monitored during short-term (A) and long-term (B) starvation. EGT was quantified by ESI LC-MS/MS. C, survival of H37Rv wildtype,  $\Delta egtD$  and  $\Delta egtD::egtD$  strains in 4-week-starved cultures. Samples were taken on a weekly basis to assess viability by CFU counts. In these three experiments (A-C), samples were taken from Mtb incubated as static cultures at 37°C and starved in PBS with 0.05% tyloxapol. The results are representative of two independent experiments (each experiment performed in triplicates)  $\pm$  S.E. \*\*\*  $P < 0.0001$ .

### 6.3 EGT Biosynthesis is Dependent on Central Carbon Metabolism

The observation that EGT is required for the survival of Mtb under conditions of long-term starvation indicates its involvement in metabolism. To further establish a link between EGT and carbon source availability, we quantified intracellular EGT levels in the presence of different concentrations of glucose, acetate and propionate (Figure 19A). Regardless of the carbon source, intracellular EGT levels decrease with increasing availability of glucose, acetate or propionate, and negligible EGT levels were observed when Mtb was grown in the presence of high glucose concentrations. Another observation from these results was the apparent influence a carbon source has on intracellular EGT levels. We therefore became interested in further examining this effect in the presence of longer chained fatty acids and cholesterol as these are Mtb's primary *in vivo* carbon sources (256). Again, we grew wildtype H37Rv in the presence of various carbon sources (0.05 mM) in 7H9 supplemented with 0.05% tyloxapol, and quantified intracellular EGT levels. Mtb that was grown in cultures containing higher (25 mM)

concentrations of the respective carbon source grew at a faster rate indicating that the tested carbon sources had not reached saturating levels in Mtb metabolism. However, Mtb grown in 0.05 mM concentrations of the examined carbon sources all grew at a similar rate as an  $A_{600} = \sim 1.0$  was reached and EGT was extracted from the cells on the same day.

From Figure 19B, it is apparent that the type of carbon source influences intracellular EGT levels; however, the carbon chain length does not. Lastly, we examined the impact of EGT on Mtb growth in the presence and absence of glucose (1 mM) over 14 days (Figure 19C). In this set of experiments, we observed that in the absence of a carbon source, the absorbance of the Mtb  $\Delta egtD$  mutant was significantly lower than that of the wildtype by day 12. However, the observed decrease in OD could be reversed by the addition of glucose to the culture (Figure 19C).



**Figure 19. Intracellular EGT Levels are Influenced by Type and Availability of Carbon Source.** H37Rv wildtype Mtb was grown in 7H9 supplemented with 0.05% tyloxapol and the respective carbon source to an  $A_{600} = \sim 1.0$  prior to quantifying EGT levels by ESI LC-MS/MS. **A)** Intracellular EGT levels monitored at different concentrations of glucose, acetate or propionate. **B)** Quantification of intracellular EGT levels in the presence of various carbon sources (0.05 mM). **C)** Absorbance readings of  $\Delta egtD$  mutant cultures relative to wildtype and  $\Delta egtD::egtD$  over a 14 day period in the presence (right) or absence (left) glucose (1 mM).

## 6.4 Chapter Summary

Through our findings on the regulation of EGT biosynthesis we hypothesized that intracellular EGT levels are related to carbon source availability. We found EGT levels to be up-regulated during long-term starvation, and for EGT to be required for Mtb survival in this state of NRP. These findings were further supported by the fact that EGT levels decrease upon increasing availability of a carbon source. In addition, we found intracellular EGT levels to be influenced by the type of carbon source present. Particularly, EGT biosynthesis is up-regulated in the presence of palmitate and cholesterol, two primary components of Mtb lipid bodies, but remains constant in the presence of other fatty acids. Taken together, these findings link EGT to Mtb metabolism and persistence.

## Chapter 7: Discussion

Although EGT was discovered over one hundred years ago (132) its role in microbial physiology and infection remained unknown. Due to its previously proposed antioxidant properties (173), and observed elevation in a *M. smegmatis* MSH deficient mutant (159) we hypothesized that EGT contributes to Mtb's resistance to oxidative stress generated by the macrophage during the early stages of infection (92). Therefore, the underlying goal of this work was to elucidate the role of EGT in the physiology and pathogenesis of Mtb.

### 7.1 *Rv3701c* Encodes for a His Methyltransferase Required for EGT Biosynthesis in Mtb

To effectively study the role of EGT in Mtb biology, we generated a strain deficient in EGT for comparative studies. We used an *in silico* analysis based off of Seebeck's work (155) to identify a putative five gene cluster (*rv3700c-rv3704c*) in Mtb. Purification of the recombinant protein encoded by *rv3701c* led us to characterize the protein as EgtD, as we found it to possess methylation activity specific to His (Figure 8A). The lack of methylation activity detected when using imidazole or histamine indicates that the substrate-binding site of EgtD recognizes both the imidazole ring and carboxylate side chain to be catalytically active. In addition, the presence of substituted methyl groups, as shown by 1-methyl-L-His, 3-methyl-L-His and  $\alpha$ -methyl-DL-His, could not be recognized by EgtD's active site, which demonstrated the importance of the spatial orientation of the imidazole ring and  $\alpha$ -carbon. These findings indicate that Mtb EgtD can only accommodate substitutions at the  $\alpha$ -nitrogen of His. This notion is further supported by recent crystallographic analysis of the substrate-binding site where the carboxylate group and imidazole ring of His form an extensive hydrogen bonding network with a number of EgtD amino acid residues. In contrast, the  $\alpha$ -amino group of His forms a single hydrogen bond permitting the accommodation of methyl groups at this site (270,271).



An earlier study by Reinhold *et al.* (272) observed the conversion of His to  $\alpha$ -*N*-methyl-L-His and  $\alpha$ -*N*-*N*-dimethyl-L-His derivatives, and hercynine in the presence of a cell-free extract of *N. crassa* mycelium. The findings from Reinhold *et al.* (272) in combination with our own substrate specificity data indicate that the methylated His products observed in Figure 8B were mono-, di- and tri-methylated on its  $\alpha$ -nitrogen. The  $\alpha$ -*N*-methyl-L-His and  $\alpha$ -*N*-*N*-dimethyl-L-His intermediates are the preferential substrates of EgtD in *N. crassa* (272) and *M. smegmatis* (155), which indicates that EgtD methylates His in a step-wise reaction. As all three EgtD-catalyzed reactions proceed with a similar turnover number ( $k_{cat}$ ) and little difference in catalytic efficiencies ( $k_{cat}/K_M$ ) these findings suggest that the observed processivity is a result of increased affinity for the methylated intermediates (270). Indeed, Vit *et al.*, (270) identified EgtD to have four- and 70-times stronger binding of  $\alpha$ -*N*-methyl His and *N*- $\alpha$ -dimethyl His, respectively, than that of His itself. The specificity of EgtD for these methylated derivatives likely acts as a regulatory mechanism limiting the quantity of His taken up for EGT biosynthesis.

Comparison of our EgtD kinetics with that of Vit *et al.* (270) showed similar  $K_M$  and  $k_{cat}/K_M$  values. Similarity, kinetic data in these two studies suggest similar structure and properties between Mtb EgtD and *M. smegmatis* (270).

From our biochemical assays we concluded that *rv3701c* encodes for a His methyltransferase that is able to generate a trimethylated His product which we predicted to be the EGT biosynthetic intermediate hercynine. Through allelic exchange using a conditionally replicating mycobacteriophage, we constructed an *rv3701c* knockout strain to determine the relevance of *rv3701c* in EGT biosynthesis (Figure 9B and C). The absence of EGT in this knockout strain confirms that *rv3701c* encodes for EgtD and is required for EGT biosynthesis in Mtb (Figure 9D). We also showed that the Mtb CDC1551 *egtD* and *egtA* transposon mutants

were unable to synthesize EGT (246), and the essentiality of *egtD* in *M. smegmatis* in EGT biosynthesis has been independently confirmed (273). Furthermore, the enzyme Egt1, a fusion protein of both EgtD and EgtB, was also found to be essential for EGT biosynthesis in *N. crassa* (143) and *S. pombe* (144).

## **7.2 Regulation of EGT Biosynthesis by Mtb STPKs**

The inherent energy cost of synthesizing EGT in mycobacteria led us to believe that biosynthesis is likely regulated as it would not be energetically economic for the cell to continuously canalize numerous metabolites into the pathway. We identified EgtD to act as a substrate for several STPKs and specifically of PknD, which negatively regulates EGT biosynthesis in Mtb. Thr-213 was shown to be the phosphorylation site both *in vitro* and inside *E. coli*, and in combination with the observed negative effect of phosphorylation on EgtD activity and EGT biosynthesis, these findings point to Thr-213 as a critical residue in regulation. Crystallization studies of *M. smegmatis* EgtD, performed by Vit *et al.* (270) and Jeong *et al.* (271) show Thr-213 to be responsible for binding the imidazole ring of His via a water-mediated hydrogen bond. Therefore, the introduction of a phosphate at this residue may impede the interaction between the methyltransferase and its substrates, resulting in the observed reduction in methylated His. Interestingly, the phosphosite Thr-213 is buried deep within EgtD's structure when bound to His (271) suggesting that phosphorylation of EgtD at this residue takes place prior to substrate-binding. Although superimposition of EgtD-His on the apo-EgtD structure did not show any significant difference in protein conformation, the authors were unable to exclude the possibility that the binding of His resulted in a conformational change. Interestingly, the imidazole and acetate molecules from the crystallization solution were both found to occupy the His binding pocket of the apo-EgtD and the interaction mode of these bound molecules highly

resembled that of His (271). Due to the low concentrations of imidazole and acetate in a physiological environment it is predicted that the apo-form of EgtD in cells will have an open conformation for easy access of PknD and/or the substrate into the substrate-binding pocket. The results of our phosphopeptide analysis (Figure 16c) further support the notion of the dynamic structure of EgtD, as we observed phosphorylation of EgtD at Thr-213 by PknD in a cell based system.

We discovered the amount of His methylation product was reduced by only 20% by phosphorylated EgtD *in vitro* (Figure 15); however, we observed a dramatic drop in EGT levels (~85%) from our genetic experiments (Figure 17). Based on our observations from the progress curve in Figure 15B, phosphorylation exhibits little effect on EgtD's catalytic efficiency in the presence of His. Therefore, we deem it likely that phosphorylation influences the affinity of the mono- and/or di-methylated His intermediates. If this is indeed the case, we would expect there to be little influence on the initial rate of EgtD's methylation activity with His, but overtime result in lower levels of methylated product due to the higher affinity of non-phosphorylated EgtD for mono- and di-methylated His. A reduced affinity for the methylated intermediates would ultimately produce inadequate amounts of hercynine limiting EGT biosynthesis in Mtb. Furthermore, a number of fungi contain two EgtD homologues, one of which contains considerable variation at position 213 (based on Mtb EgtD numbering). For example, Ybs from *Aspergillus nidulans* has an asparagine at position 213 and His is ten times less efficient as a substrate than that of Tyr (270). Further structural analysis identified this residue in recognizing Tyr as a substrate through hydrogen bonding. From these findings it is evident that position 213 plays a crucial role in not only modulating EgtD's methylation activity but also substrate specificity.

It is also plausible that, despite incubating EgtD with PknD for an extended period of time to achieve optimal phosphorylation, only a small portion of the methyltransferase population was phosphorylated. Column purification of recombinant EgtD from *E. coli* was performed using imidazole as the eluent, and despite being dialyzed in Tris following purification, may have left a significant portion of the EgtD population in a closed confirmation impeding PknD's access to Thr-213. Stoichiometric analysis of the level of phosphorylation achieved for EgtD would have aided in the clarification of whether the effect of PknD on EGT biosynthesis is due to negatively regulating EgtD's catalytic activity or altering its affinity for methylated His substrates. Regardless of the mechanism, both *in vitro* and *in vivo* phosphorylation of EgtD by PknD demonstrate a negative effect on EGT biosynthesis.

In addition to PknD, we also found that EgtD can serve as a substrate for both PknB and PknK (Figure 13). It is not uncommon for substrates to be the target of more than one Mtb STPK. The effect of phosphorylation on its substrate's enzymatic activity has been shown to remain the same, regardless of the acting STPK, and has been proposed as a means for the cell to "fine tune" enzymatic activity (274). STPKs are also found to regulate more than a single enzyme in a biosynthetic pathway, indicating that other EGT biosynthetic genes could be regulated. Our preliminary work demonstrating that EgtA can also be phosphorylated by multiple STPKs *in vitro* (Figure 14) provide evidence that this may be the case.

### **7.3 Role of EGT in the *in vitro* Growth and Intracellular Survival of Mtb**

MSH has been found to be essential in the *in vitro* growth of Mtb in the absence of exogenous catalase, indicating a role for MSH in protecting against baseline oxidative stress (244). However, we did not observe any growth defect between the  $\Delta egtD$  mutant, the wildtype and  $\Delta egtD$  complement strains when grown in the presence or absence of catalase (Figure 10).

As a result, we believe that EGT has a limited role in protecting Mtb against oxidants generated during *in vitro* growth.

Despite having limited effect on the *in vitro* growth of Mtb, we wanted to further investigate if EGT played a role in protecting Mtb against free radicals generated by the host. The oxidative burst initiated upon phagocytosis of an invading microorganism creates a highly oxidative environment within the phagosome. Previously, it was shown that MSH is not required for the survival of Mtb in immunocompetent C57Bl/6 mice and immunocompromised SCID mice following aerosol infection (244), indicating that Mtb uses an alternative scavenging system *in vivo*. As Ta *et al.* (159) observed elevated EGT levels in the *M. smegmatis*  $\Delta mshA$  mutant, we hypothesized that EGT provides Mtb with protection against the macrophage's microbiocidal molecules during infection. However, when we monitored the survival of the Mtb  $\Delta egtD$  mutant relative to wildtype in THP-1 cells no difference was observed over a 120 h time period (Figure 12A). A similar trend was observed in J774A.1 murine macrophages, with the exception of a half-log decrease in survival of the  $\Delta egtD$  strain relative to wildtype and complement at 120 h (Figure 12B). These results were further confirmed in the Mtb *egtD*:Tn where there was only a half-log reduction in survival relative to wildtype in RAW264.7 murine macrophages (246).

Differences in the oxidative response of macrophages is known to exist between species, with the production of NO (248) and superoxide (275) to be greater in mice. Furthermore, monocyte-derived macrophages also produce up to 90% less superoxide than their peripheral blood precursors (276-278). These results could explain the differences in  $\Delta egtD$  survival between THP-1 cells and murine macrophages. However, we remain open to the possibility of other cell-line or species-specific characteristics that may contribute to the observed differences,

such as rates of phagosomal acidification and degree of proteolysis (275). Still, our finding that the  $\Delta egtD$  mutant was only slightly attenuated at 120 h was surprising due to EGT's reputation as an antioxidant. Perhaps MSH or  $\gamma$ -glutamylcysteine provide a compensatory effect in Mtb in the absence of EGT *ex vivo*; however, neither we nor other authors have found increased levels of MSH in the absence of EGT (159,246,247). Furthermore, the  $pknD:Tn$  mutant showed no difference in viability when infecting J774 macrophages (173), further supporting the notion that EGT levels have limited effect on the survival of *Mtb* in murine macrophages (279). Additional studies examining the survival of *Mtb*  $\Delta egtD$  in murine gp91<sup>Phox-/-</sup> and NOS2<sup>-/-</sup> macrophages would attribute a clearer role to EGT protecting the bacilli against host generated ROS and RNS *ex vivo*.

The fact that MSH, but not EGT, is required for growth in the absence of catalase (244) indicates that EGT is not the primary redox buffer in the cell. However, we still found the Mtb  $egtD:Tn$  mutant to have reduced survival relative to the wildtype and its complement strain in presence of hydrogen peroxide, paraquat, menadione, and cumene hydroperoxide (246). Interestingly though, the  $egtA:Tn$  and  $egtD:Tn$  from this study demonstrate varying survival profiles in the presence of oxidants, again exemplifying different phenotypes for these mutants. Therefore, we caution correlating  $egtA$  mutant phenotypes with that of EGT and therefore avoid making comparisons with other studies (246) investigating the phenotype of the EgtA mutant. It is more likely that this mutant is representative of a cell absent of the antioxidant,  $\gamma$ -glutamylcysteine, rather than EGT.

How the  $egtD:Tn$  survival profile compares to that of an Mtb MSH mutant in the presence of oxidants is difficult to determine due to the *in vitro* growth restrictions of MSH deficient strains in the absence of catalase. *M. smegmatis*, which does not require MSH for *in*

*vitro* growth (237), showed an  $\Delta egtD/\Delta mshA$  double mutant to be significantly lower in survival in comparison to the  $\Delta mshA$  or  $\Delta egtD$  single mutants when challenged with cumene hydroperoxide or *tert*-butyl hydroperoxide (273). This work may indicate a redox buffer hierarchy for EGT and MSH in mycobacteria, at least for hydroperoxides. At the same time, we observed EGT to act as a cytoprotectant against exogenous hydrogen peroxide or superoxide (246), although EGT has not been found to directly scavenge these oxidants (173). Despite these observed cytoprotectant effects, EGT's low intracellular concentration relative to MSH (1:166) (159,280), extensive resistance to auto-oxidation (281) and low reduction potential (170) do not support its primary role as an antioxidant/redox buffer in the cell. Another interesting point is that the reducing power of  $\gamma$ -glutamylcysteine is significantly greater (-234 mV; (282) than that of EGT, so it becomes of interest to speculate why the cell would use a superior antioxidant as an intermediate in EGT biosynthesis. Therefore, we believe it is more likely that EGT plays an alternative role in Mtb physiology, which happens to coincide with protecting the cell against oxidative stress.

#### **7.4 EGT is Linked to Central Carbon Metabolism in Mtb**

As the overall goal of our work was to understand the role of EGT in Mtb physiology and pathogenesis, we wanted to utilize our findings on the regulation of EGT biosynthesis in Mtb to direct us toward its biological function. Our first hint came from our studies monitoring EGT levels at various stages of growth (Figure 11). Here, we observed intracellular EGT levels to rise substantially during late-logarithmic phase and to accumulate to an even greater amount in stationary phase. Elevated levels of intracellular EGT was also observed during the stationary phase of *M. smegmatis* by Ta *et al.* (159) but the reverse was shown by Sao Emani *et al.* (247).

Stationary phase is characterized by a number of growth limiting factors, one of which is nutrient deprivation. From our literature search, we were unable to identify any environmental conditions which impacted the transcript levels of the *egt* genes in Mtb. However, previous work identified *S. pombe egt1<sup>+</sup>* to be positively regulated by the transcription factor Ste11 (283). As a key regulator in the control of mating and sporulation of this microorganism (284), *Ste11* has been shown to be negatively regulated by cyclic-adenosine monophosphate (cAMP) levels in response to nutrient starvation (285). In response to unfavourable growth conditions, intracellular cAMP levels drop, down-regulating the activity of the cAMP-dependent kinase, protein kinase A (PKA) (286). PKA negatively regulates the expression of *ste11* by phosphorylating and thereby preventing the translocation of the *ste11* transcription factor, Rst2, to the nucleus (287). In addition, the STPK, target of rapamycin (TOR), also negatively regulates *ste11* expression (288). Similar to PKA, the TOR signalling pathway regulates cell growth in response to growth factors, nutrients and energy conditions. These findings indicate that EGT levels are regulated at a transcriptional level in *S. pombe* in response to nutrient availability.

An earlier study using a nutrient starvation model of persistence in Mtb identified *pknD* to be significantly down-regulated 4 h following starvation (228). Since EgtD is negatively regulated by PknD, we expected to observe increased levels of intracellular EGT during starvation. Despite being metabolically costly to the cell, Mtb maintained elevated intracellular EGT levels over six weeks in the absence of a carbon source, which indicated that EGT is needed during long-term starvation (Figure 18). Further evidence for a role of EGT during starvation arose from our findings that EGT levels were significantly greater in Mtb grown in the presence of low (0.05 mM) versus high (25 mM) concentrations of glucose, acetate or propionate (Figure 19A). In agreement with our findings, previous studies in *S. pombe* identified EGT



biosynthesis to be up-regulated during nitrogen (161) and glucose (160) growth-limiting conditions. However, EGT was found to accumulate within 1 h following nutrient limitation in *S. pombe* (289) which may represent differences between the growth rates (minimum of 2.5 h for *S. pombe*; (290) and 16 h for *Mtb*; (291)) between these organisms.

We observed a discrepancy in EGT levels between the immediately starved (week 1-6, ~300 ng/10<sup>8</sup> cells) and stationary phase (OD<sub>600</sub> = 2.0, 750 ng/10<sup>8</sup> cells) cultures. It is possible that these differences are the result of *Mtb* sensing the gradual depletion of nutrients under non-chemostatic culture conditions during stationary phase, which provided the bacilli with the opportunity to accumulate more EGT. These findings are in accordance with those of *S. pombe* where intracellular EGT levels peak when grown in the presence of low glucose concentrations (1.1-5.6 mM) in comparison to high glucose or a lack thereof (160).

As we observed that nutrient availability has an effect on EGT levels, we wanted to further examine the role of different carbon sources on EGT levels (Figure 19). We observed that EGT levels were lowest in the presence of glucose but increased in the presence of fatty acids. As  $\beta$ -oxidation of fatty acids is a major source of endogenous oxidative stress for organisms, we would expect EGT levels to increase in response to the carbon chain length of fatty acids if EGT was acting as an antioxidant. However, there was no correlation between EGT levels and carbon chain length. Furthermore, intracellular EGT levels of *Mtb* grown in the presence of the TCA cycle intermediates propionate and acetate were on par with cultures containing fatty acids that are catabolized through  $\beta$ -oxidation, again indicating no correlation with  $\beta$ -oxidation. We did observe, however, that EGT levels were highest in the presence of cholesterol. EGT was also recently found to accumulate in hepatocytes of guinea pigs fed diets high in cholesterol, and

these levels were correlated with the degree of TAG formation (hepatic steatosis) in the liver (292). Still, no role has yet to be attributed to EGT in the liver.

There is significant evidence that *Mtb* relies on fatty acids as their the primary carbon source during infection (256) as well as starvation *in vitro* (228,293). As intracellular EGT levels remain the same regardless of whether the fatty acid enters  $\beta$ -oxidation, we rationalize that EGT is not related to this metabolic process. However, elevated intracellular EGT levels are correlated with lipid-rich diets both in *Mtb* (246) and mammals (292).

In response to low blood glucose levels, the liver must generate more glucose from glycogenolysis, gluconeogenesis, or glycerol derived from TAGs. These metabolic processes are essential for mammals as glucose acts as the primary energy source of cells. Interestingly, red blood cell metabolism, which relies entirely on the anaerobic metabolism of glucose to lactate, was previously shown to be influenced by EGT (294). Daily administration of EGT over seven days to rats was found to maintain lactate levels in red blood cells when the animals were fasted for 24 h, whereas lactate levels of those animals not fed EGT decreased by 60%. Interestingly, under normal feeding conditions, EGT had no influence on red blood cell lactate levels (294). These findings indicate that EGT may be involved in one of the metabolic pathways responsible for maintaining blood glucose levels; however, no follow-up investigations have been performed.

Only recently has a new role been shown for EGT as a mediator or coenzyme in catalytic enzymatic reactions. Through the characterization of the biosynthetic pathway of the antibiotic lincomycin A in *Streptomyces lincolnensis*, EGT was demonstrated to act as a sugar carrier (295). At the start of the pathway, a glycosyltransferase (LmpT) catalyzes the reversible displacement of guanosine diphosphate (GDP) from GDP-D- $\alpha$ -D-lincosamide by EGT to

generate an EGT  $\beta$ -S-conjugated lincosamine. Formation of this intermediate mediates lincosamine's condensation with 4-propyl-L-proline, thereby driving the reaction forward. EGT is then exchanged with MSH, through a second, but irreversible glycosylation reaction generating an  $\alpha$ -S-linkage in the MSH S-conjugate which undergoes additional modifications before becoming lincomycin A. The function of EGT in the biosynthesis of lincomycin A appears to play a role in the preservation of lincosamine stereochemistry to enable the attachment of MSH in the same orientation as that of GDP. These findings are of considerable interest as they point to the possibility that EGT could be involved in more general sugar modification reactions.

Blast analysis of the lincosamide synthase complex against mycobacterial genes identified a phosphoheptose isomerase (GhmA) in Mtb to share 53% identity. This enzyme is responsible for catalyzing the isomerization of sedoheptulose 7-phosphate to D-glycero-D-manno-heptose 7-phosphate, an intermediate involved in glycan biosynthesis in Mtb. When the glycosyltransferase responsible for transferring lincosamide to EGT was used in a Blast search, we found LmbT to belong to the glycosyltransferase family 4, which exhibit a wide range of biological functions that can be observed at <http://www.cazy.org/GT4.html>. Interestingly though, these glycosyltransferases are found to be involved in glycogen and trehalose biosynthesis in many organisms. Perhaps coincidental, but taking together the work of ours and others, it appears that EGT is involved in metabolic pathways linked to carbohydrate metabolism.

Alpha-glucan, a polymer similar to glycogen with the exception of a higher degree of branching (73,296,297), is known to accumulate in Mtb under nitrogen- or sulfur-limiting conditions (298). In bacteria,  $\alpha$ -glucans are polymerized by the glycogen synthases, GlgA and GlgB, and these orthologues in mycobacteria can also synthesize glycogen (299-301). The role

of  $\alpha$ -glucans in Mtb is not limited to carbon storage. The bacilli also surrounds itself in a capsule primarily composed of  $\alpha$ -glucans, which are known to participate in the evasion of both the innate and adaptive immune response (302-304) and contributes to persistence in mice (299). Interesting, this capsule is found to accumulate when Mtb enters a non-replicating state (305). Therefore, more specifically, we suspect EGT to be involved in  $\alpha$ -glucan metabolism of Mtb, which could also have broad physiological implications.

It is important that we return to the original findings that EGT protects cells against oxidative stress (173). The plethora of studies in support of the cytoprotectant role of EGT cannot be ignored; however, based off of our knowledge of EGT's chemistry and its involvement in biochemical processes, it is necessary that we revisit our interpretation of these results. Oxidative challenge has been shown to result in the accumulation of glycogen in yeast (306-308) and yeast unable to breakdown glycogen under stress conditions exhibit higher ROS production accompanied by a shortened lifespan (309). Therefore, it appears that glycogen and the control of the glycolytic flux is essential for the cell under stress. If EGT does play a role in the metabolism of these carbohydrate reserves, it would indirectly contribute to protecting the cell against oxidative stress.

## **7.5 EGT Maintains Viability of Mtb during Quiescence**

To better understand the importance of EGT during starvation, we monitored the survival (CFU) of the  $\Delta egtD$  mutant in the absence of carbon sources. After three weeks we observed a significant loss in the viability of the  $\Delta egtD$  mutant ( $\sim 1.5$  log) relative to the wildtype and  $\Delta egtD::egtD$  strain. These findings indicate that EGT is required for the long-term survival of Mtb during starvation. Interestingly, the wildtype and complement strains did not show differences in CFUs over the four weeks of starvation, yet optical density over 14-days increased

not in direct correlation with cell numbers (Figure 19C). Mtb has been described to undergo a wide variety of morphological changes in response to entering a quiescent state (305). One of the major noted differences is the extensive cell-wall remodeling Mtb undergoes while transitioning into a state of NRP (108). Cunningham and Spreadbury (310) reported a very prominent thickening of the cell wall outer layer in Mtb which was later attributed to the gradual accumulation of loosely bound extracellular material around the bacilli in the form of a capsule (305). This matrix is primarily composed of proteins and polysaccharides (73), with only a small proportion (2-3%) containing lipids (74). Interestingly, the major carbohydrates making up 80% of the extracellular capsule are  $\alpha$ -glucans (305). Furthermore, stationary phase is known to increase cell density, and this is described to result from an accumulation of storage carbohydrates, particularly trehalose and glycogen (311). Therefore, the changes in absorbance observed in the absence of cell proliferation may be due to the enhanced synthesis of carbohydrates. In the same set of experiments, we found the absorbance of the  $\Delta egtD$  mutant to be significantly lower than that of the wildtype and  $\Delta egtD::\Delta egtD$  strains after 14-days. Coincidentally, these findings correlate with the decreased survival of the  $\Delta egtD$  mutant after two weeks. Interestingly, when the  $\Delta egtD$  mutant was provided with a low concentration of glucose, the absorbance pattern of the strain was restored to that of wildtype and the  $\Delta egtD$  complement. These findings indicate an implication for glucose, or maybe just carbon source availability, in these absorbance differences.

Similar to our studies, comparative metabolomic analysis of *S. pombe* identified quiescent cells to contain significantly more EGT than vegetative cells (144,160,161). No loss in viability of the *S. pombe*  $\Delta egtI$  was reported 20 days following media change (144), a result similar to our findings where we did not identify our Mtb  $\Delta egtD$  mutant to have a loss in

viability during early stationary phase. These findings, in conjunction with our  $\Delta egtD$  survival studies under starvation, indicate a requirement for EGT during quiescence.

Further support for a role of EGT in cellular quiescence comes from studies in fungi. Perhaps the first link between EGT and fungal spores was the identification of EGT in the honeydew (sugar rich secretion) of rye grain containing *C. purpureae* conidia (312). Later studies analyzing the EGT content of the conidia of *Beauveria bassiana* (313), *N. crassa* and *Collectotrichium graminicola* (143) were all found to have significantly higher concentrations of EGT relative to their mycelia. The role of EGT in fungal physiology was further analyzed and was not found to impact the mycelial growth or conidial germination of *N. crassa* in the presence of superoxide or cupric sulfate (143). Endogenous EGT was also not found to have any effect on the mutation rate of untreated conidia or mitigate the lethality or mutagenicity of UV light in *N. crassa* (314). The authors did, however, find the germination of the  $\Delta egtI$  mutant to be significantly more sensitive to *tert*-butyl hydroperoxide than wildtype (143). From these findings, it was suggested that EGT scavenges ROS in *N. crassa* conidia; however, due to the high reactivity of *tert*-butyl peroxide it is likely that the oxidant reacts with the conidial membrane before reaching EGT in the cytosol. Therefore, we again suggest that EGT may be playing an indirect role in protecting against ROS in the conidia.

Both the sporulation and germination process of *N. crassa* is described to have a transient hyperoxidative state to which EGT could be exerting a protective effect (315,316). However, in the absence of exogenous *tert*-butyl hydroperoxide the authors did not visualize enhanced oxidative stress in germinating conidia (143), indicating that EGT does not play a role in mitigating endogenous oxidative stress in fungal spores. However, assessment of percent germination of *N. crassa* conidia over 84 days identified the  $\Delta egtI$  mutant to be significantly

lower than that of wildtype (143). It was later observed that the  $\Delta egtI$  mutant produces fewer (~40%) ascospore progeny, and that these cells exhibit a significantly reduced lifespan (314). Previous up-regulation of *egtI* transcripts in the presence of hydrogen peroxide (317), cadmium (317), and light (318) indicated that the biosynthesis of EGT is strongly stress responsive. However, *egtI*<sup>+</sup> transcripts were found to be elevated in response to meiosis (158), and not specific to environmental conditions that induce oxidative stress (314), support a role of EGT in cell quiescence itself.

If EGT does play a role in Mtb quiescence, we might expect to see a decrease in survival of the *egtD* mutant during the later stages of infection. However, mice infected with the *egtD*:Tn strain that were sacrificed five weeks post-infection had less than a quarter-log reduction in bacillary load in the lungs in comparison to the Mtb wildtype and complement strains (246). From this work it could be concluded that EGT plays a minor role in persistence; however, the mouse is a poor model for assessing the role of Mtb in human latent infection. Due to the high bacterial load and progressive pathology associated with Mtb infection in mice, it is thus considered as more of a chronic infection (319). In addition, the human granuloma does not resemble those found in mice, which are non-necrotic, and lacking in structure and organization (320). The importance of necrosis when studying LTBI is due to the fact that this process leads to the death of the majority of tubercle bacilli, leaving behind a small population which is in a state of NRP (321). Consequentially, if EGT's role is primarily during quiescence, it would be difficult to assess EGT's role in pathogenicity in a mouse model. We suggest a more appropriate *in vivo* model to assess the role and impact of EGT in Mtb's pathogenicity would be to use rabbits as these animals undergo intragranulomatous necrosis as well as exhibit disease latency (322).

## 7.6 Targeting EGT Biosynthesis as a Strategy to Eliminate Quiescent Mtb

It could be argued that the exceptionally long treatment course that is required to prevent disease relapse is one of the most important factors limiting our control of TB. Currently, our first line drug regimen takes 6-9 months to treat active disease, yet when the same drugs are used *in vitro* we are able to rapidly kill the bacilli (108). The environment of the host is shown to cause Mtb to further slow its replication or induce a quiescent state during infection (80), and as the majority of our antitubercular drugs target functions necessary for cell growth it is not surprising that these cells are less sensitive to existing antibiotics. Furthermore, these non-replicating bacilli are responsible for generating latent disease. Therefore, the importance of understanding Mtb quiescence is paramount for identifying new strategies for eliminating non-replicating bacterial populations. In turn, targeting quiescent bacteria in our therapies would likely accelerate treatment time, reduce the risk of developing a latent infection and prevent disease reactivation.

In principle, there are three possible ways to target quiescent cells: sensitize existing cells to current antibiotics, identify new drug candidates that target pathways essential for quiescence, or alter host immune pressures that induce an antibiotic tolerant state (108). Evidence exists, although the correlation is not absolute, where drugs that retain activity against non-replicating Mtb *in vitro* have a strong sterilizing activity *in vivo* (323). These findings further reinforce the idea that TB drug development should largely focus on targeting pathways that are essential for Mtb in a quiescent state. We have shown that EGT is required for maintaining Mtb viability in the absence of replication. Despite requiring further validation in a necrotic granuloma animal model, these findings provide support for the EGT biosynthetic pathway as a novel drug target in TB treatment. In addition, the fact that the *egtD*:Tn mutant exhibited increased susceptibility



against a wide range of antitubercular reagents in actively replicating Mtb (246) indicates that targeting the EGT biosynthetic pathway would also render, and perhaps more so, quiescent cells more susceptible to current treatment.

## 7.7 Conclusion

Our investigation to elucidate the role of EGT in Mtb physiology and pathogenesis has led us down many new avenues and has provided novel insight into this molecule's function. Our studies initiated with the biochemical analysis of the gene product of *rv3701c*, which we postulated to encode for EgtD in EGT biosynthesis. We identified that EgtD is a His methyltransferase that catalyzes the first reaction step of EGT biosynthesis. When we knocked out *egtD* in Mtb we showed its essentiality in the synthesis of EGT. The absence of EGT in  $\Delta egtD$  mutant provided an additional tool to explore the environmental conditions that EGT was required for by Mtb. Results from our *ex vivo* studies indicate that endogenous EGT plays a small role in maintaining Mtb viability within 120 h post-infection. Thus, we deem protection against ROS and RNS generated by the innate immune response is not the primary role of EGT in Mtb.

Using a number of biochemical assays and genetic studies we identified that PknD negatively regulates EGT biosynthesis in Mtb through its phosphorylation of EgtD. Our findings on the regulation of EGT biosynthesis led to identification of EGT up-regulation under nutrient limited conditions and its essentiality for the survival of Mtb during quiescence *in vitro*.

Due to the apparent involvement of EGT in maintaining Mtb survival in the absence of replication, we recommend further studies be performed to confirm the role of EGT in Mtb persistence in disease latency *in vivo*. Preliminary observations from our work on EGT levels in

the presence of different carbon sources and their availability, in combination with the findings of other groups (295,324), indicates a role for EGT in sugar metabolism.

Although quiescence is characterized as a downshift in metabolism (311), Mtb never truly enters a dormant state and is required to maintain its membrane potential and continue ATP synthesis (109). Many studies have implicated the importance of storage carbohydrates and gluconeogenesis (264,325) in the survival of microorganisms during quiescence which is likely supporting energy homeostasis in Mtb. We hypothesize that the decrease in survival of the  $\Delta egtD$  mutant during quiescence is due to EGT's role in energy storage and homeostasis.

The work from this thesis contributes to our limited knowledge regarding the physiology of Mtb during quiescence, as well as proposing a novel role for EGT in biological systems. Further investigation of the dispensability and role of EGT during latent infection is warranted; however, our findings suggest the EGT biosynthetic pathway may be a promising target for eliminating non-replicating bacterial populations.

## **7.8 Future Directions**

We previously assessed the viability of the *egtD*:Tn mutant in BALB/c mice (246). The mouse model is the best characterized and most extensively used model to study Mtb pathogenesis. Despite its advantages to *in vitro* models of Mtb growth restriction, the classical murine model does not represent human LTBI. In contrast to humans, Mtb in mice continues to multiply (104) and remains metabolically active (260) during chronic infection leading to high bacillary burden and early death (326). Since we established that EGT is essential for the survival of Mtb during quiescence and is not associated with bacterial growth inside of the macrophage, it is not surprising that we did not observe a more prominent effect on the survival of the *egtD*:Tn mutant in mice. To date, rabbits are the most feasible model for studying LTBI.

Rabbits are relatively resistant to Mtb infection resulting in suppression of bacillary loads following 10 weeks infection (327,328). In addition, this model forms granulomas similar to those found in humans (326) and disease reactivation can be triggered through administration of immune-modulating agents (327,328). Therefore, to accurately assess a role of EGT in Mtb pathogenicity and disease latency, future studies should look at the viability of the *ΔegtD* mutant in the rabbit model.

As we observe the viability of Mtb during quiescence to be reduced in the *ΔegtD* mutant, it is of interest to understand the mechanism behind this reduction in survival. Having originally been proposed as an antioxidant, one of the first set of experiments we would propose is to investigate the role of EGT in protecting against endogenous and exogenous oxidative stress in quiescent Mtb. However, we would like to mention an observation that EGT protects Mtb against oxidative stress in these experiments does not necessarily imply that EGT is acting as a scavenger. As we observed a link between EGT and Mtb metabolism (246), we hypothesize that EGT is involved in energy homeostasis during quiescence, which would ultimately support Mtb's oxidative stress defense system. Therefore, monitoring the generation of ROS and ATP levels may provide us with insight as to whether EGT is protecting against oxidative stress or playing a role in ATP homeostasis, respectively.

We predict the involvement of EGT in energy storage, particularly sugar metabolism due to the previously observed role of EGT in lincomycin A biosynthesis (295). Both glycogen and trehalose are known to accumulate in microorganisms (311), including Mtb (298,305), under conditions which induce quiescence. The exact role of intracellular trehalose remains to be determined; however, it is thought that the trehalose released from glycolipids in the Mtb cell wall during infection are used for the synthesis of  $\alpha$ -glucans or acts as a reservoir for glucose

(256,329). Therefore, we recommend quantifying glycogen and trehalose levels prior to and following nutrient starvation for several weeks and comparing them in our wildtype,  $\Delta egtD$  mutant and  $\Delta egtD::egtD$  strains. Any differences observed in trehalose or glycogen levels would likely implicate a role for EGT in the metabolism of storage carbohydrates in Mtb.

TAGs are the major energy source for animals, plants and prokaryotes during and following dormancy and hibernation and are also being used by Mtb during disease latency (330). The correlation between EGT and TAG accumulation in the livers of guinea pigs (292), along with our observations that Mtb EGT levels increase in the presence of fatty acids may merit an involvement of EGT in TAG metabolism (246) and require further scrutiny as well.

Evaluating the importance of EGT for the survival of Mtb during quiescence (latent disease) is of importance as EgtD demonstrates considerable potential as a drug target for TB. Furthermore, identifying the role EGT plays in Mtb will have broader implications, as EGT is present across many life forms. If we can demonstrate EGT's involvement in energy storage or carbohydrate metabolism in Mtb, these findings may assist in our understanding of the involvement of EGT in human diseases such as diabetes, Crohn's disease and rheumatoid arthritis.

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