Developing a Cell Based Screen for Inhibitors of Two Component Signal Transduction in Mycobacteria

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ABSTRACT

The growing number of drug resistant strains of *Mycobacterium tuberculosis* appearing worldwide has had an enormous impact on the ability to control and treat Tuberculosis (TB). Discovering new anti-TB drugs is of paramount importance to the global effort for TB eradication. The success of the pathogen is largely due to its inherent ability to remain in a non-replicating or latent state for extended periods of time. In order to achieve this shift it requires tightly controlled signal transduction mechanisms to respond to its host environment. Two component systems (TCS) are one example of signalling mechanisms employed by prokaryotes and are ideal candidates for antibacterial drug targets. It is understood that many TCS are conserved in a large number of organisms, they are often essential to the virulence and persistence of pathogens and they are virtually exclusive to prokaryotes. In this study three *Mycobacterium smegmatis* TCS were selected; DevS/DevR, MtrB/MtrA and SenX3/RegX3. Promoters under the control of these systems were cloned into an optimised mycobacterial high copy number GFP reporter plasmid and subject to a number of *in vitro* stress conditions to ascertain induction conditions for these systems. As expected the DevS/DevR controlled *hspX* promoter was responsive to oxygen starvation and the SenX3/RegX3 controlled *phoA* was induced by phosphate starvation. Interestingly, *phoA* and *mtrA* were also induced by magnesium chelator EDTA in minimal media.

The *phoA* and *mtrA* promoter constructs were then used for *in vitro* high throughput bioassays with a number of compound libraries in order to screen for any inhibitory activity on each of the target systems. A phosphorylation inhibitor included in one of the screens, oleic acid, indicated that this assay could potentially be used to screen

for TCS inhibitors, but no novel compounds were found in this study. As a proof of principle, known TCS inhibitors palmitoleic and oleic acid were employed to show a dose dependent inhibition *mtrA* expression. This method could potentially be expanded to other TCS of *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG, or other signal transduction systems such as one component regulators and serine threonine kinases.

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LIST OF ABBREVIATIONS

96 wp 96 well plate

ADP Adenosine diphosphate

AFB Acid Fast Bacilli

AIP Auto Inducing Peptide

Amp Ampicillin

AMP-PNP Adenosine Monophosphate p-Nitrophenol

AraLam Arabino Lipoarabinomannan

ATP Adenosine triphosphate

ATPase Adenosine triphosphatase

AWC Alan Wilson Centre

BCG Bacille Calmette-Guérin

cAMP cyclic Adenosine Monophosphate

c-di-GMP cyclic diGuanosine Monophosphate

CIP Calf Intestinal Phosphatase

CR Complement Receptor

DOTS Directly Observed Therapy

DST Drug Susceptibility Testing

DTP Developmental Therapeutics Programme

EDDA Ethylene Diamine-N,N'-Diacetic Acid

EDTA Ethylene Diamine Tetraacetic Acid

EEA-1 Early Endosome Autoantigen 1

eGFP enhanced GFP

EGTA Ethylene Glycol Tetraacetic Acid

ESR Institute of Environmental Science and Research

F Fluorescence

FACS Fluorescence Activated Cell Sorting

FITC Fluoescein Isothiocyante

gDNA genomic DNA

GFP Green Fluorescent Protein

HdeB Hartman deBonts

HK Histidine Kinase

HPt Histidine containing Phosphotransfer domain

HTH Helix Turn Helix

HTS High Throughput Screen

IFNɣ Interferon gamma

INH Isoniazid

iNOS inducible Nitric Oxide Synthase

I**PTG** Isopropyl-β-d-thiogalactopyranoside

kan kanamycin

kan^r kanamycin resistance

LA Luria Agar

LB Luria Broth

LOPAC Library of Pharmacologically Active Compounds

LTBI Latent Tuberculosis Infection

ManLam Mannosylated lipoarabinomannan

MAP Mitogen Activated Protein

MDR Multi Drug Resistant

MIC Minimum Inhibitory Concentration

NEB New England Biolabs

NIH National Institute of Health

NIWA National Institute of Water and Atmospheric Research

OD Optical Density

oriE Origin of replication (*E. coli)*

oriM mycobacterial Origin of replication

oriM high mycobacterial Origin of replication (high copy number)

PCR Polymerase Chain Reaction

PIT Phosphate Intake Transporter

PST Phosphate Specific Transporter

PZA Pyrazinamide

RBS Ribosomal Binding Site

RD-1 Region of Difference

rF relative Fluorescence

RIF Rifampin

RR Response Regulator

SDW Standard Distilled Water

SM Streptomycin

STPK Serine Threonine Protein Kinase

TB Tuberculosis

TCS Two Component System

TCSi Two Component System Inhibitor

TEP TriEthylPhosphate

TLR Toll Like Receptor

TNFα Tumour Necrosis Factor alpha

WHO World Health Organisation

XDR Extensively Drug Resistant

X-gal X- galactosidase

CHAPTER ONE:

General Introduction

1.1 Background

Mycobacterium tuberculosis has been a significant human pathogen for thousands of years [1, 2]. Without a natural reservoir of its own, some consider it to have co-evolved with humans [2-4]. Studies have shown evidence of *M. tuberculosis* infection in Egyptian and Incan mummified remains up to 9,000 years ago [2-4] and the domestication of cattle around 10,000 years ago [5, 6] was thought to be implicated in the origins of this disease [7]. Modern molecular methods have since indicated that the causative agent of bovine tuberculosis, *Mycobacterium bovis,* is a more recent species, having evolved from human specific *M. tuberculosis* ancestry [3, 8]. Ancient *M. tuberculosis* DNA, containing none of the four splogiotypic groups used to distinguish the species from *M. bovis,* has been found in humans and animals dating back long before cattle domestication [9]. A recent paper suggests that modern *M. tuberculosis* may have evolved from a similar prehistoric pathogen found in *Homo erectus* up to 500,000 years ago [10]. While *M. tuberculosis* is clearly an ancient species, direct evidence of outbreaks of phthisis, consumption and the white plague cannot be traced back as far as the organism itself [3].

Whether pathogenically inert or otherwise, *M. tuberculosis* has infected mankind for a substantial proportion of our existence. Robert Koch, who first isolated the acid fast bacillus (AFB) in 1881, estimated at the time that one in seven humans died of Tuberculosis (TB) [11]. Later in 1898, Smith identified different types of mycobacteria that infect livestock, who shared the hardy mycolic acid cell wall [12]. Since then, our knowledge of the *Mycobacterium* genus has expanded to 71 species, 32 of which are pathogenic, these have been classified in a Runyan scheme by various features including pigmentation and rate of growth [12]. More recently, the evolutionary

distance between species has been further defined by the analysis of mycolic acid structures, genetic markers such as the 16S rRNA gene and, in the last decade, the comparison of entire genomes [8-10]. Members of the *Mycobacterium tuberculosis* complex are generally responsible for disease in humans and cattle; however a number of opportunistic Mycobacteria can give rise to infection in an immuno-compromised host. Slow growers such as *Mycobacterium celatum* and *Mycobacterium abscessus* can infect patients with Acquired Immune Deficiency Syndrome (AIDS) or chronic immune suppression, while post-traumatic infection is generally caused by fast growers such as *Mycobacterium goodie* [12]. Comparing pathogenic and non-pathogenic species has been a useful tool in understanding the evolution of the *Mycobacterium tuberculosis* complex and its host-specific virulence [13].

Mycobacterium smegmatis, a non pathogenic soil bacterium, has been extensively used as a model organism owing to its rapid doubling time of three hours compared with *M. tuberculosis'* 12hours [12, 14]. Another model organism is the vaccine strain *M. bovis* BCG (Bacille Calmette-Guérin) whose sequence is 99.95% identical to that of *M. tuberculosis* [15]. These and other models will be discussed in section 1.2.3.

1.1.1 The Development of TB Treatment

TB has been a human disease for thousands of years and a range of treatments and therapies have been explored. Many significant events have lead to the current understanding and treatment of TB and other infectious diseases today. The Royal College of Physicians was set up in 1518 by Henry VIII to fight the bubonic plague and meant that physicians of the time could monitor infectious outbreaks in order to

circumvent the devastating effects disease was having on a rapidly growing population [16]. The burden of TB was probably intensified by overcrowding and urbanisation, becoming a major cause of death by the Industrial Revolution [17]. TB was no exception to the emerging idea that living conditions play as significant a role as the disease itself, something which is still a very important factor in TB control [16]. Records show deaths attributed to TB in England and Wales fell from 62,633 to 27,871 between 1878 and 1940, testament to the effect of an improved standard of living on the disease [16].

Another common feature of infectious endemic is war [18]. Devastating outbreaks of infectious diseases in post war Europe fast tracked the antibiotic era with the discovery of penicillin in 1928 by Fleming and then streptomycin (SM) in 1944 by Waksman, Schatz and Bugie [16-18]. Prior to this point, the most common form of treatment for TB had been fresh air and bed rest [19] so Waksman's discovery in 1943 of a broad spectrum antibiotic that eliminated *M. tuberculosis* infection in most patients revolutionised its treatment and lead to a sharp decrease in mortality.

Preventative therapy by immunisation with a live attenuated strain of *M. bovis* BCG was pioneered in 1921 and first given to "at risk" groups such as nurses and medical students in 1949 [16]. In one of the first large-scale medical research trials in the United Kingdom, 52, 239 children were immunised between 1950 and 1952 with the attenuated strain of *M. bovis* BCG [16]. The incidence of TB decreased 79% between 1954 and 1965 which was probably a combination of drug and vaccine efficacy as well as an improved standard of living, which reduced the infection rate [16].

Thousands were saved by vaccination and antibiotic development, but it did not take long for SM resistant strains of *M. tuberculosis* to emerge [20]. The discovery of pyrazinamide (PZA), isoniazid (INH) and rifampin (RIF) meant that TB could be relatively well controlled and combined therapy could be administered to avoid the emergence of resistant strains [21, 22]. Eventually a growing number of patients presented strains resistant to each of the available anti-tubercular agents prompting scientists in the field to search for new classes of antibiotics.

Presently, drug resistant strains are classified as either multi drug resistant (MDR) or extensively drug resistant (XDR) based on the number and class of antibiotics to which susceptibility has been lost. MDR strains of *M. tuberculosis* are defined by the World Health Organisation (WHO) as having "resistance to at least INH and rifamycin" and XDR is described as "resistance to at least INH and RIF among first-line anti-TB drugs, resistance to any fluoroquinolone, and resistance to at least one second-line injectable aminoglycoside (amikacin, capreomycin, or kanamycin)" [23]. *M. tuberculosis*' ability to acquire genotypic and phenotypic resistance to these drugs is of great importance to physicians administering these drugs and to drug discovery efforts [23].

1.1.2 Drugs and resistance

The treatment of TB has evolved from concoctions of distilled milk infused with earth worms and snails to modern synthetic pro drugs like INH [20, 24]. It is impossible to say how effectively some of the early remedies such as cod liver oil, arsenic and iodine worked to reduce the TB burden at the time, but some interesting discoveries began to emerge at the turn of the 20th century. In 1912 studies employed the use of

cultured *Bacillus subtilis* and *Bacillus mesentericus* to inhibit the growth of *M. tuberculosis in vitro* and in a guinea pig model [20]. Later, the use of sulfonamides showed great anti-mycobacterial potential *in vitro*, but were far too toxic for use in the treatment of pulmonary TB [20].

Streptomycin (SM) was isolated from *Streptomyces griseus* in 1943 and before the end of 1944 it was being used in clinical trials against TB, showing efficacy *in vivo* and *in vitro* [20, 25]. SM was quickly moved to industrial scale production as it showed an effect on a broad spectrum of organisms including Gram negative, Gram positive and acid fast bacteria [25]. SM is an aminoglycoside antibiotic which acts on bacterial ribosomes inhibiting translation initiation and causing misreading [26]. Various strains have been sequenced to identify polymorphisms responsible for drug resistance in mycobacteria recorded by the drug resistance mutation database (http://www.tbdreamdb.com) [27]. Genes encoding the ribosomal subunits *rrs* and *rpsL* have been implicated in resistance to SM and other aminoglycoside [22, 26].

Current first line drugs include INH, rifampin, ethambutol (ETB) and pyrazinamide. INH acts on mycolic acid synthesis, inhibiting cell wall synthesis [28]. It is a nicotinamide-derived drug first discovered in the 1950s, and proved more effective than any anti TB drug of its time [24]. Resistant isolates were discovered the same year as the drug. However, it was nearly half a century before genetic analysis of these isolates lead to the elucidation its mode of action [24]. INH is a pro drug activated by the *M. tuberculosis* KatG protein and certain polymorphisms in this gene give rise to INH resistance [24]. PZA is an interesting drug, owing to its inactivity *in vitro* [29]. The mode of action of PZA is currently unknown, but its effect in an anaerobic environment is far more pronounced than in aerobic conditions [29]. Its

activity on so called “semi dormant” bacilli has reduced the treatment time of TB from 12 to 6 months [29]. A number of mutations to *pncA* gene and promoter region result in reduced susceptibility to this drug [27].

The evolution of drug resistant strains of mycobacteria is influenced by many factors. On a clinical level, the existence of many sub populations within a host, sometimes in more than one location therein, as well as an inherent ability to survive in either an active or dormant state engenders resistance [22]. Host specific factors also contribute to the likelihood of drug resistance developing such as the strength of innate immunity, HIV status and whether or not a patient has been treated for TB previously [13]. Studies have shown that the likelihood of MDR or XDR-TB arising is much higher if the patient has been previously treated for TB or is HIV positive [23, 30]. The latest WHO figures show that countries where the incidence of MDR and XDR-TB is highest include countries such as South Africa, Ukraine and Latvia where the number of people with HIV and AIDS is also high [23, 31]. Previous treatment is another important factor, as low quality drugs, incorrect prescription and poor adherence will affect the probability of resistance arising [27, 32].

On a molecular level, mutations causing polymorphisms within a population can lead to a subpopulation of resistant bacteria [33]. Mutation can arise spontaneously or as a result of exogenous agents causing DNA replication errors. Point mutations, insertions, duplications or loss of genetic material can result in a change of phenotype allowing an organism to become resistant to a drug which usually knocks out an essential process [34]. In some ways the more critical the target to cellular replication, the more likely resistance will arise. The rate of mutation in pathogenic

mycobacteria is relatively low compared with faster growing pathogens, however there are many genes which display variable mutation rates [35].

There are many reasons *M. tuberculosis* is able to develop resistance to drugs including its extended incubation and latency period as well as the fact that most drugs act on targets essential for growth and division [13, 22]. As well as these documented genotypic changes, the idea of reversible phenotypic drug resistance is a concept that may well be central to *M. tuberculosis* drug susceptibility. The idea that sub populations of bacteria can survive exposure to drugs by a phenotypic switch to a reductive metabolism, rather than a permanent genotypic change is a relevant argument for the case of mycobacteria [36].

1.1.3 Epidemiology

The WHO annual surveillance report on TB for 2009 gives a summary of the current state of TB as well as strategies to combat the disease worldwide [23]. Encouragingly the report indicates that the number of new TB cases per capita has decreased since 2003, however with 9.27 million new cases and 1.3 million deaths in 2007, these alarming statistics show there is still a long way to go before TB is completely eradicated [23]. The highest incidence per capita is in Africa with an average rate of 363 per 100,000 and 14.4 deaths in 2007 [23]. The USA and Monaco have the world's lowest prevalence rates of 3.1 and 1.9 per 100 000 respectively, while Swaziland has the highest rate with 1,226 per 100 000 [23].

The WHO gives the latest prevalence of TB in New Zealand as 7.3 per 100,000 for 2007 and ESR (Institute of Environmental Science and Research) recorded this rate as 7.2 per 100,000 in 2008 [23, 30]. Five new cases of TB were recorded in its latest

public health survey report for July and September 2008 all of which were in Wellington [30]. TB has a very strong association with poverty and age in NZ [37]. Unlike countries with a high prevalence of HIV, the majority of TB cases are found in older people, children and people in a low income bracket [37]. Although globally the rate of TB has dramatically decreased in the last hundred years, thanks to the availability of antibiotics and a higher standard of living in most countries, the emergence of MDR and XDR strains of TB is of huge concern [23]. First line TB drugs can be used in most cases to treat TB. Second line drugs are used in cases of drug resistance; however they are more expensive, must be administered for longer and display severe side effects [23, 37].

Drug resistant TB is not geographically isolated, according to the WHO 2008 report MDR TB was detected all over the world and at least one case of XDR TB was reported in all 45 countries surveyed [23]. Globally, it is estimated that 3.9 to 6.6 % of all cases of TB are MDR [23]. The highest rates of MDR-TB in the world are recorded in Eastern Europe especially in Latvia and Ukraine (up to 36 % of all cases), much higher than in Africa [23]. Data from these countries has shown that the percentage of MDR-TB is significantly different in new cases at 2.9 % compared with previously treated cases at 15.3 % [23]. Some associate this with a sharp increase in the number of HIV cases reported in this area [23], it may also be that Eastern Europeans countries have a very effective surveillance strategy for MDR and XDR-TB and are picking up more cases than other countries.

The STOP TB strategy set up in 2006 includes an expansion of the directly observed therapy (DOTS) regime to 184 countries and addresses the challenge of TB HIV co infection [32].

In addition to an increased susceptibility to TB, a positive HIV status is significant because it will affect drug resistance and the host immune response to TB, precipitating the onset of disease [23]. Testing for HIV status among TB cases in high risk areas has increased from 8% to 35% since 2002 [23]. A very important part of TB research is recording information from around the world showing trends of drug resistance. Drug susceptibility testing (DST) is one way that MDR and XDR can be monitored and managed [32]. Although mutations causing resistance to current TB drugs are unavoidable, ensuring that the right combinations of drugs are given to patients is one way that the rate of MDR and XDR-TB can be reduced.

Currently, the discovery of new anti-TB drugs is of paramount importance in the effort to stop TB. In order to combat emerging drug resistant strains new targets need to be elucidated. Our focus is on latent TB and the following section will describe the progression, persistence and virulence of TB and why targeting latent, in addition to active, TB could help to alleviate this global epidemic.

1.2 Immunology and persistence

One of the most challenging features of TB treatment is the organism's ability to maintain a dormant state within its host for long periods of time [34, 38]. This section will outline the progression of TB with an emphasis on factors which affect the disease outcome for patients and morphological and genetic changes within *M. tuberculosis* which allow it to persist for so long.

1.2.1 Disease progression

M. tuberculosis is an obligate pathogen, unlike some other mycobacterial species it has no environmental niche but can live in humans asymptomatically for long periods of time [39]. Given that one in three humans harbours latent TB, there is in fact a huge reservoir of *M. tuberculosis* worldwide (http://www.who.int/mediacentre/factsheets/fs104/en/). Infection begins with inhalation of aerosols containing *M. tuberculosis* AFB to pulmonary alveoli [36, 37]. The presence of foreign pathogenic bacteria is very quickly recognised by the host immune cells via bacterial toll like receptors (TLR) and mannose receptors [36, 38]. Macrophages and dendrites are recruited to the site of infection where they phagocytose bacterial cells. In non-pathogenic mycobacteria this results in efficient control of the infection since macrophages induce the elimination of bacilli by phagolysosome fusion, complement activation and lymphocyte mediated killing [40]. During *M. tuberculosis* infection, most of the AFB are cleared in this manner but in approximately one third of cases, the pathogen persists by interfering with macrophage maturation [38, 41] as shown in figure 1.1.

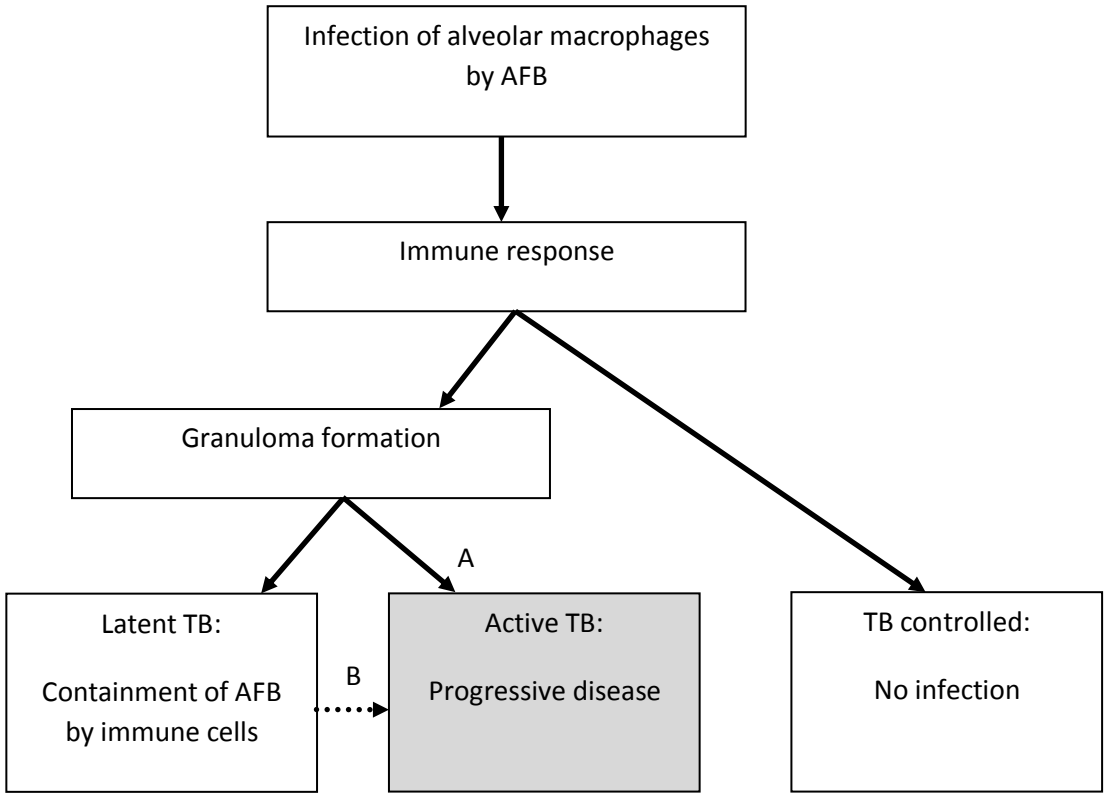


Figure 1.1: Disease Progression in TB*. In most cases AFB are cleared by the immune system. Active TB results from expansion of the primary granuloma (A) or re-activation (B) of contained AFB*

Various mechanisms of immune evasion have been proposed which are described in a number of reviews [36, 38-41]. Although the infected macrophage cannot induce the release of toxic lysosomal products to the AFB containing phagosome, there is still a large immune response from the host [40]. This leads to a gathering of immune cells forming a tubercular lesion known as a granuloma. These characteristic lesions are a hallmark in TB diagnosis and range in size and cellular makeup depending on stage of disease [42]. In about 90 % of individuals, the AFB are contained in the granuloma which consists of dead and foamy macrophages surrounded by activated macrophages and lymphocytes [41, 43]. There is often a surrounding fibrotic layer leaving the AFB in a contained environment [43, 44]. In a small number of individuals

the initial infection will lead to primary tuberculosis after an incubation period ranging from 4 to 12 weeks [43]. Dissemination of the primary granuloma results in active TB by allowing AFB to spread to other regions of lung and eventually other organs of the host [36, 37].

1.2.2 The Tubercular Granuloma

The host adaptive immune response results in the formation of a granuloma; providing protection from but not sterilization of an *M. tuberculosis* infection [40]. This is also seen in other pathogens such as those of the *Schistosoma* and *Brucella* genera [44].The large number of immune cells recruited to the site of infection is exaggerated during a TB infection, effectively encapsulating the AFB to the initial infection foci. After AFB enter a macrophage they are transported across the epithelial lining where they recruit more immune cells to the site. This recruitment requires the Region of Difference (RD1) virulence gene locus [45]. Ulrichs and others showed that the type of cell arriving at the granuloma changes with time, after 4 weeks 45 % of the cells are macrophages whereas at 6 weeks 80 % are lymphatic cells [42].

A number of reviews describe the structure of the granuloma and the general consensus is that there is a necrotic core surrounded by concentric rings of immune cells [36, 41, 42]. AFB reside in macrophages surrounded by a mass of cellular material consisting of dead macrophages and Langhans giant cells, the latter having formed by the fusion of epitheloid macrophages [42]. This layer is surrounded by a number of activated macrophages, surrounded by naive macrophages and T lymphocytes. A number of others components are involved in this organised structure including B lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix [44].

Surrounding tissue keeps the entire structure in check by secreting chemokines and cytokines [42]. The aggregation of T cells around the fibrotic layer acts as mini lymph nodes surrounding the healing granuloma [42, 43]. The conditions within the tubercular granuloma have been studied extensively using a number of models; certain areas of the granuloma have been shown to be hypoxic, nutrient and iron deprived, and contain toxic nitric oxide products [42, 44].

The cellular composition of the granuloma is altered depending on whether the lesion is healing or disseminated and the centre of the granuloma can either be casious or calcified [42]. Other differences in these states are the presence of blood supply in an active granuloma and fibrotic coating surrounding the healing granuloma keeping AFB contained to the site of infection [42]. One of the most important factors affecting the rate of healing is the bacillary load present in the lungs [45]. Other host factors will also determine how quickly the granuloma heals, in particular the health of the individual's immune system.

There is debate as to whether the granuloma is in a constant or dynamic state [42, 45]. The historical view of granuloma formation implies that after infection granulomas heal over time unless certain host factors trigger re-activation [42]. This static view implies that the dormant bacilli are contained in the granuloma, employing a reductive metabolism in order to survive in the hypoxic nutrient deprived lesion for extended periods of time until resuscitated. Numerous studies in different models have shown that TB is able to switch to a dormant state during LTBI [45-48]. There is, however, mounting evidence that this may not be the case for every single AFB. For example, the finding of *M. tuberculosis* DNA outside the granulomatous lesions of patients with LTBI indicates that AFB could be escaping from granulomas even

during an LTBI [49]. The problem here is that the presence of DNA does not necessarily prove that live *M. tuberculosis* survives in the lung. To date the presence of RNA, indicative of live *M. tuberculosis*, has only been found within macrophages in non-necrotic zones of the granuloma [44].

The dynamic hypothesis describes a state seen in the *M. marinum* model where the AFB are contained by fibrotic tissue surrounding the granuloma but can escape to other areas of the lung with the help of macrophages [45]. In this view, infection remains controlled even though the lesions are moving around the lung; more forming as older ones heal completely [45]. Unfortunately, which view you take is largely dependent on what model organism and host you are studying. As there is really no perfect model for LTBI in humans, all of the evidence obtained using different model organisms and hosts needs to be carefully considered. In any case, there is something unique about the ability of *M. tuberculosi*s to maintain a slow growing persistent state in response to the harsh environmental conditions it faces. The granuloma effectively houses the infectious agent for extended periods of time. This can lead to endogenous re-activation of the disease in a compromised host. There is much debate as to whether the majority of current TB cases are the result of exogenous re-infection of AFB or endogenous re-activation from a previous infection [41]. Given that so many humans harbour latent TB, yet only a small proportion of those exposed to the pathogen develop a latent or active infection, one could argue that both processes are very important in post primary TB.

1.2.3 Endogenous Re-activation and Exogenous Re-infection

A number of studies attest to *M. tuberculosis*' ability to survive for long periods of time in a dormant state *in vivo* and *in vitro* [16, 45-48]. There is evidence that TB can survive in a human host for up to 33 years [48]. Re-activation occurs when a granuloma disseminates and infected macrophages spread to other parts of the lung [42, 50]. The ratio of cases which arise from re-activation and re-infection is unknown but it is likely that a number of host factors are involved in the outcome. A Latent TB infection (LTBI) is maintained by a delicate balance between the host immune system and the population of AFB in the granuloma [42]. Immune suppressant drugs such as infliximab have been shown to greatly increase the rate of re-activation [51]. Malnutrition, old age and co-infection with HIV have all been shown to increase re-activation rates [13, 34]. Even genetic differences within individuals of a population can influence the odds of re-activation, for example polymorphisms on chromosome regions 2q21-2q24 and 5p13-5q22 have been linked to susceptibility to re-activation [52].

On the other hand many of these factors could also increase the likelihood that an individual is susceptible to re-infection. Consider that a malnourished, elderly or HIV positive patient is probably much more likely to be unable to control a new infection if they live in an area with a high risk of contagion. As we learn more about the granuloma status in latent and active TB it seems likely that both re-activation and re-infection contribute to the current epidemiology of the disease. There may be an entire spectrum of disease states in these lesions. The AFB may be replicating very slowly and causing new lesions which heal without ever causing active disease until there is a break down in the immune system control causing re-activation or an

increase in AFB number as a result of re-infection [45]. In either case, the fact that TB can survive in this latent phase, providing such a large reservoir of AFB developing drug tolerance and resistance, make it an extremely dangerous pathogen.

1.2.4 Models of Persistence

Before discussing the ability of *M. tuberculosis* to enter dormancy, it is necessary to explain some of the models used to explore the dynamics of mycobacterial gene expression and host responses to the AFB during LTBI. H37Rv, a common lab strain of *M. tuberculosis,* is restricted to physical containment level 3 facilities in New Zealand. This precaution and its long doubling time of 12 hours make it a difficult organism to work with, thus other species of the mycobacterium genus have been used in its place. *M. smegmatis* and *M. bovis* BCG are commonly used to explore changes involved in dormancy [48, 53]. Other related mycobacterial model organisms include zoonotic pathogens *M. avium* and *M. marinum* [12].

The Wayne model is an *in vitro* model used to explore changes in gene expression during stationary phase [54]. It involves a slow starvation of oxygen to a point where the optical density (OD) of the cell culture is constant but the cells remain viable [55]. This model was originally established in the 1970s and the authors discovered that genes involved in the glycoxylate shunt were up-regulated during this growth phase [56]. Three markers of dormancy were described namely, tolerance to anaerobiosis, unique antigen production and a significant increase in the production of glycine dehydrogenase and isocitrate lyase enzymes [56]. These changes mean that *M. tuberculosis* can use up less energy and persist in a slow or non-replicating state. Many of these findings have been replicated by two dimensional gel and microarray

analysis showing upregulation of *icl* and *aceA* genes during stationary phase and during *in vivo* infection [57]. Other variations on this model achieve stationary phase persistence by altering temperature, pH, nutrient sources and nitric oxide levels [44].

While *in vitro* models cannot replicate the hostile environment of the granuloma, many mycobacterial species exhibit similar changes as a result of oxygen starvation to those seen *in vivo* such as antibiotic tolerance, down regulation of a number of genes involved in active metabolism and induction of a set of genes known as the dormancy regulon [47]. *In vivo* induced genes have also been categorised by infection with human macrophage cells [44]. Some of these can be further broken down to groups of macrophage and granuloma activated genes [44]. The different subsets show that there are some intrinsic host factors required to fully appreciate dormancy in human LTBI [44].

The Cornell model has been used extensively to discover more about *in vivo* adaptation by *M. tuberculosis*. This model involves drug induced immunosupression in mice [36, 65, 66]. After infection, a mouse will generally clear most of the AFB without causing disease until treatment with steroids causes *M. tuberculosis* re-activation. While much of what we know about *in vivo* gene expression and host response is derived from this model, it does have its limitations. The bacterial load required for a TB infection in mice is much higher than what is usually seen in humans [44]. Consequently, the mice end up with a chronic infection causing severe damage of the lung, rather than the anaerobic calcified granuloma seen in humans [36, 43]. Another problem is that the administration of drugs is not standardised across the field which may cause discrepancies in results [44].

These problems have lead to the exploration of other host organisms. Guinea pigs are much more susceptible to *M. tuberculosis* infection and produce granulomas of similar pathology to those in humans [36, 67]. Rabbits also show a similar pathology with caseous and calcified healing lesions however, they are resistant to *M. tuberculosis* infection and will only be infected by *M.bovis* [44].

Using other species of mycobacteria in their specific host is another way of determining how persistence can be achieved. Although they will not fully represent the human disease, many of the mechanisms used to survive intracellular conditions are similar. Members of the *M. avium* subspecies are obligate pathogens which infect chickens and cause Crohn's disease in immune compromised humans [12]. *M. bovis* is 99.95 % similar to *M. tuberculosis* on a nucleotide level and infects a number of animals including goats, pigs and sheep [15]. Interestingly *M. bovis,* which is more recently evolved than *M. tuberculosis* from the original progenitor of the complex, the loss of certain genetic elements exhibits a broader host range [15].

Avirulent *M. bovis* BCG has been used as a vaccine strain for many years and a particular region that was lost in BCG is the previously mentioned RD1 region [36, 68]. This genetic region was recently studied in *M. marinum,* which can cause disease in frogs and fish [45]. It is a particularly useful model as it infects macrophages and gives a similar granuloma structure to the closely related *M. tuberculosis*. The aforementioned work used this species in zebrafish embryos to determine formation and stability of granulomas in this species.

1.2.5 The Dormancy response

Using the models described, significant advances have been made in elucidating the dormancy response during persistence in a granuloma. Transposon mutagenesis studies have implicated a number of genes for survival of *M. tuberculosis* at various stages of infection [46]. The pathogen must respond rapidly to environmental cues in order to alter its proteome and adapt to these harsh conditions using tightly regulated signal transduction mechanisms. Some of the important cellular changes will be described.

The RD1 Region

The development of the *M. bovis* BCG vaccine strain involved serial passage of the strain until it reached an avirulent state [58]. Genotyping the vaccine strain revealed the loss of two significant genetic regions, esat6 and cfp10 collectively known as the RD1 region. A BCG:RD1 knock in strain displayed an increase in virulence that was not entirely restored with respect to the wild type strain [58]. RD1 mutants have a different morphology to wild type and do not form a granuloma structure. A recent study confirmed that this genetic region was vital in maintaining persistence in a granuloma using the *M. marinum* zebrafish model [45].

The distinct layers of a granuloma suggest that these lesions are not a random mass of cellular material but an organised structure which forms as macrophages are activated by AFB, leading to aggregation of mononuclear phagocytes [59]. Davis *et al*. found that the *M. marinum* RD1 mutant was attenuated in its ability to form a granuloma. The main distinctions they found in macrophage aggregation from the RD1 mutant compared with wild type included differences in the speed and

morphology of macrophages recruited to the infection foci. The granuloma expansion was also different; in wild type cells a continual expansion of the granuloma was observed through cycles of apoptosis and phagocytosis, while the mutant showed no chemotaxic recruitment of macrophages.

Other changes involved in the dormancy response include the induction of the so called dormancy regulon. Over 40 genes are induced by the DevS/ DevR two component system (TCS) in response to conditions of low oxygen and nitric oxide [60-62]. These include enzymes required for glycoxylate metabolism, shown in the previously mentioned Wayne model [56], a number of other stress response genes are induced by this TCS, including the *hspX* gene [62-64].

Changes to the cell wall structure of the pathogen involving the PhoP/ PhoR system are also noted during latency [57, 70-73]. A review on the potential of cell wall components as drug targets describes multiple changes in the cell wall composition as a result of stationary phase in mycobacteria [65]. The author describes possible changes to the amount and conformation of mycolic acid, a change in the ratio of cross-linked peptidoglycan and a decrease in the level of arabinogalactan present during dormancy [65].

All of the mechanisms which *M. tuberculosis* uses to evade immune responses and survive latently in an intracellular environment rely on adapting to life in the macrophage. The AFB is able to do this via a number of signal transduction mechanisms which tightly control gene expression in response to extra and intra cellular changes it encounters. The current understanding of changes to the mycolic acid structure of the AFB cell wall during infection raises questions about the efficacy

of cell wall related drug targets, such as INH, for treating latent TB [65]. The AFB's switch to a more reductive metabolism will also render drugs which target active replication poor candidates against latent infection. In order to eliminate the sizable reservoir of latent TB worldwide, new candidates that act on targets utilised during this dormancy phase are required.

1.3 Cellular Signalling

Every living organism must have tightly controlled sensing mechanisms in order to survive in and respond to its particular niche. Both prokaryotic and eukaryotic cells employ a number of responsive systems and processes, without which they would be rendered defenceless in the face of any changes in their respective environments. Organisms adapt to life in specific conditions with relation to temperature, pH, and the availability of nutrients and oxygen, to name a few. How well an organism adapts to a changing environment determines its survival and evolutionary fitness.

In *M. tuberculosis,* signalling molecules and systems allow the organism to live in the hostile environment of the human immune system induced granuloma for extended periods of time, making these structures attractive drug targets. Their relative distance, in terms of homology, from eukaryotic signalling systems means that inhibitors of prokaryote signal transduction are less likely to evoke a negative response in the human host. This section will highlight important prokaryote signalling mechanisms seen in mycobacteria, with an emphasis on the two component systems (TCS) which are the focus of this study.

1.3.1 Classes of Prokaryote Signalling Molecules found in mycobacteria

In response to external signals, prokaryotes employ single and multi protein phosphorelay systems to interpret and respond to external ligands. This results in control of gene expression by either transcriptional control or post translational modification of proteins. Histidine kinases (HKs) are generally part of two component system signal transduction. Other sorts of protein kinases include serine threonine

and tyrosine kinases. The earliest evidence for reversible phosphorylation in prokaryotes was unearthed in the 1970s [66]. This sort of mechanism had only been revealed in eukaryotes and the bacteriophage T7, so the discovery of serine threonine kinases in *Salmonella typhimurium* [66] preceded a surge of interest in this area.

One Component Regulators

One component signalling, as the name implies, requires just one protein with sensor and effector domains [67]. Intracellular changes are recognised by the sensing domain which results in DNA binding of the effector, which acts as a transcriptional activator or repressor [67]. Some well known examples include the TetR and AraC proteins in *E.coli*, which respond to L-arabinonse and tetracycline respectively [67]. The simplest AraC type regulators do not require phosphorylation or communication between proteins [67]. Thousands of bacterial proteins share motifs with the AraC regulator but none have been found in eukaryotes [67]. An example of this sort of protein in mycbacteria is the iron dependent regulator IdeR. The protein contains an N terminal helix turn helix motif for DNA binding and two metal binding sites surrounded by the dimerization domain [68]. When bound to iron, the protein represses the *fxbA* gene product [79-81], which is an important enzyme in the synthesis of the *M. smegmatis* siderophore exochelin [69], and activates iron storage gene *bfrA* [70]. The ability to acquire and store iron is very important during intracellular infection and is noted as a virulence factor for *M. tuberculosis* [82, 83]. The *ideR* gene has been deemed essential [71], probably because loss of this gene product results in an overload of iron accumulation which is toxic to the organism [72].

Serine Threonine Kinases

The first mycobacterial serine threonine protein kinase (STPK) was discovered in 1997 [73], since then 11 Mycobacterial STPKs have been identified named Pkn A to L, which are implicated in development, pathogenicity and stress responses [74]. STPKs are often implicated in development from one state to another such as in sporulation of *M. xanthus* [75]. They are also thought to interfere with Mitogen Activated Protein (MAP) kinases of epithelial cells in *Listeria monocytogenes* [76]. Mycobacteria, especially *M. marinum*, have a large number of STPKs; it is possible that they are complementing a relatively smaller number of TCS compared with other bacteria [77]. PknG and PknK are the only *M. tuberculosis* STPKs that do not posses transmembrane regions, all the other STPKs are grouped based on homology clusters or function.

Adenylyl cyclases and diguanylyl cyclases

Other transcriptional regulators are influenced by global regulators such as cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic diguanosine monophosphate (c-di-GMP). Bai *et al* have shown that cAMP has an important role in macrophage interaction as its concentration increases 50 fold during *M. tuberculosis* and *M. bovis* BCG infection [78]. At least 15 class III adenylyl cyclases exist in *M. tuberculosis* and 31 in *M. leprae*, a relatively high number compared with other bacterial genomes [79, 80].

1.3.2 Prokaryote Two Component Signal Transduction

The concept of two component signal transduction, whereby one component recognises some external change and another component modulates an appropriate response, is now a hallmark of prokaryote signal transduction. Phosphorylation of histidine kinases was recognized in *E.coli* by Kundig and Roseman in 1971 who were studying mechanisms of sugar transport [81]. Later it was found that phosphorylation of such proteins can modulate changes in gene expression [82] when coupled with a response regulator in a process outlined by figure 1.3. The term two component system is something of a misnomer, as it includes multistep kinases with intermediate proteins to regulate and insulate signals [83]. In *E. coli* there are 32 response regulators (RR), 23 histidine kinases (HKs) and 5 hybrid sensory kinases [83]. These were identified by Mizuno *et al* using OmpR, NarL, NtrC and CheY systems as probes to search for similar open reading frames [83]. They described a number of common “signature sequences” of HKs and RRs.

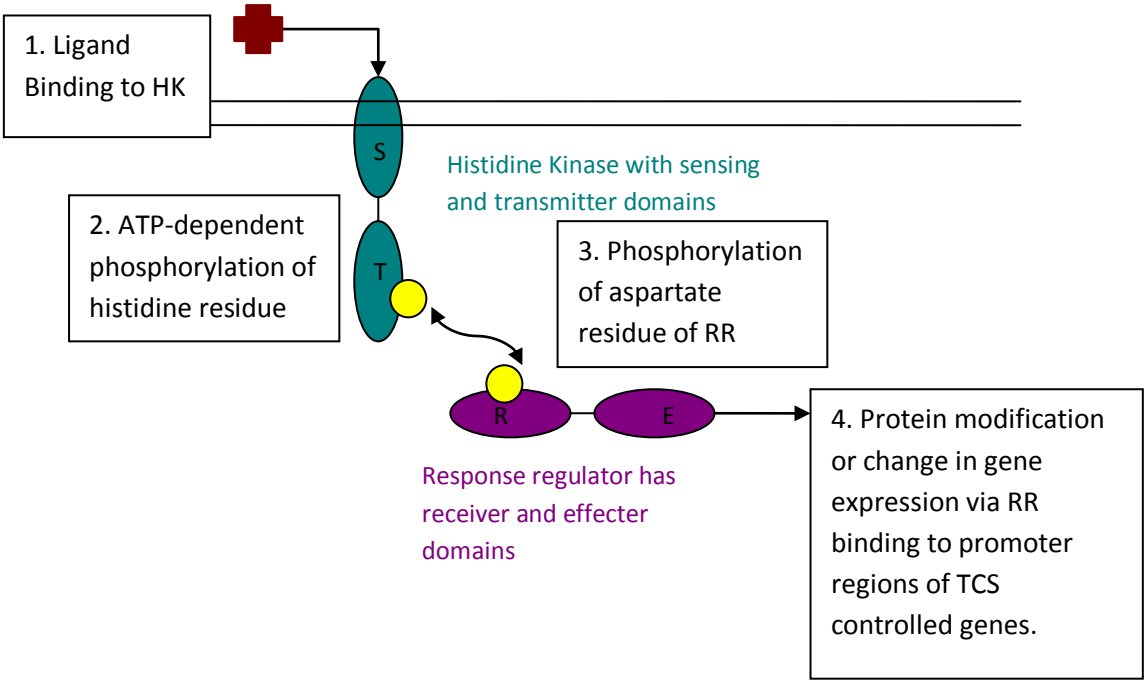


Figure 1.2: Two Component Signal Transduction. *Prokaryote two component systems. Signals are relayed from membrane bound Histidine Kinase to Response Regulator via phosphorylation of conserved amino acids ○. Apart from ligand-specific binding domain of Histidine Kinase and effector domain of Response Regulator, many regions of these systems are well conserved among prokaryotes.*

1.3.3 Mechanisms of two component signalling

Histidine kinases possess diverse sensing domains as well as a number of conserved transmitter domains [84, 85]. They have been divided into one of 11 groups based on their protein sequence surrounding conserved residues on their transmitter domain namely the H box, N box, D box, F box and G box [85]. The HK usually includes one to five N terminal transmembrane regions responsible for ligand binding [86]. This part of the protein can also be periplasmic, or intracellular and connected to other transmembrane receptors [85].

As mentioned, this part of the protein is extremely variable amongst TCS. Some studies have shown the sensory domain to have a specific ligand binding preference, such as divalent cations [87], others imply that certain conditions lead to a change in conformation of the protein [88]. In either case, this results in an ATP-dependent autophosphorylation of the H box of the protein. The HKs occur as dimers with catalytic domains connected by a HAMP linker motif [84]. The catalytic domain forms a complex with a cognate RR containing a conserved aspartate residue [89]. The phosphoryl group is transferred from the histidine to the aspartate residues resulting in a switch to the RR's active state [89].

The RR usually contains around 120 amino acids, including the crucial aspartate and two other conserved residues [83]. The RR carboxy terminal effector domain is responsible for initiating the cellular responses to the ligand [83]. OmpR, NarL and NtrC type RRs have DNA binding domains and act as transcription factors in that they directly induce or repress gene expression [83]. Others have an enzymatic role in the cell or directly interact with other proteins [85].

There are essentially four steps common to all TCS which are illustrated in figure 1.3. An environmental stimulus is recognised, usually in the form of ligand binding. This results in a conformational change to the HK, causing autophosphorylation of the histidine residue of the HK transmitter domain by the conversion of an ATP to ADP (adenosine diphosphate). Then there is a transfer of the phosphoryl group from a histidine residue on the HK to an aspartate residue on a response regulator, and finally dephosphorylation of the phosphoryl group to inorganic phosphate [89].

This is a simplified model of this mechanism because there are a number of variations on this theme. Some systems involve intermediate steps for greater control and networking between signals. Multi-step phosphotransfer signalling works on the same principles as two component signalling, with an added level of complexity. HPts are histidine containing phosphotransfer domains found in some signalling systems [84]. This higher level of complexity is often found in eukaryote signalling systems, either to ensure that there are multiple regulatory checkpoints, to amplify a signal or so that the HPt can be an alternate target [84].

1.3.4 Cross talk and Networks in TCS Signal Transduction

As well as having intermediate proteins, these systems can form networks to integrate signal transduction [90]. Pathways can be branched, in other words one HK can phosphorylate many RRs, such as is the case in the CheA/CheY/CheB system or multiple HKs can phosphorylate one RR [91]. It is somewhat surprising, given the conserved mechanism of phosphotransfer across signalling networks, that crosstalk is not a problem [91]. Since there are often dozens of these systems in a cell, responding to a vast array of different signals, it is important that these phosphorelays are insulated from each other. There is evidence that, in the absence of their cognate HK, a RR can be phosphorylated by other HKS [91]. For example, in a PhoR deletion mutant, its native RR PhoB can be phosphorylated by another HK named VanS [91]. Another way to induce cross talk of signals is to over-express the HK, presumably making the protein more likely to phosphorylate another RR. This has not been shown in wild type cells, indicating that there is a high level of control over signal insulation. It is likely that RRs, which are generally more abundant in the cell than their cognate HKs, have a preference for their own HK [90, 91]. Also, the

observation that HKs often negatively control RRs by de phosphorylation means that there is a smaller chance of signals going astray [91].

In eukaryotic *Arabidopsis thaliana*, a TCS network has been analysed giving a map of hundreds of interactions between proteins involved in these systems [92]. A similar study in *E. coli* concluded that the most significant interaction between TCS was in the overlap of gene expression resulting from different systems [93, 94].

1.3.5 The Importance of TCSs in Prokaryotes

The signal relays described usually culminate in an alteration of gene expression. Large numbers of genes are either switched on or off as a result of the RR activation. Control of cell osmolarity in *E. coli* by EnvZ/ OmpR is a well understood example of how the cell is capable of maintaining turgor pressure [84]. Controlling the expression of membrane proteins OmpC (during high osmolarity) and OmpF (during low osmolarity) is crucial in a changing environment [84].

As well as general survival of the cell in harsh conditions, pathogenic bacteria also use these signal systems to express virulence factors, some of which are shown [89] in table 1.1.

TCS	Species	Response
PilS/PilR	*P. aeroginosa*	Pilus production
FleS/FleR	*P. aeroginosa*	Adhesion
KinA/KinB/SpoF	*B. subtilis*	Sporulation
PhoP/PhoQ	*S. typhimurum*	Pathogenesis
PhoP/PhoR	*E.coli*	Phosphate sensing

Table 1.1: Virulence and survival associated TCS in prokaryotes. *(as reviewed by Fontan 2004 [89])*

Another concern is the ability of certain TCS to induce drug resistance in a pathogen. Polymixin resistance in *Salmonella typhimurium* for example is aided by its PhoP/PhoQ TCS, which senses membrane disturbances and activates lipid A modifications to evade to the effects of the drug on the cell [95]. Another example is *E. coli*'s ability to alter gene expression under the control of the OmpF/ OmpC system which mediates entry of tetracycline to the cell [95]. Vancomycin resistance is seen in *Enterococcus faecalis* and *Staphylococcus aureus* as a result of the VanS/ VanR and VncS/ VncR systems respectively [95, 96]. Vancomycin binds to precursors in peptidoglycan synthesis preventing cell wall assembly [96]. The VanS/ VanR system is able to modify peptidoglycans giving them a lower affinity for the drug [97].

The fact that various TCS have been deemed essential to the organism is another reason they would make potential drug targets; to date Cck/ CtrA of *Caulobacter crescentus*, MtrB/ MtrA of *Mycobacterium tuberculosis*, HP166/ HP165 of *Helicobacter pylori* and YycG/YycF of *Bacillus subtilis* and *Streptococcus pneumonia* are considered to be essential [98].

1.4 Two Component Systems in mycobacteria

The *M. tuberculosis* genome encodes 11 TCS and seven orphan HKs and RRs [86]. All of these belong to either the OmpR or NarL family of TCS defined by the residues surrounding their H box. *M. bovis* shares all of these TCS while the *M. smegmatis* genome contains five out of the seven orphan HK and RR and nine out of the eleven complete TCS [86]. Table 1.2 names all of these systems and their *M. smegmatis* homologues with any known conditions regarding their expression, based on articles by Tyagi, Fontan and Haydel [113, 116, 125]. These TCS are found in a majority of mycobacterial species, with the exception of *M. leprae* which has only 4 out of 12; SenX3/ RegX3, PrrB/ PrrA, MprA/ MprB and MtrB/ MtrA.

Some of the TCS of *M. tuberculosis* have been well studied and their activating factor or factors proposed. Others have been shown to be upregulated during intra-macrophage infection, indicating an important function during infection and latency, but the specific condition they are responsive to remains elusive. The systems used in this study were MtrB/ MtrA, DevS/ DevR and SenX3/ RegX3 which will be described..

Name (HK/ RR)	Gene	*M.Smegmatis* homologue	Similarity	Proposed function
SenX3/ RegX3	Rv0490/	MSMEG_0936	0.695	Phosphate
	Rv0491	MSMEG_0937	0.933	starvation
KdpE/ KdpD	Rv1028/	MSMEG_5395	0.773	Nutrient
	Rv1027	MSMEG_5396	0.858	starvation
MprB/ MprA	Rv0982/	MSMEG_5487	0.763	
	Rv0981	MSMEG_5488	0.9	
MtrB/ MtrA	Rv3245/	MSMEG_1875	0.828	Iron
	Rv3246	MSMEG_1874	0.969	starvation
PrrB/ PrrA	Rv0902/	MSMEG_5663	0.813	
	Rv0903	MSMEG_5662	0.932	
TrcS/ TcrR	Rv1033/	MSMEG_2916	0.715	
	Rv1032	MSMEG_2915	0.507	
DevS/ DevR*	Rv3133/	MSMEG_5244	0.852	Hypoxia,
	Rv3132	MSMEG_5241	0.664	nitric oxide
PhoP/ PhoR	Rv0758/	MSMEG_5870	0.798	
	Rv0757	MSMEG_5872	0.927	
TcrY/ TcrX	Rv3765/	MSMEG_4989	0.589	
	Rv3764	MSMEG_4990	0.859	
NarL/ NarS (NarL family)	Rv0844/ Rv0845	MSMEG_0105 -	0.781	
TcrA/-/-	Rv0600-Rv0602	-		
Orphan RR, NasT	Rv1626	MSMEG_3246	0.872	
Orphan RR	Rv0260c	MSMEG_0432	0.747	
Orphan RR	Rv0818	MSMEG_5784	0.747	
Orphan RR	Rv3143	MSMEG_2064	0.736	
Orphan RR	Rv2884	-		
Orphan HK	Rv3220	MSMEG_1918	0.757	
Orphan HK	Rv2027c	-		

Table 1.2: HKs and RR of M. tuberculosis. *(All HKs belong to the OmpR family except * which is a NarL type HK)*

1.4.2 1.4.1 MtrB/ MtrA

The first TCS to be discovered in *M. tuberculosis* was the MtrB/MtrA system [99]. Curic et al used a *xylE* promoter fusion reporter system to show that the *mtrA* gene expression varied depending on the media used to culture *M. smegmatis and M. bovis* [99]. Another group used a Green Fluorescent Protein (GFP) promoter fusion to

show this gene was expressed during macrophage infection with *M. bovis* BCG [127] which was later complemented by work from Via and co-workers [100]. One interesting quality of this TCS is the fact that it has not been knocked out, indicating that it may be essential to *M. tuberculosis* [101]. This has however made it more difficult to investigate its function during infection. In 2006 a group over expressed the MtrA RR showing that overproduction of this protein prevented multiplication during macrophage infection, but not in broth [102]. They concluded that the ratio of phosphorylated to unphosphorylated MtrA was vital for intra macrophage replication, since simultaneously upregulating its HK MtrB had no effect [102].They supported this theory by showing that MtrA binds to the promoter of a replication gene *dnaA* [102]. They also suggested that it may be involved in the AFB's ability to block phagolysome fusion. Friedland et al solved the crystal structure of MtrA and demonstrated that it was similar to the PrrA RR and may have multiple conformations [103].

Studies of this RR in other species may also give clues as to its function in *M. tuberculosis.* A study of *M. leprae* cell wall associated proteins unearthed an *mtrA* homologue in this species, highlighting its importance for intracellular growth [104]. In *M. avium* the *mtrB* locus has been knocked out using transposon mutagenesis, resulting in a strain with attenuated virulence which cannot make the red white switch on Congo red agar [105]. In this species the TCS is required for the control of cell surface proteins and regulates permeability and composition of cell wall components [105]. In a more distantly related species *Cornebacterium glutamicum* an MtrA knockout showed an effect on antibiotic susceptibility and osmo-regulation of the cell [106]. In this species the *mtrA* gene is probably involved in peptidoglycan metabolism and osmotic protection [106]. Interestingly, a similar TCS the AmrA/ AmkA

system of *Amycolatopsis mediterranei* has been knocked out showing a 20% decrease in RIF production by the organism [107].

MtrB/ MtrA is well conserved in other mycobacterial species including *M. smegmatis*, which makes this system easy to manipulate for this study. Since MtrB/ MtrA appears to be induced during macrophage entry and has not yet been knocked out, this system shows great potential as a drug target in *M. tuberculosis.*

1.4.2 SenX3/ RegX3

The SenX3/ RegX3 TCS has been well characterised in *M. tuberculosis* [135-137]. It is involved in the phosphate transport system and induces the *M.smegmatis phoA* gene, whose protein product is an alkaline phosphatase required for transport of inorganic phosphate into the cell [108]. It is proposed that this system controls the regulation of around 50 genes as well as the *regX3* gene product [109]. This positive autoregulation of the RR is important in TCS for signal amplification [110]. The *phoA* gene is present in *M. smegmatis* and *E. coli* but not *M. tuberculosis* [111]. This gene is involved in inorganic phosphate transport in *E. coli,* a mechanism which has been well characterised in this species [135, 139, 140]. *PhoA* is induced by SenX3/ RegX3 in *M. smegmatis* and by PhoP/ PhoR in *E.coli* in a phosphate dependent manner [135, 139]. It functions as a cell surface associated lipoprotein which liberates inorganic phosphate from macromolecules [111].

The phosphate specific transporter PST and phosphate intake transporter PIT are responsible for maintaining cellular levels of inorganic phosphate [111]. The PST proteins act as a sensor of phosphate concentration, keeping RegX3 in an unphosphorylated state [108]. When phosphate is limited the PST complex causes

dimerization of the SenX3 HK, which activates the RegX3 RR to induce *phoA* and other genes required for phosphate production [108].

To summarise, this TCS is implicated in the phosphate transport system, its expression is increased during early macrophage infection [112] and its knockout displays attenuation of growth in a mouse and macrophage model. This suggests that this TCS may be an important virulence factor [109]. Specific sequence differences between virulent and avirulent strains of the *M. tuberculosis* complex species have been shown in the sequences of this TCS, indicating that virulence phenotypes may be affected by SenX3/ RegX3 [113].

1.4.3 DevS/ DevR

The DevS/ DevR system is responsible for inducing the dormancy response of *M. tuberculosis* during hypoxia and nitric oxide stress [71, 72, 142-144] making it a valuable target in preventing latency [114]. Unlike most other TCS of *M. tuberculosis*, the DevS/ DevR system is part of the NarS/ NarL family [89]. The DevS HK contains two GAF domains in its sensing core named GAF A and GAF B [115]. It has been proposed that GAF A binds to heme which changes from an oxygenated to a deoxygenated state during oxygen starvation, this results in DevR activation and transcription of a number of dormancy related genes [115]. This assumption was based on the observation that cyanide, which binds with heme, hinders the dormancy response in *M. tuberculosis* usually induced by nitric oxide stress and oxygen starvation. The importance of this TCS during hypoxic stress has been illustrated by its knockout mutant, which cannot survive oxygen starvation and is more sensitive to heat shock [62]. As previously mentioned, the granumloma formed in the human lung during *M.*

tuberculosis infection leaves the AFB in a hypoxic environment [116]. It is proposed that this oxygen based sensor could be a switch from aerobic to anaerobic metabolism.

A mouse model has shown knockouts of this system to be hyper virulent [112] while rabbit and guinea pig models show it is required for persistence [117]. Converse *et al* [117] showed marked differences in results depending on the model host used. Considering the different pathology shown in different host organisms, it is possible the DevS/ DevR system is required to shut down active metabolism and therefore the knockout appears hyper virulent in mice because this host does not naturally show latency.

During the *M. tuberculosis* dormancy period a number of genes required for active metabolism are repressed, while stress response genes, including *hspX,* are induced [47, 144, 148]. The *hspX* gene product is an alpha crystallin antigen which may play a role in stabilizing proteins during persistence [63]. As well as *hspX*, DevS/ DevR induces 3 universal stress proteins (UspL,M and N) and a nitroreductase (Rv3131) [118].

The signal transduction mechanisms of *M.* tuberculosis undoubtedly have an overall effect on its virulence and pathogenesis, in particular the switch from active to latent infection. It is in this light that these systems are viewed as excellent potential drug targets in mycobacteria and other pathogenic prokaryotes. Consequently, a number of groups have attempted to develop inhibitors to these signalling systems.

1.4.4 Two Component Systems as Drug Targets

The search for new anti-TB drugs is a particularly important component of the STOP TB strategy, given the rising threat of MDR and XDR-TB. Using growth inhibition assays to discover anti-tubercular agents from a vast array of compounds by measuring the effect on the whole cell, may lead to the discovery of effective agents against the pathogen. On the other hand, a more targeted approach to specific components of the cells dormancy program is more likely to yield an agent with a detrimental effect on *M. tuberculosis* during a latent TB infection.

Since the discovery of TCS and their inferences to virulence and persistence, they have been labelled as critical targets in pathogenic bacteria [98]. Generally speaking signal transduction mechanisms allow bacteria to persist and cause disease in a human host; TCSs in particular are of great interest for four main reasons [98]:

- Their ubiquity among prokaryote organisms and selective presence in higher eukaryotes, indicates a TCS inhibitor would be less likely to exert a negative effect on a human host
- Principle active sites in TCS display a high level of homology, so a TCSi could potentially act on a number of TCS across a broad spectrum of bacteria
- At least four TCS are essential to their host organisms
- TCS are often used by prokaryotes to regulate the expression of virulence factors

More specifically, in terms of *M. tuberculosis,* current drug targets act on processes such as cell wall and protein assembly, which are only effective in the case of an active TB infection. In targeting the mechanisms *M. tuberculosis* employs to sustain

an ongoing latent infection, problems encountered with many of the current anti-TB agents such as toxicity, lengthy treatment duration and the emergence of resistant strains could be prevented [114].

1.5 Inhibitors of TCS

There are multiple stages of the TCS phosphorelay that could be targeted to inhibit this type of signal transduction [119]. Figure 1.3 shows some of the reactions of the phosphorelay which could potentially be inhibited as well as examples of known TCS inhibitors and their proposed targets. In theory, targeting a conserved mechanism such as the phospho transfer between the histidine and aspartate would give rise to a broad spectrum inhibitor. On the other hand, targeting a specific ligand binding mechanism of the HK, or DNA binding of the RR, would render TCSi that is fairly specific to that TCS.

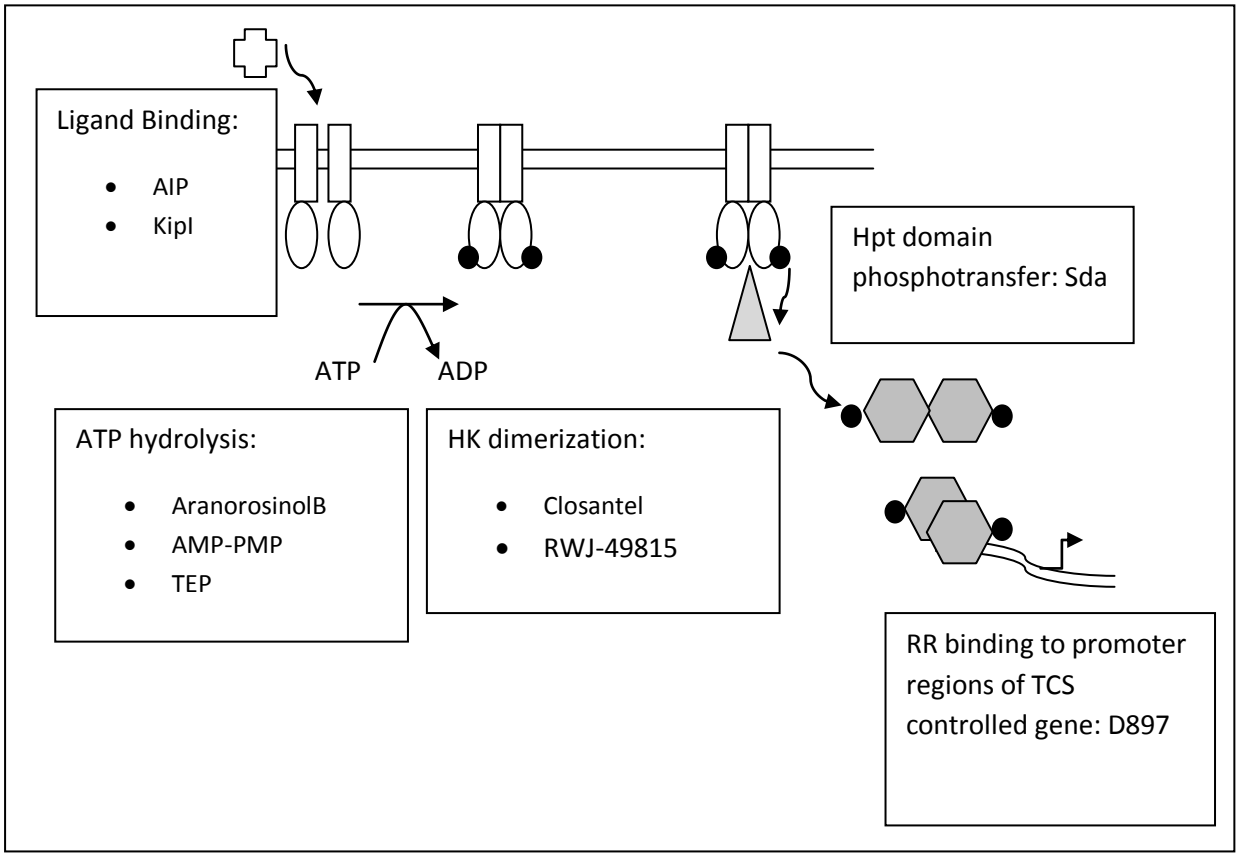


Figure 1.3: TCS Inhibitors*. A summary of known TCSi and their proposed mechanisms of action. Agents have been found which inhibit a number of the steps involved in TCS signal transduction including ligand binding to HK, ATP hydrolysis, HK dimerization and RR phosphotransfer and promoter binding.*

To date there have been a number of compounds introduced to the field which act on specific components of the signal transduction machinery, but none that do so in a way that affects signalling independently of other prokaryote and eukaryote cellular processes [98].

1.5.1 Inhibition of the Histidine Kinase

Compounds which affect ligand binding to HK sensor domains have been developed for specific targets [98]. These are usually derivatives of naturally occurring ligands which are modified at specific residues in order to compete with naturally occurring ligands of the TCS.

Streptomyces produce autoinducing peptide (AIP) to induce virulence factor Agr required for quorum sensing [119]. When the population reaches a threshold density, secretion of AIP binds the HK of the ArgC/ ArgA TCS effectively inducing virulence genes and repressing surface proteins [119]. Replacing the sulphur of the thiolactone ring structure of AIP with O or N provides a competitive inhibitor of their quorum sensing mechanism [119].

KipI is a protein inhibitor of *B. subtilis* sporulation TCS KinA/SpoOA [120]. This protein inhibits the autophosphorylation of KinA, but not the phospho transfer step. Another gene in its operon, *kipA*, counters its activity [120].

A number of chemicals have been found to inhibit the ATP to ADP hydrolysis shown in figure 1.4. These can act in either a competitive or non competitive fashion. AMP-PNP (adenosine monophosphate p-Nitrophenol), TEP (triethyl phosphate) and aranorosinol B for instance competitively inhibit this hydrolysis reaction while cis

unsaturated fatty acids such as oleic and palmitoleic act in a non competitive fashion [98]. While they effectively inhibit this signal transduction process, they would clearly affect a number of other processes in both the host and pathogen.

Most TCS inhibitors to date interfere with the dimerization domain of a HK, exposing hydrophobic amino acids resulting in an aggregation of HKs at the cell membrane [121]. Closantel and RWJ-49815 act in this way, denaturing the dimer of KinA by interfering with its 4 helix bundle between the sensor and effecter domains [122]. Without suitable dimerization, signal transduction cannot be relayed to the RR. These compounds have the same effect in a sensor kinase in *E. coli* reflecting a broad spectrum of activity [96]. Unfortunately, many of these HK dimerization inhibitors also have an effect on membrane intergrity in both bacterial and mammalian cells and thus have not been developed as antibiotics.

The Sda protein inhibits the sporulation system KinA/KinB through binding of the histidine phosphotranfer domain (HPt) of KinB [121]. This compound binds with the alpha helices, not the conserved H box or catalytic domain, and acts as a molecular barricade, hindering communication between KinB and KinA [121].

1.5.2 Inhibition of Response Regulator

A less common mechanism of action is the inhibition of the RR activity. A modified protein can alter a HK/ RR interaction via competition for their binding site. Modifications to the DrrA peptide of the HpkA/DrrA system in *Thermotoga maritima* caused competitive binding for HpkA resulting in a loss of dimer formation [123]. A compound named D897 is able to inhibit DNA binding of RR to the promoter regions of induced genes by preventing the dimerization of RR required for DNA binding [98].

Although natural and synthetic compounds have been shown to inhibit stages of this process, research in the field has yet to provide a compound which affects these processes exclusively. A major shortcoming of many TCS inhibitors described is an adverse effect on membrane integrity of both bacterial and human cell membranes [98]. A study of 24 TCS inhibitors showed that 23/24 had adverse effects on membrane integrity. This suggests that their activity may be the result of another mechanism altogether, such as membrane interference or macromolecule synthesis [124]. Unfortunately the only compound free of this defect had a relatively high minimum inhibitory concentration (MIC) [124]. Other TCS inhibitors exhibit severe toxic side effects, closantel for example adversely affects energy metabolism in the mitochondria [125].

That being said, the organism *Staphylococcus epidermis* causes major problems with its ability to form biofilms on medical instruments [126]. It has been suggested that an inhibitor of the YycG HK of this organism, required for biofilm formation, could easily be used as a sterilization agent on medical equipment if it were cost effective [126]. Another suggestion is the use of non toxic HK inhibitors in combination with other drugs to improve a therapeutic outcome. Isothiazolone and imidazolin salts were the first TCS inhibitors to be discovered [127]. These aromatic structures showed activity against the AlgR2/ AlgR1 system, an important virulence factor of *P. aeroginosa* which causes major problems in cystic fibrosis patients [127]. These could be used to improve the therapeutic outcome of patients with cystic fibrosis [98] even though they do no inhibit the organism outright. Despite work by several groups to discover and develop TCS inhibitors, none been made commercially available as antibiotics or bacteriostatic agents in a medical context. The aim of this study was to develop a way in which one can screen for novel TCSi, which inhibit mycobacterial signal

transduction exclusively of other cellular processes, by incorporating a whole cell based approach as opposed to inhibiting individual proteins.

1.5.3 Screening for TCS inhibitors

High throughput screening (HTS) methods allow researchers to test thousands of compounds at a time for activity against TCS. There are two approaches here, either random screening of a large number of compounds from a library [119] or rational design using virtual screening methods based on the crystal structure of a target protein [126]. The latter method allows one to screen a much larger database of compounds, however all hits must be validated by another method to show that the compound specifically inhibits the putative target *in vitro*.

A direct approach involves the use of purified, truncated or altered HK to screen for specific inhibitors. Inhibition can be demonstrated using radio labelled phosphate as a donor in the ATP dependent autophosphorylation of purified HKs and RR [98, 128]. This sort of assay has been established by Saini *et al* with *M. tuberculosis* DevS /DevR system proteins [128]. The Taz-1 assay was used to find inhibitors of the EnvZ/ OmpR system [129]. In this screen the HK receptor signal is replaced by an aspartate chemoreceptor which stimulates autophosphorylation of the HK in the presence of aspartate. A *lacZ* promoter fusion of the *ompC* gene was also used to determine OmpR binding to this DNA region [129, 130]. The homodimerization assay exploits the capacity of certain truncated HKs to dimerise *in vitro* [98]. Using a plasmid with GFP under the control of the *E. coli iclR* promoter, truncated Yyc proteins expressed with the IclR repressor can only induce GFP expression when present as a dimer [98].

These sophisticated screening methods are limited in that they deal with altered forms of proteins which do not naturally occur in the cell. Using the native forms of these proteins in a whole cell based assay may deliver TCS inhibitors which represent a higher level of efficiency in a whole cell model.

Whole cell based methods have a number of advantages over isolated protein methods . For one thing, TCS inhibitors found using protein based methods may be potent inhibitors of signal transduction, but their ability to permeate the cell membrane is not taken into account. What is more, other metabolic activities of the cell are accounted for, such as proteins and enzymes which could decrease the activity of these compounds. As previously mentioned, all of the current known TCSi have failed in that they cause adverse affects on bacterial and mammalian cell walls. Many bacterial species also have efflux pumps which could potentially remove these inhibitors from the cell before it reaches its target.

Another consideration is the specificity of the target. Using isolated protein methods confines the researcher to finding inhibitors of a specific molecule or reaction in the chain of events involved in signal transduction. Using a whole cell based method, which measures the end result of the signalling mechanism, i.e. the up-regulation of a particular gene under the control of that system, increases the likelihood of an inhibitor of any part of this phosphorelay being picked up in the screen.

In 1993 Roychoudhury et al discovered the first TCSi by screening around 25,000 synthetic compounds using a whole cell *xylE* reporter system assay [127]. Inhibition of the RR binding activity to the *algD* promoter was demonstrated in 15 compounds using this colourmetric assay [127]. Differential growth assays are another method that

can be applied to whole cell screening. Temperature sensitive mutants of the YycG HK showed hypersensitivity to 11 compounds in a screen of 4000 acetone extracts [131]. These hits were further characterised, leading to the detection of aranorosinol B's ability to inhibit the autophosphorylation of YycG in *S. aureus and B. subtilis* [131].

Measuring expression of a TCS target gene is a valuable method because it accounts for everything that is going on in the cell at once. These methods show that a compound is capable of reaching its target and that gene expression is being inhibited in spite of whatever else is going on in the cell. However, it is an indirect method of detecting TCS inhibition. Usually validation either at a protein level or by measuring the expression of an unrelated gene is required in order to show that a compound does not simply have a global effect on gene expression. It is also important to consider any implications that reporter systems may have on both the cell and compounds in the screen and vice versa.

In this study a GFP reporter was used to measure the expression of promoters under the control of mycobacterial TCS to evaluate conditions which induce these systems and screen for TCS inhibitors using available drug libraries.

1.5.4 Methods used in this Study

The aim of this study was to apply a promoter reporter method to demonstrate appropriate conditions under which selected TCS genes are induced, so they can be used in a screen for two component system inhibitors. GFP, isolated from *Aequorea victoria,* has been used to monitor gene expression since 1994 [132, 133]. It is an ideal reporter as it does not require substrate activation [133] and numerous enhanced variants of the original wildtype GFP exist for specific purposes [134-136]. GFP mut2 will

be used to scrutinise the expression of TCS-regulated genes, and examine changes that occur to these expression profiles as a result of exposure to compound libraries. The constitutively expressed *hsp60* promoter will be included to verify that a shift in GFP fluorescence is the result of some mechanism other than a reduction in global gene expression on the cell. The model organism *M. smegmatis* will be used so that these assays can be carried out to test as many compounds as possible in a high throughput manner as it grows more rapidly and can be safely manipulated in a PC2 laboratory. The resultant experimental procedure could lead to further studies of the more pathologically relevant *M. bovis* BCG once the assay is optimised for *M. smegmatis.*

1.6 Aims

The overall objective of this research was to design a screen which identifies inhibitors of mycobacterial TCS related to dormancy and intra macrophage survival. This required four main objectives to be carried out:

1. Create a list of suitable *M. tuberculosis* and *M. smegmatis* TCS dependent genes and identify their promoter sequences.
2. Clone these promoter sequences and an optimised GFP reporter gene into a plasmid which can be electroporated into *M. smegmatis*.
3. Demonstrate conditions under which the greatest expression of these promoters is found.
4. Develop a bioassay to screen compounds for mycobacterial TCS inhibitors based on the expression of these TCS regulated genes.

CHAPTER TWO:
Materials and Methods

2.1 Strains and culture conditions

The plasmid construction was carried out in *E. coli* DH5α grown in Luria Broth (LB) or on Luria agar (LA) from Sigma Aldrich, with either kan_{50} (kanamycin 50 µg/ml) or amp_{200} (ampicillin 200 µg/ml). *M. smegmatis* MC^2 155 was growth on LA or in LB supplemented with 0.1 % Tween $_{80}$ and 1 % D- arabinose or Hartmans deBonts (HdeB) minimal media as described [53] using kan_{50} for plasmid selection. Further modification to culture media for stress condition assays shall be discussed in section 2.6.

2.2 Plasmids and Primers

This work involved the use of a number of published and unpublished plasmids from various sources as well as the creation of a new pSHigh construct. The plasmids are briefly described and their sources displayed in table 2.1. Primers used to clone and sequence elements of the new reporter construct are displayed in table 2.2.

Plasmids	Description	Source
pcr 2.1	TA cloning vector, Amp^r , Kan^r	Invitrogen
pLUG	TA cloning vector, Amp^r	Bioline
pTKmx	pAL500, pUCori, Kan^r, *xylE*	[137]
pHS201	P_{hsp60}, GFPmut2, oriM high, pUCori, Kan^r	[138]
pOT11	$P_{tet,}$ GFPmut2, pMMB207 backbone	[139]
pOT71	P_{hsp60}GFPmut2, pAL5000, pUCori, Kan^r	O'Toole (unpublished)
pOT72	P_{hsp60}HSP60, GFPmut2, pAL5000, pUCori Kan^r	O'Toole (unpublished)
pOT62	P_{hsp60}, GFP RBS, GFPmut2, pAL5000, pUCori, Kan^r	O'Toole (unpublished)
pSHigh	GFP RBS, GFPmut2, oriM high, pUCori, Kan^r	This study
pHigh100	GFPmut2, oriM high, pUCori, Kan^r	[138]

Table 2.1: Plasmids used in this study.

Primer Name	Sequence
GFP(RBS)_F	GGGGGGTACCTTTAAGAAGATATACATATGAGTAAAGGAGAA
GFP_R	GGGGGCATGCTTATTATTTGTATAGTTCATCCATGCC
oriM_F	GGGGGCTAGCAACGAGGACAGTCGCACGAC
oriM_R	GGGGGCTAGCAT CGAGCCGAGAACGTTATC
M. tuberculosis promoters	
P_{hsp60} Rv0440_F	GGGGGAATTCCGCACCGAGTCCAGCGAG
P_{hsp60} Rv0440_R	GGGGGGATCCGCAATTGTCTTGGCCATTGCG
P_{hspX} Rv2031c_F	GGGGGAATTCTCGGTACGGCGCAGTAGGAT
P_{hspX} Rv2031c_R	GGGGGGATCCAAACTCGGGGAAGAGGGACC
M. smegmatis promoters	
P_{MtrA} MSMEG_1872 _F	GGGGGGTACCCGAGACCAACCACAA
P_{MtrA} MSMEG_1872 _R	GGGGGGTACCTCCATGGTGTCACCA
P_{phoA} MSMEG_1012_F	GGGGGGTACCCAGCCGTGCAGACCA
P_{phoA} MSMEG_1012_R	GGGGGGTACCCAGCCGTGCAGACCA
P_{hspX} MSMEG_3932_F	GGGGGGTACCAACCGTGCGGCACGGGGAGATCTG
P_{hspX} MSMEG_3932_F	GGGGGGTACCTTCAGGAAGTTTGGTCATCGGTCCTCCTCA
Sequencing Primers	
oriM(-100)_F	GGGGAACTTAATCGCCTTGCAGCA
oriM(+600)_F	GGGGCCTGTTCTGCCGCACGCTCT
oriM(-100)_R	CCCCCCAGATAGCCCAGTAGCTG
oriM(+600)_R	CCCCCAGGTGGTTGGGGGTGCTCG
pTKmx_F	

Table 2.2: Primers used in this study. *Restriction sites of primers are underlined*

2.3 Identification of TCS dependent genes in *M. tuberculosis* and *M. smegmatis*

A list of candidate *M. tuberculosis* and *M. smegmatis* TCS controlled genes was established after a literature search based on the following criteria:

1. The candidate must contain a non-coding region upstream of the gene or have its promoter region identified, preferably with a gel mobility shift assay consistent with the ability to bind directly with a RR.
2. Factors or conditions inducing expression of the gene should be postulated or proven.
3. A high fold induction of gene expression upon stimulation, or a significant decrease in expression of that gene displayed in the TCS mutant.

Sequences of candidate genes and their organisation within an operon were obtained from www.genome.jp. A promoter region of approximately 600 base pairs was selected directly upstream of each candidate gene for primer design.

2.4 Plasmid Manipulation

Cloning procedures were carried out with T4 ligase from Bioline and restriction enzymes and their respective buffer solutions from New England Biolabs (NEB). Polymerase chain reactions (PCR) were carried out with Taq polymerases from either Bioline or Invitrogen limited.

2.4.1 Amplification of Promoters from Genomic DNA

Genomic DNA (gDNA) from heat treated *M. tuberculosis* H37Rv was provided by AgResearch (Upper Hutt). *M. smegmatis* gDNA was prepared by standard genomic preparation as described by Sambrook [140]. Primers were designed to amplify around 600 base pairs of a promoter region upstream of each gene of interest, including at least the start codon of each gene. Primers contained *Eco*RI and *Bam*HI restriction sites for cloning into low copy number pOT constructs, or *Kpn*I for cloning into

pSHigh constructs. In addition, the highly conserved promoter of the *hsp60* gene (Rv0440) was cloned as a constitutively expressed positive control. Promoters were amplified from gDNA using the high fidelity polymerase Platinum Taq (Invitrogen Ltd.) according to manufacturer's instructions with an Eppendorf thermocycler. Appendix 1 displays a summary of all PCR conditions used in this study. PCR products were visualised on a 1% agarose gel to check they were the correct size, before the PCR products were isolated using a clean and concentrate kit from Zymo Ltd.

2.4.2 Ligation of promoters into Cloning Vectors and heat shock Transformation into *E.coli*

Purified PCR products were ligated into either pCR2.1 of pLUG TA cloning vectors using T4 ligase by incubating promoter, vector, ligase enzyme and 1 x ligase buffer for at least 4 hours at 16 ^{o}C. A summary of ligation reactions in this study is shown in appendix 2.

Ligation reactions were then transformed into calcium competent *E. coli* DH5α by heat shock transformation. For notes on preparation of calcium competent DH5α see appendix 4. The optimal ratio of cells to ligation product is 10:1 so for a 10 µL ligation reaction, aliquots of 100 µL of DH5α cells were used. Cells were first thawed on ice for 5 minutes before adding the ligation product. Following a 20 minute incubation period on ice, cells were heat shocked at 42 ^{o}C in a heat block for 2 minutes and then returned to ice for 5 minutes. The cells were then recovered at 37 ^{o}C without agitation, for at least 45 minutes in 600 µL of LB, without antibiotics, to allow expression of the antibiotic cassettes contained on the plasmid. Following recovery,

cells were centrifuged for 30 seconds at 12000 rpm and the supernatant removed to leave 100 µL of cells which were re-suspending by gentle pipetting.

For blue white colony selection, cells were spread on Luria agar with Isopropyl-Beta-d-Thiogalactopyranoside (IPTG) at 100 µg/ml, X-galactosidase (Xgal) at 40 µg/ml and ampicillin at 200 µg/ml and incubated at 37^{o}C overnight. At least 5 white colonies for each ligation reaction were selected and inoculated in 3 mL overnight cultures of LB with ampicillin (200 µg/ml). 600 µL from each culture was reserved for a -80 ^{o}C freezer stock, made with an equal volume of 80 % glycerol to give a final concentration of 40 % gylcerol.

2.4.3 Plasmid Isolation and Digestion

The remainder of the liquid culture was used for plasmid preparation. In this study plasmid DNA was isolated using either alkaline lysis with phenol chloroform extraction as described [140] or using a Zymo plasmid preparation kit according to manufacturer's instructions. The pellet free Zymo kit method was preferred when plasmids were to be sequenced or electroporated, since this method gives a cleaner plasmid product. The alkaline lysis method was used when a concentrated DNA preparation was required, for example before removal of a fragment by restriction digest.

The presence of the inserted promoter fragment was verified with a small amount of the plasmid preparation by a restriction enzyme digest with *Eco*RI. A summary of restriction enzyme digestions can be found in appendix 3. *Eco*RI sites surrounding the cloning region of the TA vector result in a drop-out of the fragment after digestion giving two distinct bands on a 1% agarose gel. Plasmid preparations which displayed this fragment drop out were sequenced by the Alan Wilson Centre (AWC) in

Palmerston North using primers M13 forward and reverse, which are present on the TA cloning vector surrounding the insertion site.

2.4.4 Sequence Alignment

Chromatogram data were analysed for fidelity using Vector NTI software (Invitrogen). The sequence displayed between the M13 forward and reverse primers was then aligned with the published sequence of the candidate promoters using AlignX software (Invitrogen). It was important that these sequences were accurate since the exact location of response regulator binding site on the promoter was not always known. In some cases this process was repeated until the sequence of each promoter was identical to the published genome. Clones with correct promoter sequences were identified and used to construct GFP reporter plasmids.

2.4.5 Low Copy Number Reporter Constructs

TCS regulated promoters were cloned into low copy number mycobacterial plasmid pOT71 with *Eco*RI and *Bam*HI restriction enzymes sites. The reporter plasmids were developed based on pTKmx plasmids from Kenney *et al* [137]. These contain a kanamycin resistance cassette, mycobacterial origin of replication pAL5000, *E. coli* origin of replication pUCori (also known as oriE) and *gfpmut2* cloned from the pKEN plasmid created by Cormack et al [136] with a multiple cloning site in front of the GFP.

Plasmid DNA of the pOT71 plasmid was digested with *Eco*RI and *Bam*HI with 1 x *Bam*HI buffer overnight at 37 ^{o}C. Around 3 µL of this reaction was visualised on 1 % agarose gel to ensure a single band was obtained. Restriction enzymes were removed by column purification using a Zymo clean and concentrator kit.

Sequenced promoters were concurrently digested out of their cloning vectors using the same restriction enzymes. Restriction enzyme reactions were run through a 1% agarose gel to separate plasmid and promoter fragments. These were then excised and the DNA purified using a Zymo gel extraction kit according to the manufacturer's instructions. To ensure DNA was not lost in this process, the DNA was visualised by running a small amount of the sample on a 1% agarose gel.

Promoters were then inserted into pOT71 by incubation with T4 ligase and T4 ligase buffer at 16 ^{o}C for at least 6 hours. A small amount of *Kpn*I, which cuts between the two cloning sites (*Eco*RI and *Bam*HI), was added to the reaction to prevent unwanted uncut pOT72 from remaining in the reaction. The ligation reaction was then transformed into DH5α by heat shock as previously described and plated on LB supplemented with kan_{50}.

The primer pTKmx F1 corresponding to a region on the plasmid about 100 bp upstream of the cloning site, was used in combination with each promoter's reverse primer to screen for clones with the promoter correctly inserted into the pOT71 plasmid. This was carried out by colony PCR screen, according to the manufacturer's guidelines for using Mastermix from Bioline, except that cells from a single colony were used as template DNA, and added using a toothpick or pipette tip. The same tip was then added to a 3 mL falcon tube containing LB and kanamycin for an overnight incubation at 37 ^{o}C.

The 3 mL overnight cultures for all positive clones were used to create freezer stocks and plasmid preparations of the pOT71 + promoter constructs. These were then

sequenced using the pTKmx F primer to ensure promoters were correctly inserted in frame with GFP in the plasmid.

2.4.6 High Copy Number Reporter Constructs

While this work with the pOT71 plasmids was being carried out, Bourn *et al* published a paper on a high copy number plasmid for mycobacteria involving the use of a modified pAL5000 origin of replication which will henceforth be known as oriM high [138]. They showed a seven fold increase in plasmid copy number compared with the original pAL5000 origin of replication. A modification of 3 base pairs, causing an alanine deletion, lead to a significant increase in *gfp* expression on their pHS201 plasmid [138]. The authors kindly gifted us this plasmid to use in our work. Unfortunately their plasmid contained an *Eco*RI site in front of the GFP, which was also found in their modified oriM so it was impossible to clone promoters directly into this plasmid. A new plasmid was constructed using their modified high copy number origin of replication named pSHigh.

Initial experiments involving the pOT plasmids indicated that pOT62, containing a ribosomal binding site (RBS) upstream of the *gfp* gene, displayed a higher fluorescent intensity than the pOT71 plasmids. In order to include this RBS sequence in front of the GFP in the new plasmid pSHigh, a forward primer was designed to include this sequence in front of the GFP start codon. We reasoned that having a terminator upstream of the GFP would also improve the vector by avoiding leakage and unwanted GFP expression. The pTKmx plasmid contains a terminator site upstream of the multiple cloning site, so this plasmid was used to create the new high copy number vector. In summary, three improvements were made to optimise the potential of this plasmid as a reporter of gene expression:

- High copy number origin of replication (*oriM* high from pHS201).
- Ribosomal binding site for GFP (added to the primer sequence).
- Terminator site upstream of cloning site (trp from pTkmx).

GFP RBS insertion

The *gfpmut2* gene, as well as its RBS, was cloned from pOT11 with GFP_RBS_F and GFP_R primers by PCR using a high fidelity polymerase, Platinum Taq (Invitrogen). As described for the TCS controlled promoters in section 2.3, this gene was cloned into a TA vector, sequenced and then excised with *KpnI* and *SphI*. The pTKmx plasmid was also digested with these enzymes to replace the *xylE* component of this plasmid with the *gfpmut2* by ligation and heat shock transformation onto LB agar with kan_{50}. Screening colonies of DH5α during this step was facilitated by briefly placing the plate on a UV light box and selecting brighter colonies for the PCR screen, which increased the number of positive clones containing the pTKMx_F and Gfp_R primed amplicon. Correct orientation was verified by sequencing with pTKmx F primer.

pAL5000/ oriM high *switch*

The original origin of replication was removed from pTKmx by digestion with *Nhe*I and separated from the rest of the plasmid by gel extraction. The high copy oriM high was amplified from Bourn's pHS201 plasmid using primers oriM_F and oriM_R with a modified PCR protocol for amplification of long fragments (see Appendix 1).

The PCR product was visualised on a 1 % agarose gel and then purified using a clean and concentrator column to remove PCR enzymes before ligation into pTKmx –pAL5000. Ligation products were transformed by heat shock into DH5α and

screened using oriM-100_F and oriM-100_R primers, which are located 100 bp upstream and downstream of the insertion site. PCR products were visualised on 1% agarose and positive clones were sequenced by AWC with the oriM -100 primer set to make the plasmid pTkmx_oriM.

Ligation of Promoters

Promoters were amplified from gDNA with re designed oligonucleotides containing *Kpn*I sites on both forward and reverse primers. These were ligated and cloned into either pLUG or pCR2.1 TA cloning vectors, sequenced and cut out using *Kpn*I.

Ligations with one restriction site

A number of steps were taken to improve the efficiency of these ligations

- A much higher concentration of promoter DNA was obtained by preparation with alkaline lysis instead of using the pellet free plasmid prep kit.
- During gel extraction as much of the unstained agarose (containing no DNA) was removed before the sample was melted in the ADB buffer provided in the kit, to improve the DNA yield.
- The pSHigh plasmid was incubated with *Kpn*I for at least 15 hours and then run on 0.8% agarose to visualise the different band size in cut and uncut DNA and separate the two.
- *Kpn*I cut pSHigh was extracted from the agarose gel and then incubated with calf intestinal phosphatase (CIP) at 37 ^{o}C for 30 minutes; CIP was removed from the sample by column purification before ligation.

- A pre-ligation heat step involving incubation of plasmid and insert at 65 ^{o}C for ten minutes to break weak bonds between DNA fragments which may have formed dimers.
- A negative control containing SDW instead of the promoter DNA was included. The number of colonies on the negative control plate, containing cut plasmid and SDW rather than insertion fragment, gives an indication of the efficiency of the ligation.

2.5 Electroporation of constructs into *M. smegmatis*

Reporter constructs were transformed into *M. smegmatis* mc^{2}155 by electroporation with a Biorad gene pulser. Plasmid preparations were desalted by column purification and quantified by gel electrophoresis because unclean plasmid preparations result in the electroporator arcing. The method for preparation of electro-competent *M. smegmatis* is summarised in appendix 5. Competent cells were thawed on ice for 5 minutes before 200 µL of cold 10% glycerol was added to each 100 µL aliquot. Between 7 and 10 µL of DNA was added to the cells and glycerol, this solution was mixed by gentle pipetting and moved to a cold electroporation cuvette. Cells and DNA were then stored on ice for at least 2 hours before electroporation. Cuvettes containing cells, DNA and glycerol were pulsed at the following settings according to Mycobacteria Protocols [141]:

- Voltage 1250 V
- Capacitance 25 µF
- Resistance 1000 Ω
- Cuvette 2 mm

After electroporation, cells were allowed to recover overnight in 600 mL LB without antibiotics or shaking in eppendorf tubes and then plated on LB kan_{50} to select for plasmid containing clones. *M. smegmatis* generally takes around 3 days to appear on plates, at which point freezer stocks and overnight cultures were made.

2.5.1 Visualisation of cells

Cells were visualised under oil immersion for fluorescence using a microscope with a FITC (Fluorescein Isothiocyanate) filter. Around 500 µL of cells were removed from overnight cultures and washed with equal amounts of 1 x PBS. Cells were then spun down for 30 seconds at 12,000 rpm and 400 µL of supernatant removed before re-suspension. Around 10µL of this concentrated cell solution was used to make slides without staining.

2.6 Stress Condition Assays

2.6.1 Oxygen Starvation

2.6.1.1 Oxygen Starvation Assay with low Copy number constructs

A method for growing *M. smegmatis* in oxygen starved conditions has been previously described [62].

For oxygen starvation, HdeB minimal media was made from a stock of metal salts, 100x nitrates (N), Glycerol and 100x phosphates. 15 mL overnight cultures were incubated with agitation of *M. smegmatis* containing three different plasmid constructs:

- pOT71 (negative control plasmid containing no promoter)
- pOT71_Rv0440 (positive control promoter containing constitutively expressed hsp60 promoter)
- pOT71_Rv2031 (oxygen sensitive *hspX* promoter)

Overnight cultures were diluted to an OD_{600} of 1.0 in 50 mL HdeB minimal media with Kan_{50} and grown in 250 ml flasks with airtight rubber seals [53]. Triplicate cultures were grown at 37 ^{o}C with shaking and 1 mL samples of the culture were removed periodically with a syringe to avoid oxygen being let into the flasks. These samples were measured for OD_{600} using a spectrophotometer and fluorescence using a Wallac envision plate reader with excitation and emission set at 488 nm and 509 nm respectively.

2.6.1.2 Optimised Oxygen Starvation Assay

Smaller 15 mL flasks were used with 13 mL of cells and media using up more of the total volume. A number of identical flasks were set up so that different cultures could be used to measure OD and F at different time points, without letting air in. Cells in this assay were grown in rich media instead of HdeB and a final concentration of 1.5 µg/µL of methylene blue was added to a fourth tube.

The loss of blue colour in these flasks over 4 days indicated that the level of oxygen was being depleted. In addition, the newly made high copy number plasmid pSHigh was used.

2.6.2 High throughput stress conditions

Overnight cultures of each reporter construct were grown and then diluted to an OD_{600} of 0.2. 50 µL of cells were added to equal volumes of media in a 96 wp, giving a final cell turbidity of OD_{600} 0.1. The 96 wps were set up with a gradient of each stress condition as summarised in figure 2.1 and table 2.3, each plate containing different reporter construct.

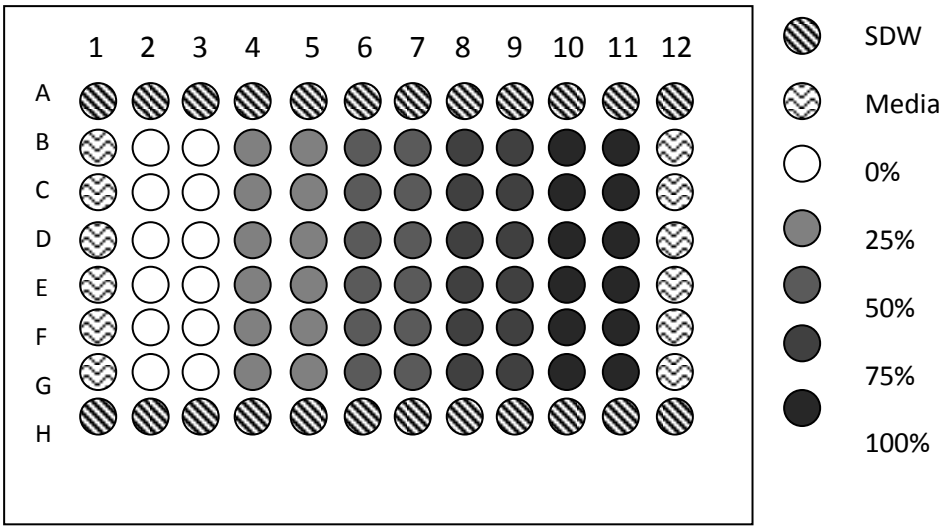


Figure 2.1: 96 wp set up for stress condition assays*. Each promoter construct was tested for changes in expression in response to a range of stress factors, displayed in table 2.3. SDW and media in the outside wells prevented evaporation and provided a media control to establish contamination and background fluorescence.*

Stress	Final Concentration					Media
	0%	**25%**	**50%**	**75%**	**100%**	
Chelators (mM):						HdeB
EDTA	0	0.25	0.5	0.75	1	
EGTA	0	0.25	0.5	0.75	1	
EDDA	0	0.25	0.5	0.75	1	
Antibiotics (µM)						LB
SM	0	0.19	0.39	0.78	1.56	
TC	0	0.1	0.2	0.35	0.75	
RIF	0	0.39	0.78	1.56	3.125	
INH	0	3.125	6.25	12.5	25	
Nutrient Starvation (mLs see appendix 7)						HdeB
Carbon	6	8	12	16	20	
Nitrogen	0.1	2.5	5	7.5	10	
Phosphate	0.1	2.5	5	7.5	10	
Others (%)						LB
EtOH	0	2	3	4	5	
SDS	0	0.02	0.03	0.04	0.05	

Table 2.3: Stress Conditions.

2.6.2.1 Nutrient starvation

15mL overnight cultures of *M. smegmatis* containing the three TCS controlled promoters (*phoA, hspX* and *MtrA*) as well as the negative control pSHigh construct were grown at 37^{o}C with agitation in HdeB minimal media. These were then diluted to an OD_{600} of 0.1 in a range of 5 different media starvation conditions (0%, 25%, 50%, 75% and 100% of each nutrient) and transferred to a 96 wp, sealed and incubated at 37 ^{o}C with agitation for 48 hours. Plates were then removed and read for OD and F. A typical 96 wp set up is displayed in figure 2.2. Column 1 of the 96 wp was filled with SDW to prevent evaporation in other wells and column 12 was used for media controls.

LB was used to grow 15 mL overnight starter cultures, with the exception of the phosphate starvation and chelator experiments for which HdeB minimal media was used. These were then diluted to an OD_{600} of 0.2, transferred to 96 wps, sealed and incubated with agitation at 37 ^{o}C for 48 hours.

2.6.2.2 Antibiotic stress

Sub-inhibitory concentrations of 4 antibiotics were tested in this manner namely, RIF, INH, SM and tetracycline. The concentration of each antibiotic ranged from half the minimum inhibitory concentration (MIC) of each drug in *M. smegmatis* so that the antibiotics would not inhibit growth of the cells in the assay. Table 2.5 indicates final concentrations in LB used in this experiment for each antibiotic.

2.6.2.3 Chelators

A number of commercially available chelators from Sigma Aldrich were used in this experiment to starve the media of cations; EDTA (Ethylene Diamine Tetraacetic Acid), EGTA (Ethylene Glycol Tetraacetic Acid) and EDDA (Ethylene Diamine-N,N'-Diacetic Acid) all of which quench media of metal ions. Up to 1 mM of each chelator was added to the media in a 96 wp before cells were added. For final concentrations used see table 2.3.

2.6.2.4 Additional Stressors

Cells were grown to mid exponential phase in LB and then transferred to three 96 wps where they incubated for half an hour at 4 ^{o}C, 37 ^{o}C and 45 ^{o}C without shaking. Plates were then returned to 37^{o}C for half an hour before being read for OD and F. Other stress conditions include low levels of SDS (0 – 0.05 %) and ethanol (0-5 %) in the LB.

2.6.3 Validation of Expression Conditions

Initial findings from the 96 wp assays were repeated with an increased culture volume of 50 mL in 250 mL flasks. Triplicate overnight cultures were inoculated and diluted in the same way, then incubated with agitation at 37^{o}C for 48 hours before OD and F were measured.

2.7 Screening For TCS Inhibitors

2.7.1 Screening Compound Libraries

Overnight cultures of *M. smegmatis* containing pShigh_*mtrA* were grown in 15mL rich media with agitation and then diluted to an OD_{600} of 0.2. An appropriate number of 96 wps for each library were dispensed with 50 µL of rich media supplemented with Kan_{50}, 0.1 % $Tween_{80}$ and D-$arabinose_{100}$ in each well excluding one column of SDW to prevent evaporation, as shown in figure 2.6. Using a CyBio liquid handler, 1 µL of each compound, from a 1 mM stock dissolved in DMSO, was dispensed to 50 µL of media giving a final concentration of 20 µM of each compound in a final volume of 100 µL, after the addition of cells. Diluted cells were aliquotted to each well giving an optical density of 0.1 at 600 nm. Controls were added to column 12 including cells in rich media with and without DMSO as well as two antibiotic controls usually rifampin and capreomycin to show background media fluorescence.

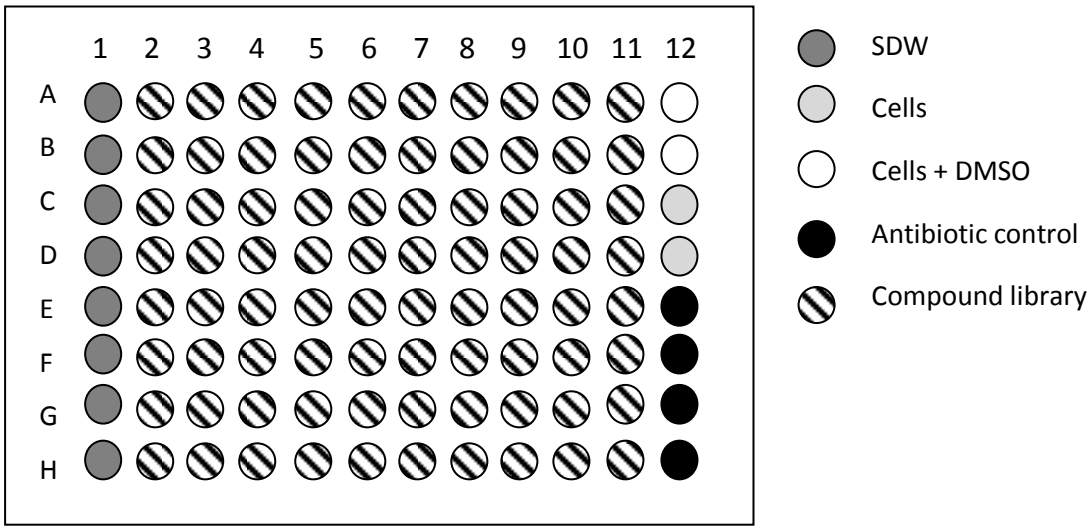


Figure 2.2: 96 well plate set up for compound library screening. *Compounds were transferred by a liquid handler from stock solutions to 96 wps. Every well represents a different compound, except for the outside wells which contain water, on the left and controls on the right*.

Plates were then sealed with plate seals and parafilm and wrapped in gladwrap before incubation at 37^{o}C with shaking for 96 hours. Plates were read by a Wallac Envision plate reader for OD_{580} and F (excitation 488 nm and emission 509 nm).

The *phoA* promoter driven expression was also tested in a similar way except that minimal media was used, supplemented with Kan_{50} and 0.5 mM EDTA. For these plates an additional control of promoterless pShigh was added, instead of having 4 antibiotic controls.

2.7.2 Palmitoleic and Oleic Acid Serial Dilutions

Three 2 x dilutions of each of the fatty acids and a solvent control of ethanol were set up in rich media starting from a final concentration of 50 µM. An ethanol solvent control was included and these values are shown in the results as 0 µM of each type of fatty acid. Cells with an initial OD_{600} of 0.1 were added to this media, including strains containing the plasmids pSHigh, pSHigh_mtrA and pSHigh_hsp60. These were incubated and measured for F and OD as described in HTP screen.

CHAPTER THREE:

Construction of a GFP reporter plasmid

3.1 Introduction

In order to screen for potential TCS inhibitors, this study required a method of determining the level of TCS controlled gene expression under different stress conditions, which could be modified to a 96 wp format for high throughput screening. The green fluorescent protein has been used for over a decade, as a reporter of gene expression [133] in a number of organisms including mycobacteria [134]. By inserting the promoter sequence of a gene of interest in front of *gfp* on a plasmid, the amount of protein expressed under the control of this promoter will be indicative of promoter activation, which can be measured by the fluorescence intensity.

Since its isolation from *A. victoria,* GFP has been engineered to suit a number of applications across many fields in biology. A number of enhanced variants, known as eGFPs, were developed by Cormack *et al* in order to increase the signal strength and stability of the protein [136]. Taking a rational engineering approach, they used site directed mutagenesis to vary the 20 amino acids surrounding the central chromophore and screened for improved signal strength with fluorescence activated cell sorting (FACS). The result was GFPmut2, with a 100 fold increase in fluorescence compared with wild type GFP and a red shift in its natural excitation and emission spectra from 395 nm to 488 nm, making it more compatible with techniques such as FACS and FITC filtration [136].

In this study GFPmut2 was chosen as a reporter over other methods, such as *xylE* expression, for a number of reasons. Firstly, as GFP is activated by light of a specific wavelength there is no substrate required, which can interfere with other cellular processes. Secondly, the intensity of a fluorescent signal can be easily measured using a FITC filter under a microscope, and quantified in a 96 wp fluorescence

reader. Finally, this reporter is well established as a measure of gene expression in mycobacteria [134].

A number of mycobacterial promoters are constitutively expressed and induce protein formation all the time; these are sometimes known as “housekeeping” genes. GFP expression under the control of the constitutively expressed promoter from the *hsp60* gene, for example, gives off relatively high levels of fluorescence. Many others, such as the TCS-controlled genes which were selected in this study, induce GFP expression under certain conditions, but never at the same level as the *hsp60* promoter. In order to monitor relatively small changes in gene expression as a result of stress conditions, a very sensitive reporter system was required. The GFPmut2 variant has been well adapted to measure bacterial gene expression; however another means of improving signal strength is to increase the number of plasmids per cell.

Bourn *et al* showed that a modification to the pAL5000 mycobacterial origin of replication on a plasmid resulted in a seven fold increase in plasmid copy number per cell [138]. In this chapter, the development of the new plasmid pSHigh containing *gfpmut2* and the modified pAL5000, will be described. Its relative fluorescence will be established and compared with that of low copy number plasmids containing the same GFPmut2 under the control of the *hsp60* promoter. Having demonstrated an increase in signal strength, the *phoA, hspX* and *mtrA* promoters from *M. smegmatis* were amplified and used to replace the *hsp60* promoter for further analysis of the TCS they control.

3.2 Results

Construction of the reporter plasmid pSHigh involved a number of steps [142]. An overview of the process in shown in figure 3.1

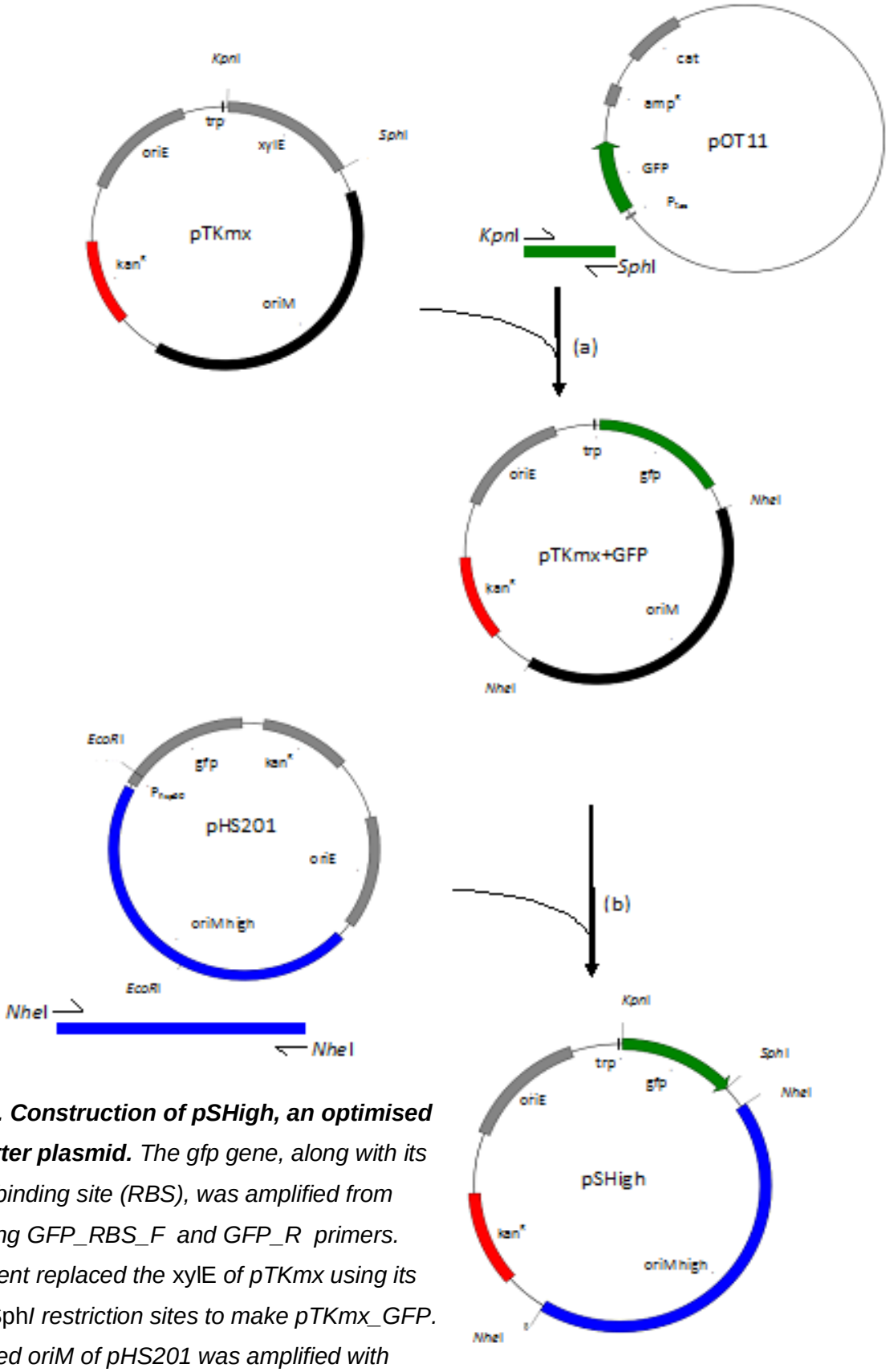


Figure 3.1. Construction of pSHigh, an optimised GFP reporter plasmid. *The gfp gene, along with its ribosomal binding site (RBS), was amplified from pOT11 using GFP_RBS_F and GFP_R primers. This fragment replaced the* xylE *of pTKmx using its* KpnI *and* SphI *restriction sites to make pTKmx_GFP. The modified oriM of pHS201 was amplified with oriM_F and oriM_R primers and this modified origin of replication replaced the oriM region of pTKmx_GFP using the surrounding* NheI *sites.*

3.2.1 Amplification of GFPmut2 and OriM by PCR

The optimised reporter plasmid pSHigh was developed by cloning a new origin of replication oriM high and *gfpmut2* into a mycobacterial plasmid pTKmx created by Kenney and Churchwood [137]. Figure 3.2 shows the PCR products of the amplification of *gfpmut2* from pOT11 and oriM high from pHS201 on 1 % agarose. A gradient PCR showed that the optimal annealing temperature for cloning the oriM high fragment was 63 ^{o}C, as shown in figure 3.2 (a).

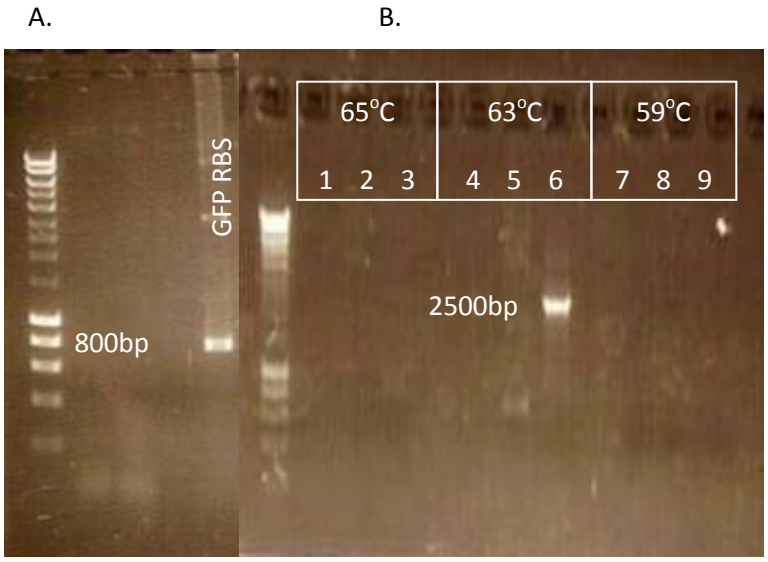


Figure 3.2: Visualisation of* gfpmut2 *and oriM high amplified PCR products. *A)* gfpmut2 *along with its RBS was amplified by PCR using pOT11 as a template, giving an 800bp product on the agarose gel. B) OriM was amplified effectively from pHS201 using an annealing temperature of 63^{o}C, giving a 2500 bp product shown in the sixth lane.*

3.2.2 Switching *xylE* and pAL5000 with GFPmut2 and oriM high in pTKmx

The mycobacterial plasmid pTKmx was used as the backbone for pSHigh. Kenney *et al* used this plasmid to monitor promoter activity with the *xylE* reporter system [137], which we replaced with *gfpmut2*. OriM high was substituted for pAL5000 to increase the copy number of the plasmid per cell. Figure 3.3 illustrates how this plasmid was cut with restriction enzymes *Kpn*I and *Sph*I to remove *xylE* and *Nhe*I to remove pAL5000. The white boxes on the gel show the bands which were removed and gel purified, pTKmx – xylE and pTKmx –pAL5000.

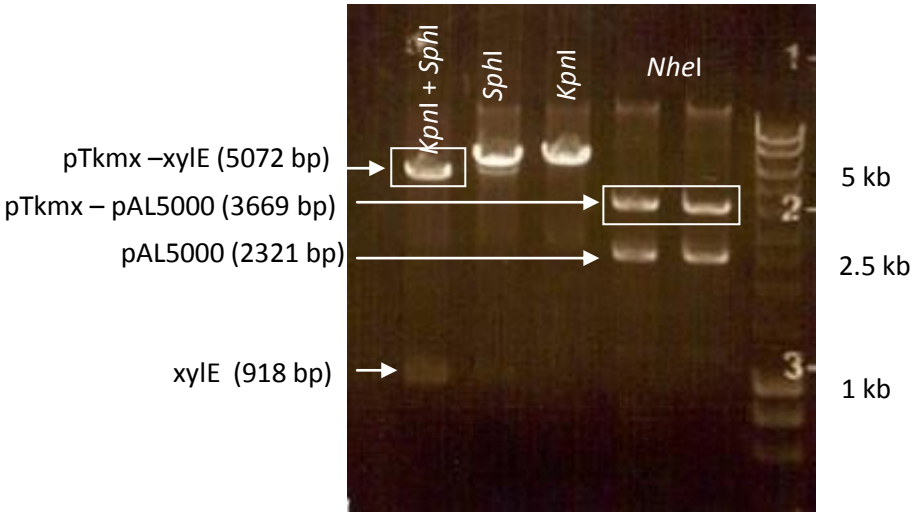
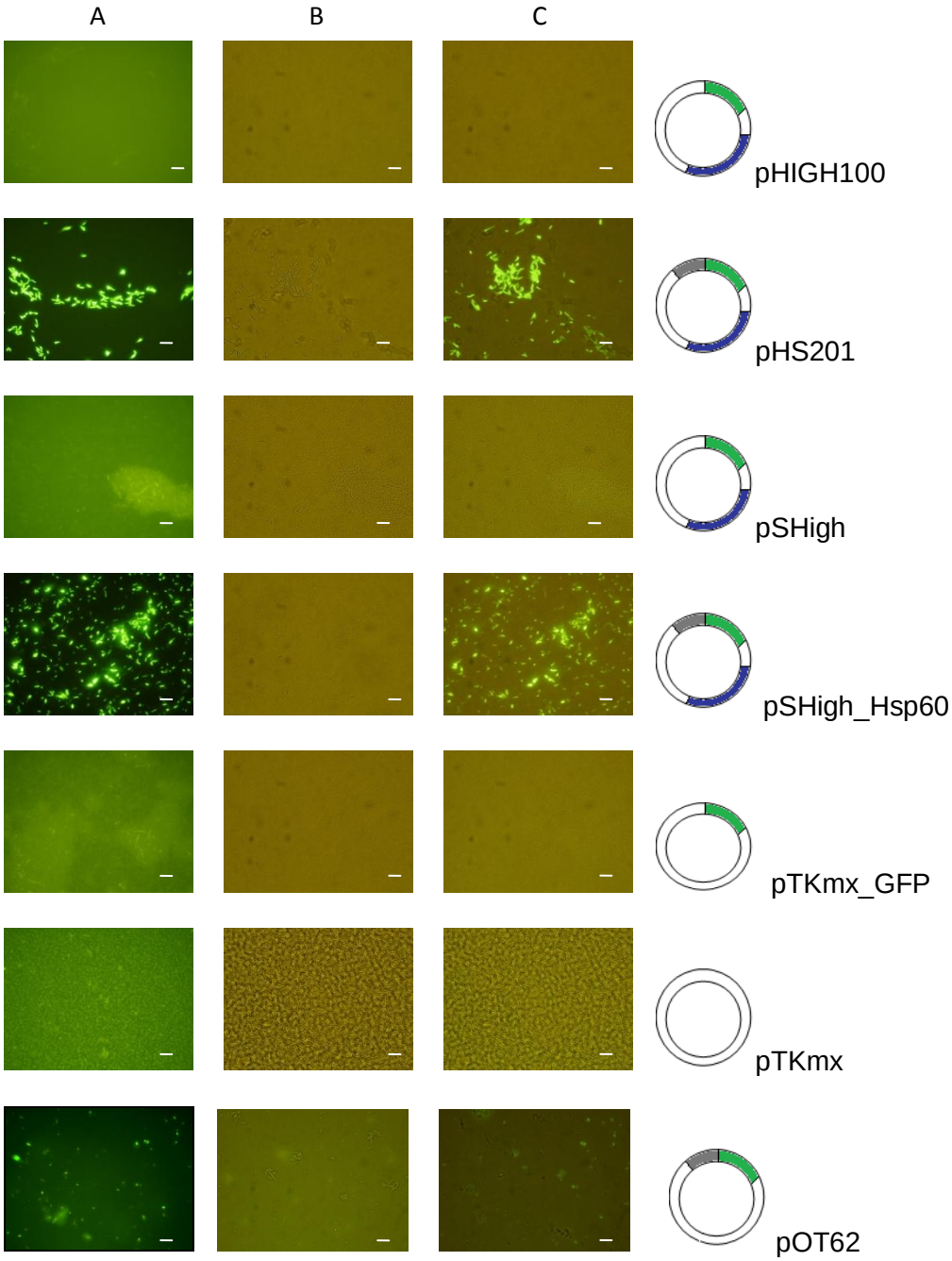
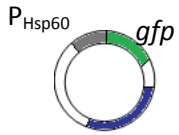


***Figure 3.3: Removal of* xylE *and pAL5000 from pTKmx by restriction enzyme digest**. KpnI and SphI were used to remove the* xylE *fragment and NheI to remove pAL5000 from pTKmx giving band sizes corresponding to the size of these fragments in relation to the plasmid These fragments were then replaced with* gfpmut2 *and oriM high to make pSHigh*

3.2.3 Comparing the Fluorescence of high and low copy number plasmids in *M. smegmatis*

The fluorescence of overnight cultures of *M. smegmatis* containing high and low copy number plasmids were compared on a single cell basis, using a microscope with a FITC filter, and on a population basis, using a 96 wp fluorescence reader. In figure 3.4 *M. smegmatis* cells containing different plasmids are displayed along with a map of essential elements contained on each plasmid. The high copy number plasmids, pHS201 and pSHigh_hsp60 display a much higher level of fluorescence compared with pOT62, with exactly the same GFPmut2 and RBS sequence as pSHigh_Hsp60. Images of the pHIGH100 and pSHigh plasmids show little fluorescence indicating that GFP expression is a result of the *hsp60* promoter induction. The pSHigh reporter constructs were used over the pHS201 and pHIGH100 plasmids because they contained a unique cloning site directly upstream of the *gfp* gene and the trp terminator site from pTKmx. The cloning site allowed for the insertion of other promoters upstream of the *gfp* gene. It was hoped that the terminator site would reduce any background fluorescence from the *gfp* gene.





OriM (high copy number)

Figure 3.4: Visualisation of reporter constructs in M. smegmatis.

Cells containing plasmids used in this study were visualised for gfp expression using a blue light with a FITC filter (475 nm) (column A), white light (column B) and both (column C). Cells containing the high copy number origin of replication shown on plasmids maps in blue were significantly more fluorescence than those with the original pAL5000 origin. Low level fluorescence of the promoterless plasmids indicates no significant leakage of GFP expression. Scale bar shown in white on all photographs represents 2 µm.

To explore whether the dynamic range of GFP expression was increased in a population of transformed cells we measured the fluorescence in relation to OD of the cultures. As figure 3.5 shows, the level of fluorescence in the high copy number plasmids pHS201 and pSHigh_Hsp60 is significantly higher than that of pOT62. The low level of fluorescence from pTKmx indicates that a small amount of fluorescence is picked up from cells without any GFP expression.

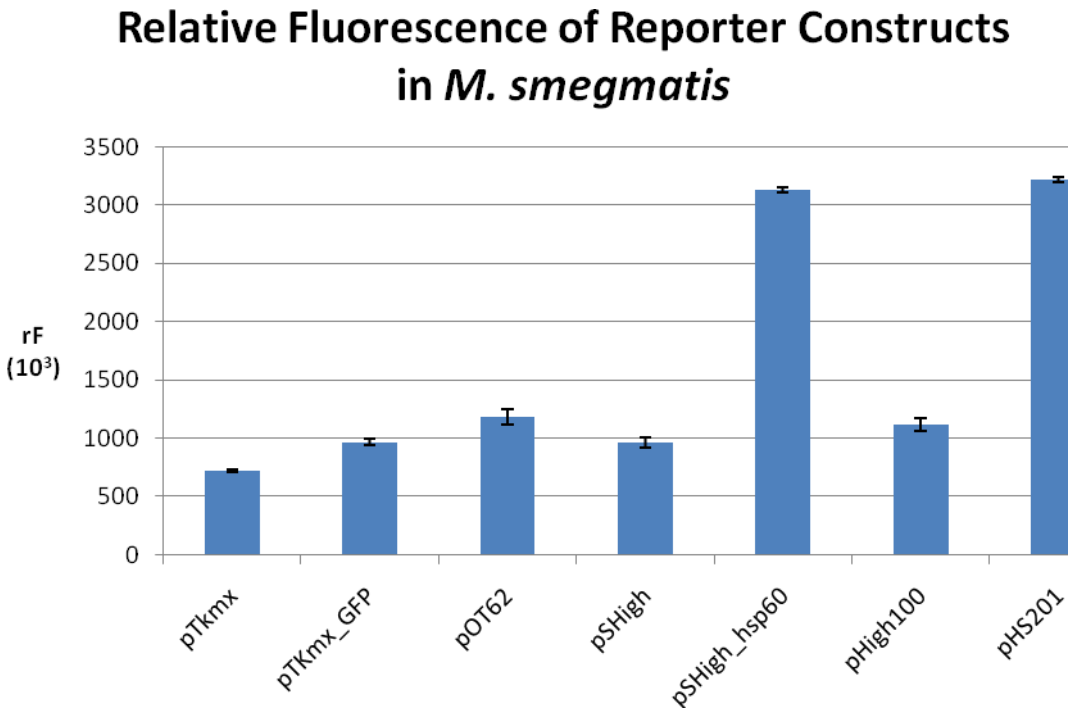


***Figure 3.5: Relative Fluorescence of Reporter plasmids in* M. smegmatis.** *High copy number plasmids pHS201 and pShigh_hsp60 display significantly higher levels of fluorescence than their promoterless counter-parts pHIGH100 and pSHigh. pOT62 containing the same promoter, RBS and gfp sequence as pSHigh_Hsp60 gives off less fluorescence, presumably as a result of the number of plasmids per cell. Error bars indicate the standard deviation between triplicate cultures.*

In order to calculate the level of GFP expression the *hsp60* promoter conferred to a cell, the readings were normalised to show the fluorescence in relation to OD minus the background fluorescence given off by normal cells, as indicated by pTKmx.

Table 3.1 displays the calculated normalised fluorescence and the fold induction as a result of the *hsp60* promoter.

	Normalised promoterless *gfp* expression	**Normalised P_{hsp60} driven *gfp* expression**	**Fold induction**
Low copy number pOT62, pTkmx_GFP	245783.2	731867.7	3
High Copy number pHS201, pHIGH100	398280.5	2500922	6.3
High Copy number pSHigh_Hsp60, pSHigh	241118.2	2412564	10.0

Table 3.1: Normalised fluorescence of high and low copy number plasmids.

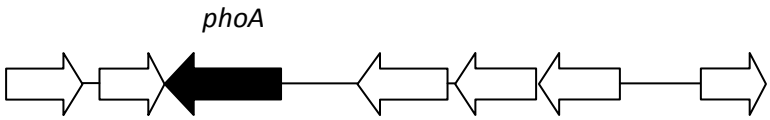
3.3 Inserting TCS controlled gene promoters into pSHigh

A literature search for TCS controlled genes in mycobacteria revealed a number of candidates. These were narrowed down to three *M. smegmatis* genes with non coding sequences upstream which could be amplified from genomic DNA and cloned into the pSHigh plasmid. A number of *M. tuberculosis* promoters were also amplified for future work in *M. bovis* BCG. Table 3.2 shows known TCS controlled genes from *M. smegmatis* and *M. tuberculosis* and what conditions are proposed for their induction.

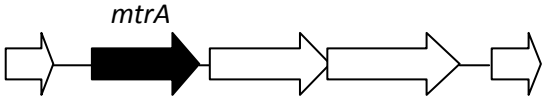
Gene name	TCS	Conditions of expression	Function if known
Current Work			
MSMEG_3932 (*hspX*)	DevS/DevR	Oxygen starvation, nitric oxide stress	Induction of dormancy regulon [62, 63]
MSMEG_1872* (*mtrA*)	MtrB/ MtrA	Possibly iron starvation	Macrophage infection [101]
MSMEG_1012 (*phoA*)	SenX3/ RegX3	Phosphate starvation	Phosphate transport and metabolism [108]
Rv0440 (GroEL2/Hsp60)	-	Constitutively expressed positive control	
Future Work in *Mycobacterium tuberculosis*			
Rv2031 (*hspX*)	DevS/DevR	Oxygen starvation, nitric oxide stress, SDS	Induction of dormancy regulon [143]
Rv1221 (*sigE*)	MprB/MprA	heat shock, tissue specific ligands?	Host inflammatory response [89, 144] intracellular persistence [145]
Rv2396	PhoP/PhoR	Low magnesium and other cations [89]	Complex lipid biosynthesis [87, 146]
Rv1033 (*tcrR*)*	TrcR/TcrS	unknown	
Rv0903 (*prrA*)*	PrrB/ PrrA	unknown	Early intracellular growth[86, 147]
Rv3246 (*mtrA*)*	MtrB/ MtrA	Possibly iron starvation [71]	Intramacrophage replication [148]

Table 3.2. Two Component system controlled genes cloned for this study and future work. ** indicates autoregulated response regulators of the TCS.*

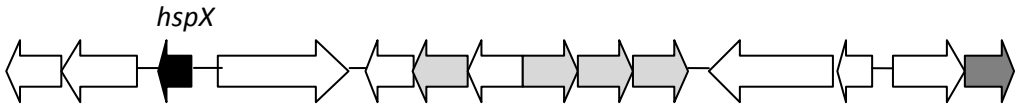
Three selected *M. smegmatis* genes, *hspx, mtrA* and *phoA,* were used in this study. The gene organisation and function of surrounding genes is described in figure 3.6. In order to use these genes in a promoter reporter plasmid it was important that a defined non-coding region directly upstream could be demonstrated for each.



Gene Name	Function
MSMEG_1010	Transcriptional regulator, TetR family
MSMEG_1011	Short chain dehydrogenase
MSMEG_1012	*phoA*: Alkaline phosphatase
MSMEG_1013	Hypothetical protein
MSMEG_1014	DNA polymerase IV
MSMEG_1015	Transcriptional regulator, TetR family
MSMEG_1016	Secreted protein



Gene Name	Function
MSMEG_1873	Thymidylate kinase
MSMEG_1874	*mtrA*: Two component system response regulator
MSMEG_1875	*mtrB*: Two component system histidine kinase, OmpR family
MSMEG_1876	Lipoprotein
MSMEG_177	Hypothetical protein



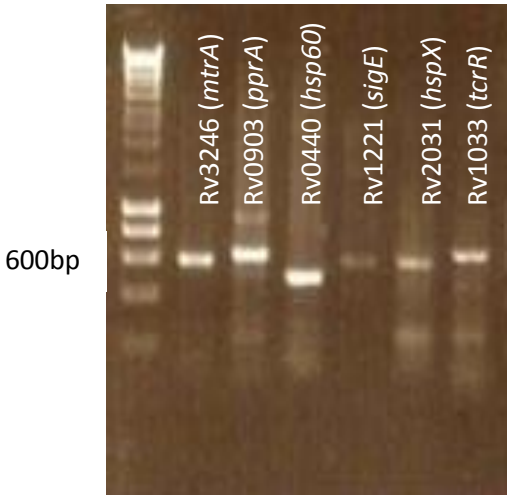
Gene Name	Function
MSMEG_3926 to MSMEG_3931	Subunits of hydrogenase enzyme
MSMEG_3932	*hspX* homologue: 14kD antigen
MSMEG_3933	Phophophenolpyruvate synthase
MSMEG_3934, 3935 and 3937	Hypothetical protein
MSMEG_3936, 3938, 3939, 3940	Universal stress proteins
MSMEG_3941	GAF family protein
MSMEG_3942	Hypothetical protein
MSMEG_3943	Hypothetical protein
MSMEG_3944	*DevR*: Two component system response regulator DevR

Figure 3.6 Gene organisation and function of selected TCS controlled genes. *All selected genes possess non-coding regions directly upstream of their start codon. The genes surrounding these are named and labelled in the order that they appear from left to right. This information was downloaded from the website www.genome.jp.*

3.3.1 Promoter Amplification by PCR from Genomic DNA

Promoter sequences upstream of each gene were amplified from *M. tuberculosis* and *M. smegmatis* genomic DNA to give fragments of approximately 600 bp. Figure 3.7 shows the 1 % agarose gels of the promoters amplified by PCR.

A.



B.

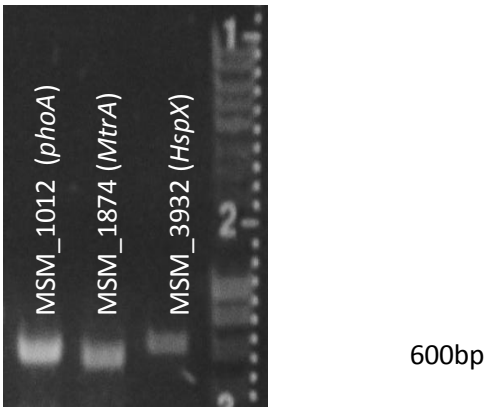


Figure 3.7: Promoters amplified from M. smegmatis and M. tuberculosis gDNA. *A) displays five TCS controlled gene promoters of around 600bp in size, as well as the constitutively expressed hsp60 promoter amplified from M. tuberculosis genomic DNA. In B) three bands represent M. smegmatis promoters from selected TCS controlled genes.*

3.3.2 Ligation of promoters into pSHigh

In order to improve the efficiency of the ligation of promoters into the reporter plasmid, the *Kpn*I cut pSHigh was gel extracted to reduce the amount of uncut plasmid DNA during each ligation reaction. Figure 3.8 shows the pSHigh plasmid in its uncut state and after digestion with *Kpn*I, the band removed from the gel is highlighted by a white box.

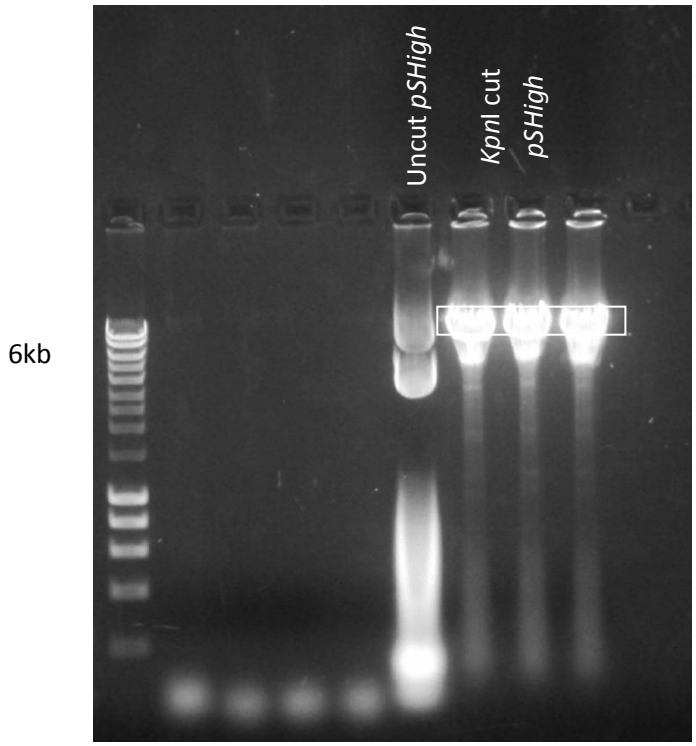


Figure 3.8: KpnI cut and uncut pSHigh plasmid*. The sixth lane shows coiled formations of the pSHigh plasmid in its uncut state. The three following lanes display pSHigh after being cut with KpnI. The cut plasmid DNA, shown in a box, was removed by gel extraction.*

3.4 Discussion

3.4.1 Optimisation of the reporter plasmid

The principal aim of this work was to generate a cell based screen for TCS inhibitors. This required a highly sensitive reporter system which could be manipulated to identify small changes in gene expression as a result of TCS-specific conditions. The results of section 3.2.3 clearly show that pSHigh_Hsp60 is a highly fluorescent reporter construct and this is a result of the change in the origin of replication of the plasmid from pAL5000 to oriM high. Only a limited amount of background fluorescence is produced from the promoterless pSHigh plasmid, when compared with the pTKmx strain, indicating the plasmid does not express the protein independently of an adjacent promoter sequence upstream of *gfpmut2*. The plasmid also contains a kanamycin resistance cassette for antibiotic selection and an *E. coli* origin of replication oriE. This pSHigh_hsp60 plasmid was used as a constitutively expressed control in this study, and has also been used for screening compound libraries against mycobacteria. The plasmid displays high levels of measurable fluorescence which can also indicate cell viability when OD readings cannot be used due to interference by coloured compounds.

The authors who created the pHS201 vector describe a significant increase in plasmid copy number from around five per cell to between 32 and 64 copies of the same plasmid [138]. The advantage of the pSHigh plasmid over the pHS201 vector is its unique *Kpn*I restriction site, directly upstream from the *gfpmut2* gene, allowing other promoters to be cloned in frame with the ribosomal binding site to monitor gene expression. In addition the trpA terminator sequence from pTKmx, located

downstream of this cloning site lead to a lower level of background fluorescence from the promoterless plasmid control.

When using a reporter to monitor gene expression, a number of assumptions are made. Firstly, any changes in fluorescence demonstrated are a result of activation or repression of a particular promoter. Therefore any post translational modification to proteins in a cell which are not directly affected by activation or repression of that promoter will not be demonstrated by the reporter system. Fortunately, the three promoters selected for this work have been previously studied and RR promoter binding sites have been demonstrated. This type of method may not be suitable for other target genes if their protein concentration or activity within the cell is controlled by some mechanism other than promoter activation.

One also assumes that the number of plasmids per cell remains relatively consistent between cultures. Measuring the fluorescence of a cell indicates the amount of protein expressed therein and is dependent on the number of plasmids present. We calculate the relative fluorescence (rF) as a measure of fluorescence divided by the OD of a culture of cells, to generate a number which is relative to the number of cells present, but we cannot account for the number of plasmids in each cell and assume that this remains relatively constant.

Another concern with this methodology is the effect a reporter may have on cellular function. The use of GFPmut2 has been established in a number of species and no significant adverse reactions to the cell have been noted. It is shown in figure 3.3 that cells expressing GFP appear normal and seem as healthy as those not expressing the protein. The benefits of this reporter over substrate based systems such as *xylE* are clear in this regard, although some work has indicated that

exposure to certain wavelengths of light can cause photo bleaching of the protein, reducing its fluorescence [149].

3.4.2 Selection of TCS promoters

As not all TCS controlled genes have had their promoters sequences published, it was important that a substantial intergenic region could be identified directly upstream of selected genes for RR binding. It is known that certain genes are controlled as part of an operon rather than direct binding at each one's promoter site, which meant some of the TCSs could not be included. Figure 3.5 indicates that genes selected for this study all qualify in this regard. We included genes such as *HspX* and *PhoA*, whose promoter binding sites are known [108, 150]. *MtrA* is an auto-regulated gene, in that induction of this TCS confers expression of its own response regulator [89].

The importance of these TCS in terms of dormancy (*hspX*), phosphate starvation (*phoA*) and survival in the macrophage (*mtrA*) has been discussed in some detail in the opening chapter. The conditions for *hspX* and *phoA* induction have been demonstrated in previous work, but the control of the *mtrA* gene is something of a mystery despite its proposed essentiality in *M. tuberculosis.* The idea of using the *hspX* and *mtrA* promoters as drug targets came from the fact that their *M. tuberculosis* homologues are involved in signal transduction mechanisms which are vital to mycobacterial survival and latency. *PhoA, o*n the other hand is absent in *M. tuberculosis*. Its established induction during phosphate starvation in *M. smegmatis* and *E. coli*, however, make it an attractive target in terms of screening for a broad class of TCS inhibitors which affect a conserved part of the phosphorylation transfer and may inhibit a number of TCS mycobacteria and other species of bacteria.

CHAPTER FOUR:

Conditions of TCS Regulated Gene Expression

4.1 Introduction

The basis of TCS signal transduction relies on the recognition of some environmental or internal change in conditions by the sensor kinase, leading to a congruent change in gene expression. We aimed to imitate *in vivo* stress conditions with a number of *in vitro* stressors in order to determine any changes in the expression of the TCS regulated genes.

Our assumption is that during macrophage infection and granuloma-induced dormancy the AFB is subject to considerable environmental stress; from the host immune response, nutrient starvation and a limited supply of oxygen. Although it is difficult to accurately reproduce the whole spectrum of these conditions *in vitro*, studies of the granuloma have suggested a number of states which can be simulated. Importantly, AFB are often found in locations within the granuloma which exhibit low oxygen tension in relevant animal models of the disease [116]. Despite the fact that their prominent site of infection is the lung, the AFB survive for extended periods of time in a relatively hypoxic environment.

Another key feature of the granuloma is the fact that it is made up of and constantly surrounded by host immune cells. These cells secrete chemokines, cytokines and nitric oxide products intended to rid the host of pathogenic bacilli. The host response mounted to counter a mycobacterial infection will differ from person to person, however, some of these hostile host responses can be imitated *in vitro.*. Heat and cold shock, changes in pH, addition of low levels of SDS, ethanol or antibiotics to media at low levels are predicted to activate cellular stress responses which may induce the expression of TCS controlled genes involved in macrophage or granuloma survival.

Finally, the use of HdeB minimal media can effectively control the level of particular nutrients available to cells. This media can be modified to alter the concentration of available carbon, nitrogen and phosphate. In addition to the direct removal of elements from the media, chelators can be used to remove cations such as calcium, magnesium and iron.

By measuring the effect of these stress conditions on TCS controlled gene expression we can elucidate the possible activators of these systems. As a proof of principal, *hspX* expression was measured during oxygen starvation, a known activator of the DevS/ DevR TCS dormancy response. This was followed by a number of other stress conditions described in the materials and methods section, which were applied to all three TCS controlled promoters *phoA*, *mtrA* and *hspX*.

The remainder of this chapter will describe the significant findings on the influence of stress condition on TCS regulated gene expression.

4.2 Results

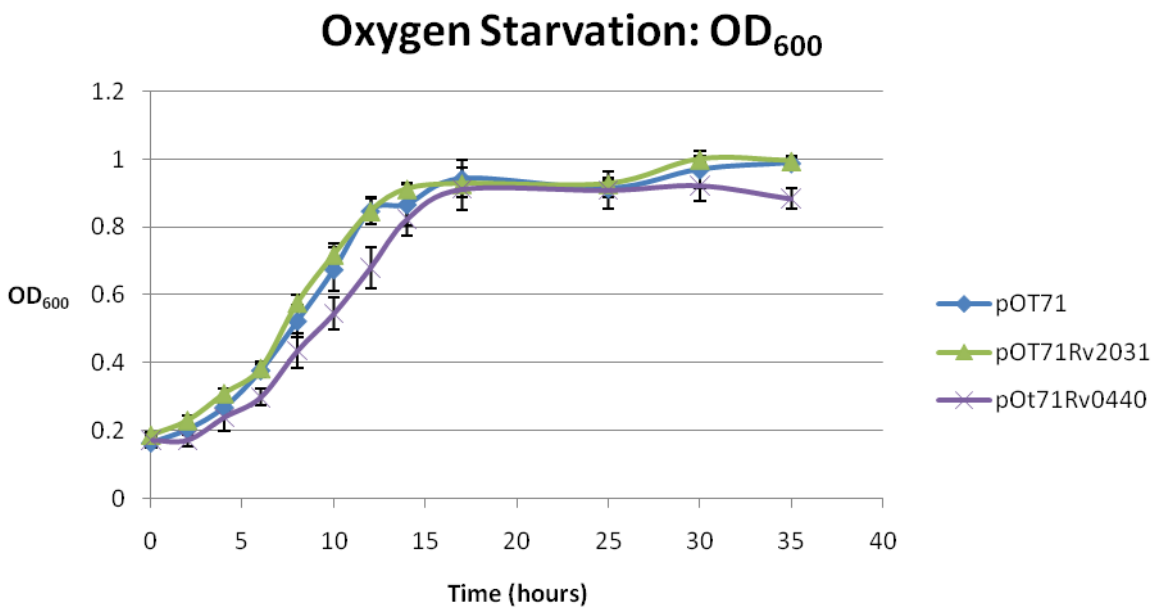
Cells containing GFP reporter constructs were exposed to a number of stress conditions throughout this study. For simplicity, only those with a reproducible outcome will be described here.

4.2.1 Expression of the DevS/DevR dependent *hspX* promoter

The DevS/DevR TCS is known to promote the expression of *hspX* under conditions of oxygen starvation and nitric oxide stress [143, 151]. Other authors have shown an increase in *hspX* mRNA during growth in media with 5% ethanol and to a lesser extent hydrogen peroxide [152].

In order to demonstrate that the *hspX* reporter plasmid was oxygen dependent two oxygen starvation assays were carried out. The first was using a low copy number pOT71 plasmid and a method described in a previous publication [62], shown in figure 4.1. After 35 hours, the *hspX*-driven expression increased to a level that was higher than that of the promoterless pSHigh plasmid. The positive control, *hsp60* driven gene expression, is generally much higher and is variable over different time points.

A



B

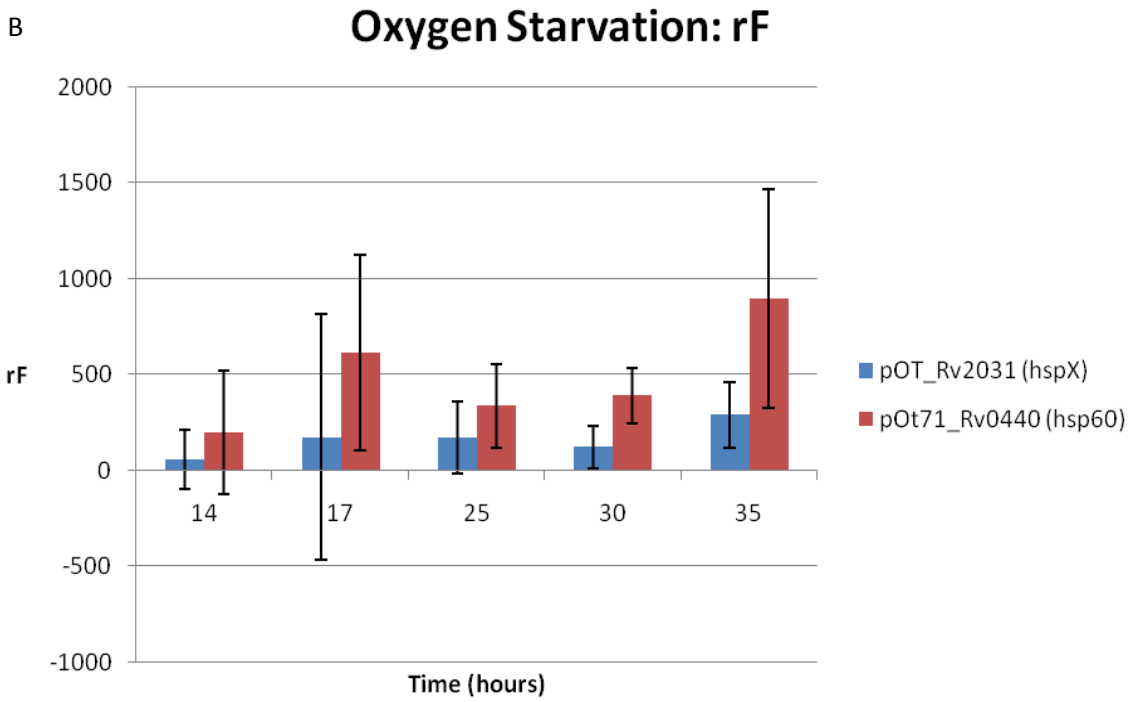


Figure 4.1:* HspX *expression during oxygen starvation using low copy number plasmids. *In figure A) the OD is shown over 35 hours of growth. When cultures reached stationary phase, at around 14 hours, cells were measured for fluorescence as shown in B).Only at 35 hours is there an increase in* hspX *expression. Error bars indicate the standard deviation between triplicate cultures.*

Another means of oxygen starvation was achieved based on a number of other methods used in current literature [56, 153]. We reasoned that there were a number of problems with the original assay and made appropriate alterations to our method.

Smaller flasks with a larger volume of media were used to reduce the amount of air in each culture to start with. Another alteration to the original method was the use of rich rather than minimal media. We argued that cells are less likely to reach stationary phase as a result of some limiting factor other than oxygen, if they are living in rich media.

Using an optimised oxygen starvation method and the improved high copy number pSHigh plasmid (amongst other changes to this method described in section 2.7), a higher level of fluorescence was produced (figure 4.2). A number of changes were made to this assay to increase the stringency of oxygen starvation and observe a higher level of GFP expression under the control of the *hspX* promoter after 4 days. Contrasting these results with figure 4.1 indicates that a combination of the method of oxygen starvation and choice of plasmid constructs results in higher *hspX* promoter induction after 4 days.

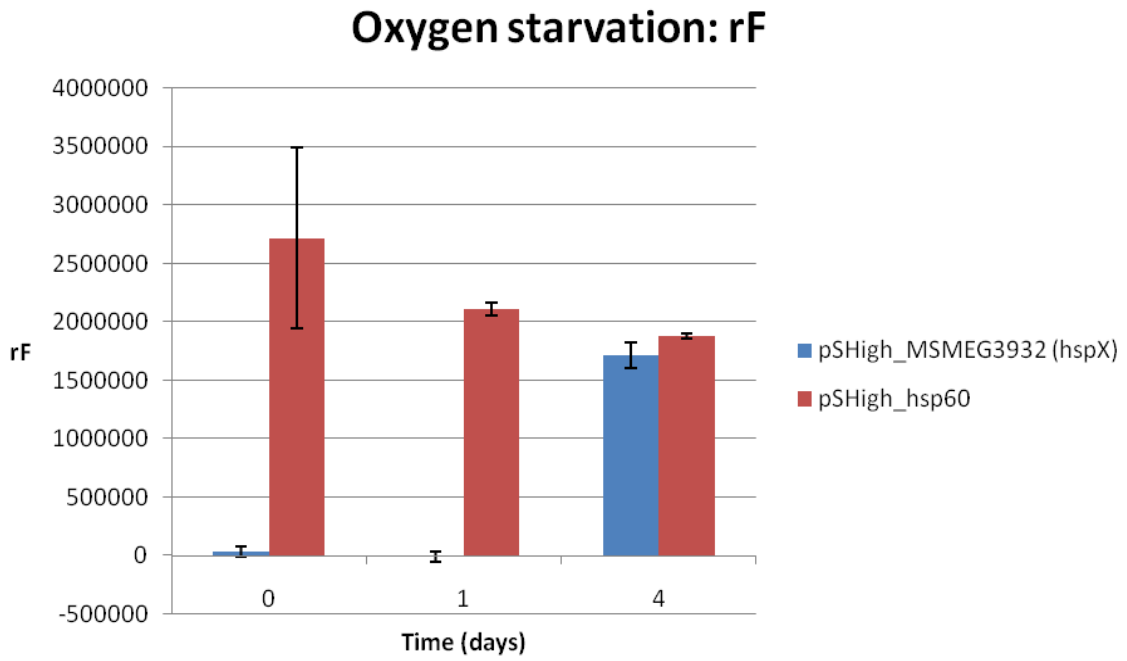


Figure 4.2:* HspX *expression using an optimised oxygen starvation method and high copy number plasmids. *An increase in* hspX *expression is observed after 4 days of growth without shaking. Changes in* hsp60 *expression do not appear to change significantly with time. Error bars indicate the standard deviation between triplicate cultures.*

4.2.2 Expression of the SenX3/ RegX3 dependent *phoA* promoter

The *phoA* gene of *M. smegmatis* is induced by the SenX3/ RegX3 TCS under conditions of phosphate starvation [108]. It was demonstrated that both phosphate and magnesium concentration influence the expression of the gene in our *in vitro* model. Using HdeB minimal media, the level of phosphate in culture media was altered to bring about stationary phase growth in a population of cells as a result of phosphate starvation. The source of phosphate in HdeB minimal media is a solution of K_2HPO_4 and NaH_2PO_4 added at 10 mM and 7 mM respectively (for more details see

appendix 7). As figure 4.3 indicates, lower levels of phosphate in the media bring about an increase in *phoA* promoter activation compared with the promoterless pSHigh plasmid grown in the same condition.

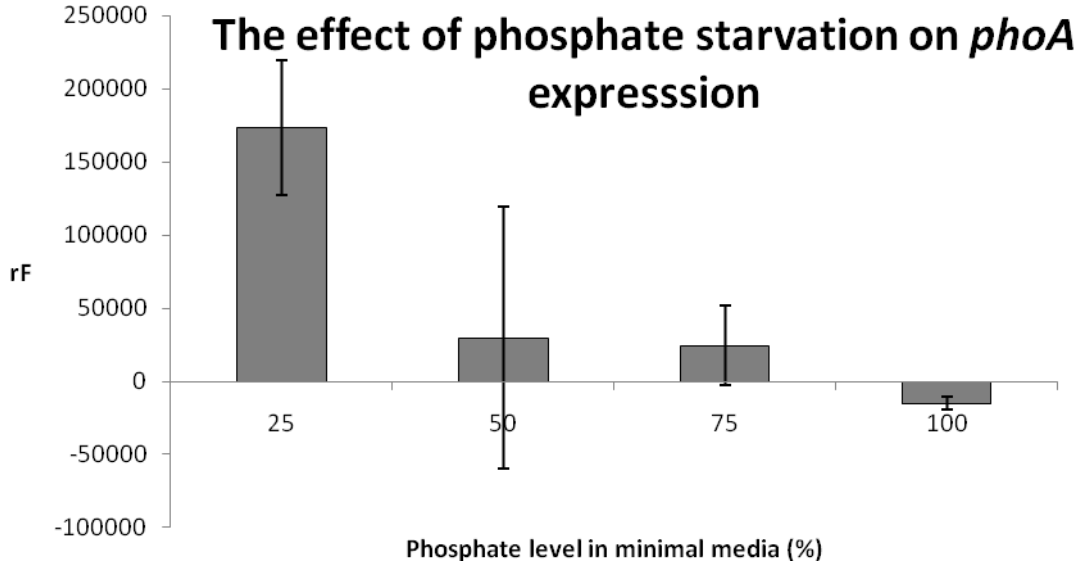


Figure 4.3: The effect of phosphate starvation on* phoA *expression. *The amount of 100 x phosphate solution added to HdeB minimal media was altered to adjust the percentage of phosphates available to cells whereby 100 % is equivalent to that of minimal media. Using media with only 20 % of the required phosphate leads to a change in* phoA *expression. Error bars indicate the standard deviation between triplicate cultures.*

Figure 4.4 demonstrates that a range of EDTA concentrations also had an effect on *phoA* expression. A final concentration of 0.5 mM EDTA added to minimal media seems to induce the expression of this gene. It was observed that a final concentration of more than 1.0 mM EDTA resulted in a slow growth rate of cells, increasing the variation of the data collected, which is perhaps why fluorescence does not increase with concentration.

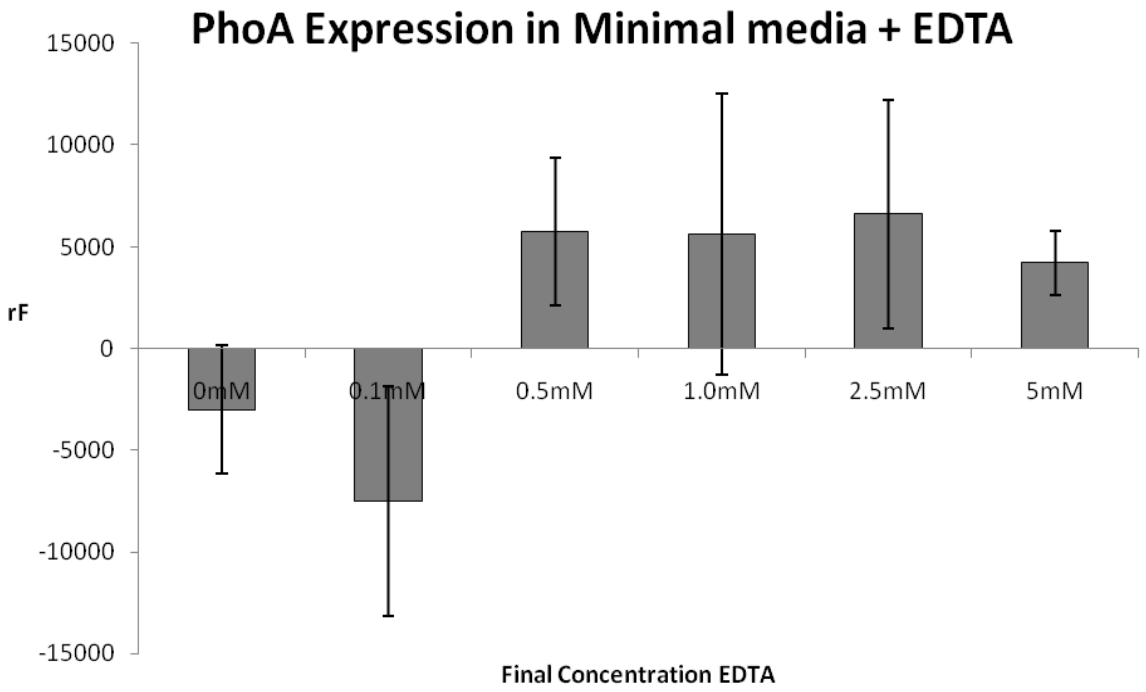


Figure 4.4: The effect of magnesium starvation on* phoA *expression. *At a final concentration of 0.5 mM EDTA, the fluorescence produced by the* phoA *promoter is increased compared with the promoterless pSHigh plasmid. Error bars indicate the standard deviation between triplicate cultures.*

4.2.3 Expression of the MtrB/MtrA dependent *mtrA* promoter

The GFP expression resulting from the *mtrA* promoter activation was higher than any other TCS used in this study. A higher level of fluorescence was observed in rich media and the *mtrA* promoter expressed GFP remained relatively consistent across all the degrees of stress that it was exposed to, with the exception of EDTA. In rich media, a control plasmid containing the reverse orientation of the promoter shows little fluorescence compared with the same sequence in the correct orientation with relation to *gfp*. As shown below in figure 4.5.

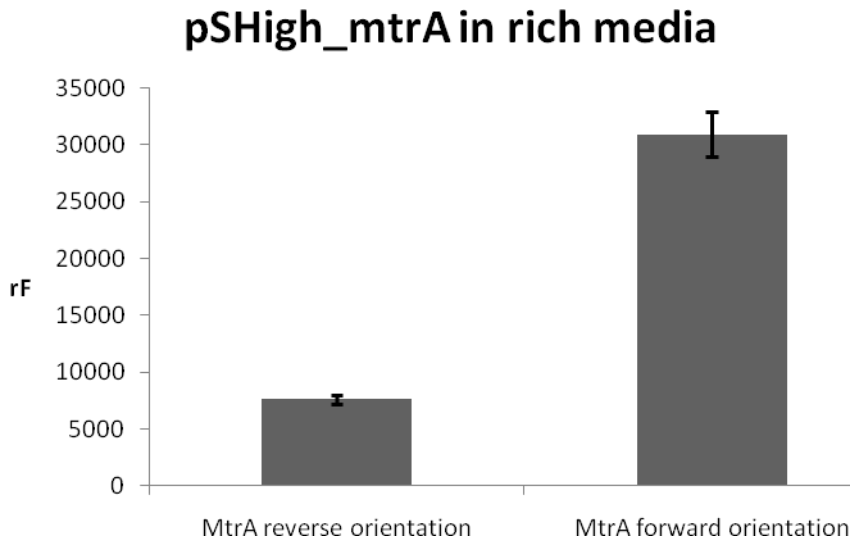


***Figure 4.5: The expression of* mtrA *in rich media**. The correct orientation of the promoter in relation the gfp on the reporter plasmid was required to give a high level of expression. Error bars indicate the standard deviation between triplicate cultures.*

There was also a change in fluorescence as a result of increasing EDTA concentration. Figure 4.6 indicates that a final concentration of 1 mM EDTA induced a higher level of fluorescence than other concentrations. This indicates that the addition of EDTA at this concentration has an effect on the *mtrA* promoter activation, possibly due to an effect of changes to the magnesium concentration of the media.

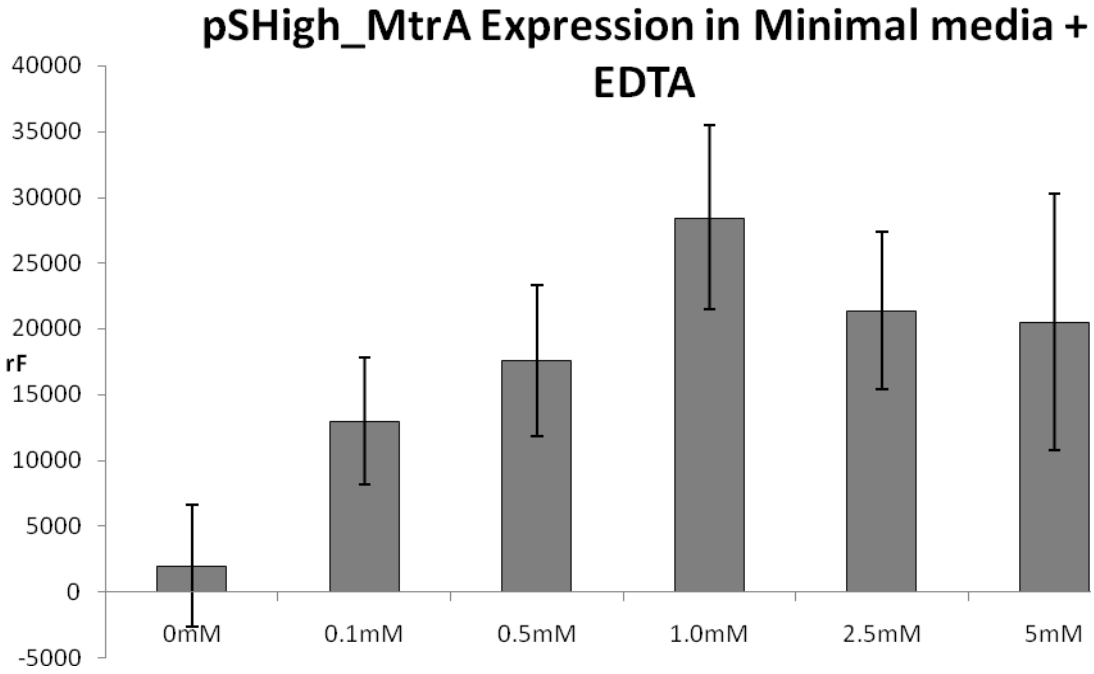


Figure 4.6: The effect of magnesium starvation on* mtrA *expression. *A high level of expression is seen at a final concentration of 1 mM EDTA in the HdeB media. Error bars indicate the standard deviation between triplicate cultures.*

4.3 Discussion

4.3 1 Expression of the DevS/DevR dependent *hspX* promoter

The oxygen starvation growth curves gave the highest *hspX*-induced rF for any condition using this promoter construct, especially with the optimised method. As a number of variables were changed, including the time allowed for oxygen starvation, the copy number of the plasmid, the media used and volume of air in the flask to begin with, we cannot specify exactly what caused the improved results. None the less, figure 4.2 shows that *hspX* was induced by oxygen starvation. This provided a proof of principle that using a promoter reporter system to monitor gene expression of a TCS-controlled gene accurately.

Surprisingly, other reported activators of expression such as, SDS and ethanol stress did not give a reproducible result for this assay. There are a number of possible reasons for this, mainly relating to differences in the method used. The up regulation of *hspX* found by Kendall et al [152] was shown by rtPCR. This method may be more effective at recognising small changes in gene expression which are a consequence of downstream effects of the stressors. Studies suggests that the sensor kinase DevS contains heme binding GAF domains, and these are likely to respond to oxygen tension [60]. Other authors suggest that carbon monoxide is the critical ligand of this sensor kinase [154, 155]. The response seen to nitric oxide may result from its ability to inhibit respiration rather than direct binding ligand binding [156]. Similarly, ethanol and SDS may be detrimental to cells in a way that reduces aerobic respiration and induces a dormancy response.

Another possibility is that these alternate inducers of the *hspX* gene occur as an effect of another stress response, rather than the hypoxia induced dormancy regulon [151]. As mentioned in the introduction, the highest level of cross talk between TCS signal transduction mechanisms is the overlap in genes under their control [90]. *HspX* may be induced by a number of other signal transduction mechanisms controlled by other stressors, at a lower level than that seen during oxygen starvation.

One problem involved in this type of assay is the effect stressful conditions have on the OD of a cell culture. Adding stressors like ethanol and SDS, even at a low concentration will limit the cell growth in a solution. A small difference in concentration of a stress inducing agent between wells of a 96 wp will equate to a large difference in cell density and a high degree of variability. The only quantifiable way of showing a change is to compare the difference in fluorescence between pShigh_hspX and pSHigh, with and without that stress condition. Herein lies another problem in that any differences in growth rate between the strains used in the experiment will mask a significant result. There are several reasons that this may occur, such as how long the strain has been stored at -80 ^{o}C, the copy number of the plasmid (in other words how much of the antibiotic resistance gene is expressed) and the burden of expressed protein on a cell.

A more relative and labour intensive method would have been to use FACS to determine fluorescence of each cell rather than an entire large culture, when using conditions which impaired growth. However relating this to a HTS assay would be near impossible.

4.3.2 Expression of the SenX3/ RegX3 dependent *phoA* promoter

The activation of the SenX3/RegX3 controlled *phoA* promoter was demonstrated in two conditions; phosphate starvation and magnesium depletion. The former condition was anticipated based on f what is known about *phoA*'s role in phosphate metabolism; however the result from adding magnesium chelator EDTA was not. There are a number of theories as to why this may be the case, none of which we can validate from this experiment alone.

Firstly, EDTA is a major chelator of magnesium, but it also chelates a number of other cations in media including calcium. To conclude that this effect is a direct result of magnesium starvation is an over-simplification. One could conclusively show this by setting up a minimal media depleted of magnesium and compare and contrast the result.

Another theory is that EDTA may have some effect on the cell wall integrity of the mycobacteria, or the phosphate importer/exporter pump system which is affecting the expression of this gene in some way. It was suggested by Ryndak *et al* that *phoP* mutants were unable to survive in EDTA supplemented media as a result of detrimental changes in the cell wall caused by this chelator, which could also explain what is happening in this case [157].

The level of EDTA in media can have an effect on cell density, indicating it presents a certain amount of stress on a cell. It could be that this gene is upregulated as a more globally controlled stress response by the cell in addition to the SenX3/RegX3 phosphate specific induction. Another signalling system may well control the expression of this gene. Once again, the possibility of overlap in genes expressed by isolated signal transduction systems may be worth considering here [90]. The PhoP/

PhoR system is thought to be controlled by magnesium concentration and is involved in cell wall maintenance, *phoA* or SenX3/RegX3 could easily be influenced by this TCS as well as the PST PIT phosphate transport systems.

It is also plausible that this TCS is controlled by more than just phosphate starvation via multiple ligand binding sites. The RegX3 sensor kinase is not itself membrane bound, interacting with a phosphate specific transporter (PST) which senses the external phosphate concentration [108] . Presumably, it is the result of a change in this transporter which causes dimerization of the sensor kinase and not a direct response to a reduction in phosphate acting on the kinase itself. So any other ligand affecting this phosphate transporter, the SenX3 sensor kinase or the interaction between the two may have downstream effects on the expression of *phoA*. A protein phosphatase assay using only the isolated HK and RR of SenX3/ RegX3 would be one way to show if the histidine kinase responded to magnesium independently of the PST.

4.3.3 Expression of the MtrB/ MtrA dependent *mtrA*

The activation of the *mtrA* promoter was not higher in any of the stress conditions than in rich media. Since this gene is thought to be essential to *M. tuberculosis* [101] *,* it may also be essential to *M. smegmatis.* By definition an essential gene is one required for cell growth; therefore some constitutive expression would be expected in rich media.

Studies have shown that this gene is induced during intra-macrophage growth. Only one of the *in vitro* models of the macrophage or granuloma environment presented here induced an effect on *mtrA* expression; EDTA. Activation of *mtrA* was generally

much lower in minimal media than that observed in rich media. As figure 4.6 shows, there is very little fluorescence without the addition of EDTA in minimal media. While this data indicates that EDTA-dependent ion depletion may have an effect on mtrA activation, this does not conclusively demonstrate that the sensor kinase directly responds to magnesium.

The inducer of this TCS is still unclear [112, 158] but one suggestion is iron starvation [71]. Rodrigues *et al*, who profiled iron-responsive gene expression using microarrays, saw an increase in the expression of *mtrA* during iron starvation [71]. In the current study, however, the use of an iron chelator EDDA did not seem to render any increase in *mtrA* expression. This would be worth exploring, by omitting iron from the minimal media rather than using a chelator in case the EDDA concentration used in this study was not optimal and did not chelate all the iron in solution. In order to show that magnesium or iron is a ligand of the MtrB histidine kinase, further work on the crystal structure and binding properties of this protein is required. EDTA was used in this assay to deplete free magnesium from the media, but the possibility exists that it causes a multitude of effects on cellular morphology independent of specific ion starvation.

The reporter protein GFPmut2 is relatively stable; consequently endpoint readings in such an assay will not disclose exactly when the gene is induced. If *mtrA* is expressed at a particular point of the growth phase our assay picks up the GFP expression at the end of the growth assay. Using an unstable variant of GFP, such as that developed by Blokpoel *et al.* 2003, may give a better idea of phase dependent expression of this gene [134].

Our aim of finding a high level of GFP expression for each TCS controlled promoter was fulfilled with rich media for this TCS. Even though EDTA induced a high level of expression, its use in high throughput assays was not advantageous as minimal media gives a much higher level of variance in a 96 wp format.

Another concern was the addition of EDTA to media as this may have a profound effect on the action of compounds used in library screens. In the end it was decided that rich media could be used for high throughput (HTP) screening because a more stable rate of growth could be achieved. Another benefit was that this data could be compared with other screens using the *hsp60* promoter which were also carried out in rich media.

CHAPTER FIVE:
Screening Compound Libraries for TCS Inhibitors

5.1 Introduction

The overall aim of this project was to develop a GFP reporter and optimize conditions under which TCS controlled genes were highly expressedto develop an assay which identified compounds which inhibit this sort of signal transduction. In order to test as many compounds as possible, a high throughput method was employed, whereby thousands of compounds could be tested for their effect on the expression of these genes. Using a liquid handler, a large number of compounds could be added to separate wells of a 96 wp and incubated with *M. smegmatis* cells to observe changes in fluorescence. In theory a compound affecting any of the steps in the phospho transfer from a histidine kinase to response regulator and consequential DNA binding could cause a reduction in fluorescence driven by the TCS controlled promoter.

There are numerous benefits of using this whole cell approach to target TCS compared with isolated protein methods, as mentioned in the introductory chapter. The main advantage is that a compound inhibiting any reaction in the signal transduction mechanism may be identified, rather than targeting a single protein. Compounds which inhibit another target, resulting in a decrease in cell growth, will also display a decrease in fluorescence. By observing the OD of each well we are able to identify general cell growth inhibitors and eliminate these from the list of positive findings.

Another important point is the fact that all known TCSi affect the cell membrane in addition to any signal transduction inhibitory action. Using this screen, the OD of the cell suspension is taken into account so compounds which act on the cell in some

other fashion, such as a detrimental effects on membrane integrity, will not be picked up.

A number of compound libraries available were tested for an effect on the activation of the *mtrA* promoter, including the LOPAC, Spectrum Collection, NIH diversity collections and a library of natural compounds isolated from marine organisms which will be known as the NIWA collection. MtrB/ MtrA was selected as it showed high levels of expression in rich media without the addition of growth inhibitors such as EDTA. This meant that we could expect a uniform growth rate and limited interaction between compounds and the chelator. One compound library, the LOPAC library, was screened against *phoA* driven expression, but as the results will show, the difference between *phoA*-induced expression and promoterless expression of GFP from pSHigh was too variable in this high throughput format to discern differences in fluorescence as a result of a compound interaction with the SenX3/ RegX3 TCS. The oxygen starvation assay, which was described in chapter 4, did not translate well to a 96 wp format. It was not possible to obtain a high level of fluorescence from the *hspX* promoter that was uniform across the plate by increasing the volume of liquid in each well and creating an airtight seal.

In order to ascertain that there was no inhibition from the solvent used to dissolve the compound libraries, a negative control was included containing the same volume of DMSO added to two wells of each plate instead of a compound. As a positive control, to show the effect of compounds which inhibit cell growth outright, antibiotics were added to two wells of each plate.

5.2 Results

5.2.1 Compound Library Screens against *mtrA* expression

5.2.1.1 Spectrum Library against *mtrA* expression

Fluorescence (F) and OD data were plotted on a scatter graph to reveal any changes in F without a change in OD, shown in figure 5.1. If a compound inhibits growth of the cells it will also lead to a decrease in F due to the reduction in cell number per well so we were looking for compounds which act on F alone. For each library, the median Fand OD readings are shown as the X and Y axes respectively so that as a rough guide compounds in the top left quarter of each graph could be considered to have an effect on promoter driven GFP expression levels. It was hoped that positive control values, displaying no effect on OD or F, would be presented in the top right quarter and negative controls, affecting OD and F would be observed in the bottom left corner.

Figure 5.1 shows a high level of variance in F, indicating that a number of compounds may affect either cell density or *mtrA* expression. In order to select only those with an effect on *mtrA* expression, ten outliers from the group of points were manually selected for re-testing. These are displayed by a circle on the graph and named in table 5.1.

There is a large group of compounds centred on the median mark for both F and OD. They display a lower level of both F and OD than the positive control values in the top right.

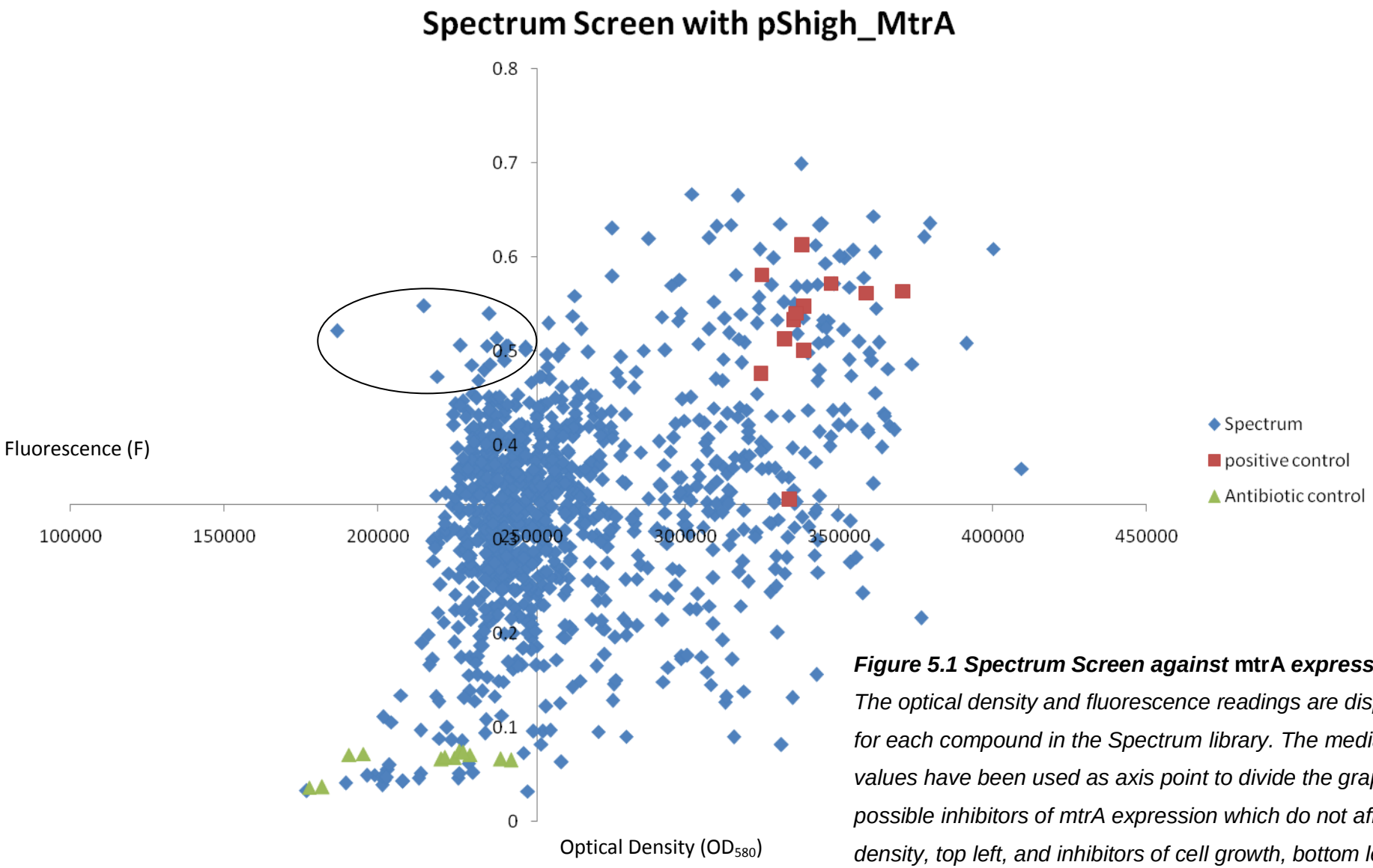


Figure 5.1 Spectrum Screen against* mtrA *expression. *The optical density and fluorescence readings are displayed for each compound in the Spectrum library. The median values have been used as axis point to divide the graph into possible inhibitors of mtrA expression which do not affect cell density, top left, and inhibitors of cell growth, bottom left. Data points which were separate from the main group were selected (circled) and re-tested. These are names in table 5.1*

Because the *mtrA* gene target is fairly specific, we assume that not all of these compounds are affecting the TCS. It is more likely that they affect cell growth in some other way giving a lower reading of F. The values circled stand out from the main group in that they do not display a lower OD than the positive control values, but display a lower F and are not part of the large group of compounds in the median range. The names, formulae and sources of these compounds are displayed in table 5.1, none of which are known to be signal transduction inhibitors.

NAME	Formula	Bioactivity	Source	F	OD_{580}
STIGMASTEROL	C29H48O		soya and calabar beans;	186735	0.521
CITICOLINE	C14H26N4O11P2	cognition enhancer,	cytidine-5'-diphosphocholine	214927	0.547
CARNOSIC ACID	C20H28O4		Salvia spp, Rosmarinus officinalis	219330	0.472
TETRAHYDRO GAMBOGIC ACID	C38H48O8		derivative	226869	0.506
PRISTIMEROL	C30H42O4		derivative of pristimerin	230552	0.485
7-OXO CHOLESTERYL ACETATE	C29H46O3		Cliona copiosa	232658	0.469
RHODOMYRTOXIN B	C24H28O7		Rhodomyrtus macrocarpa	234508	0.479
AUSTRICINE	C15H18O4	Anti inflammatory	Compositae	235476	0.505
BAICALEIN	C15H10O5	antiviral (HIV)	Scutellaria baicalensis	236264	0.539
METHOPRENE (S)	C19H34O3	Ecto parasiticide	synthetic	236431	0.486
POSITIVE CONTROL				339962	0.528
NEGATIVE CONTROL				214974	0.064

Table 5.1: Spectrum Library Candidates. *Names and descriptions of compounds considered for re-testing from Spectrum collection with their F and OD values from the initial screen.*

5.2.1.2 LOPAC Library against *mtrA* expression

Using the established assay, we then screened the LOPAC library. Fortunately, this library contained an intrinsic positive control that we were not previously aware of. Oleic acid, which was briefly discussed as a TCS inhibitor in the introduction, was identified as a hit in the LOPAC screen, confirming our assay. Figure 5.2 displays the OD and F readings for each well in this screen with oleic acid labelled in a different colour to show where hits of this screen should sit. As with the previous figure, the median values are shown as the axis lines to divide the graph into four quarters. The "hits" observed are displayed in table 5.2. Unfortunately in this assay, the variance in the negative controls was far more pronounced. Positive control wells displayed a huge range of variance in OD. However a trend can be seen from the top right quarter to the bottom left, in terms of the positive control readings, so the hits were selected according to their position away from this diagonal line.

The basis for choosing hits from this screen was their position in relation to the oleic acid, which is known to inhibit the phosphorylation step of HK dimerization. Ten compounds, named in table 5.2, were selected and re-tested in triplicate.

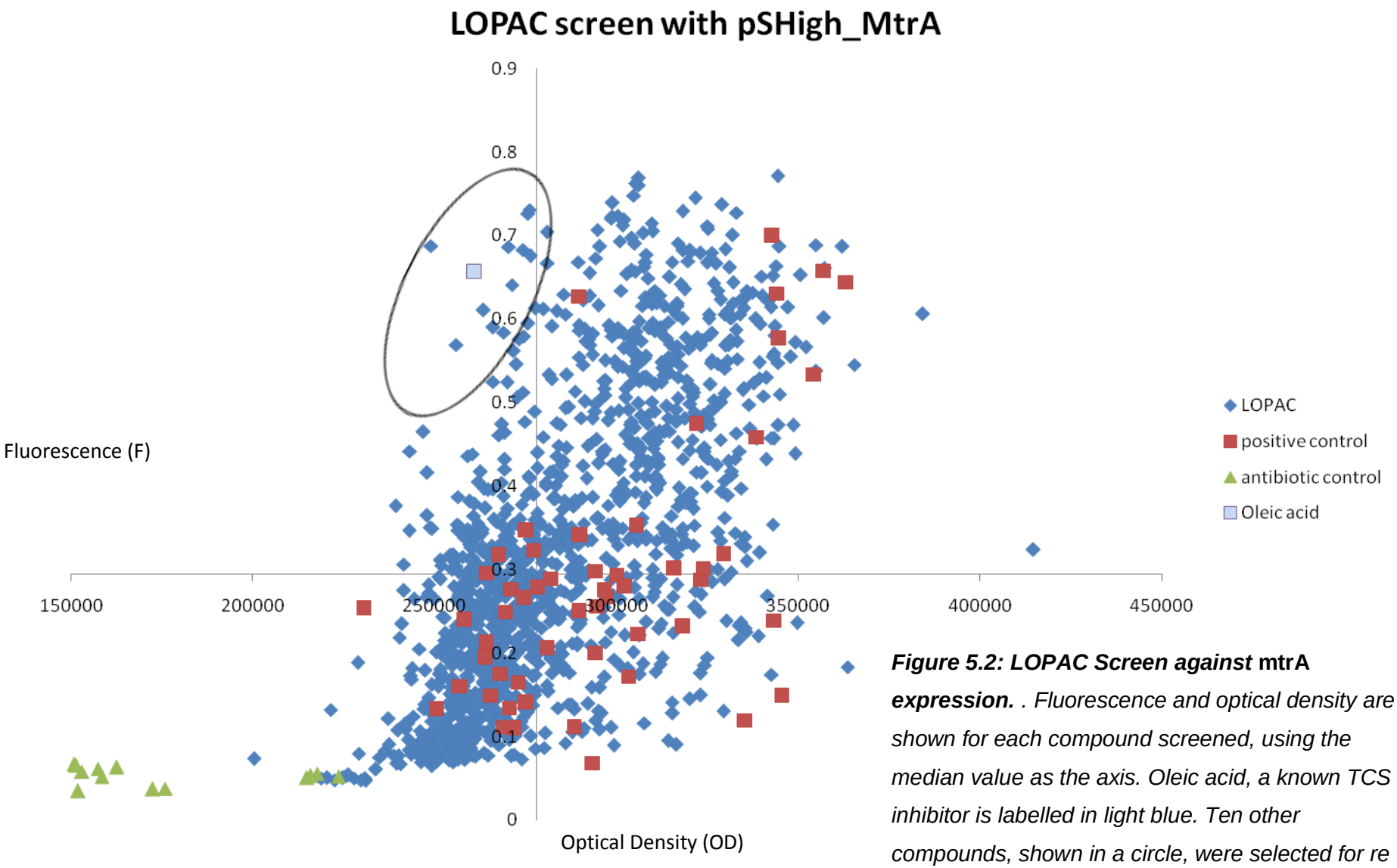


Figure 5.2: LOPAC Screen against* mtrA *expression. *. Fluorescence and optical density are shown for each compound screened, using the median value as the axis. Oleic acid, a known TCS inhibitor is labelled in light blue. Ten other compounds, shown in a circle, were selected for re testing. Their names, a brief description as well as their values for F and OD are shown in table 5.2*

NAME	Class	Description	F	OD
PROTRIPTYLINE HYDROCHLORIDE	Adrenoceptor	Norepinephrine reuptake blocker	243046	0.441
ODQ	Cyclic Nucleotides	Potent and selective NO-sensitive guanylyl cyclase inhibitor	246851	0.464
N - ACETYLPROCAINAMIDE HYDROCHLORIDE	Na+ Channel	Blocks Na+ channels and appears to have Class III anti-arrhythmic activity; metabolite of Procainamide	247850	0.416
PARGYLINE HYDROCHLORIDE	Neurotransmission	MAO inhibitor, relatively selective for MAO-B	249006	0.687
NILUTAMIDE	Hormone	Antiandrogen	255868	0.569
OLEIC ACID	Phosphorylation	Activates protein kinase C in hepatocytes; uncouples oxidative phosphorylation	260912	0.656
XANTHINE AMINE CONGENER	Adenosine	A1 Adenosine receptor antagonist	263300	0.610
L-ARGININE	Nitric Oxide	Nitric oxide precursor	266069	0.590
OXOTREMORINE METHIODIDE	Cholinergic	Nonselective muscarinic acetylcholine receptor agonist	266106	0.52
S- (P-AZIDOPHENACYL) GLUTATHIONE	Multi-Drug Resistance	Glyoxalase and glutathione S-transferase inhibitor	269002	0.583
Positive Control			***296366***	***0.290***
Negative Control			***171356***	***0.053***

Table 5.2 LOPAC Library Candidates. *Names and descriptions of compounds considered for re-testing from LOPAC collection with their F and OD values from the initial screen. This information was obtained from Sigma.*

5.2.1.3 NIH Library against *mtrA* expression

The NIH screen was the largest compound library tested in this study. This library has mostly been used to test potential anti-neoplastic agents and can be obtained from the NIH. It includes a large number of unknown compounds as well as a number of natural products. Figure 5.3 displays the F and OD of each compound tested with median values used as the X and Y axis in each case. Positive control values are mostly isolated to the top right hand corner, while antibiotic control values are in the bottom left. Hits from the top left hand corner were selected for re-testing.

Table 5.3 shows the names (if known), formula, molecular weight and F and OD values of those compounds labelled “hits” for this screen. This information was obtained from the Developmental Therapeutics Programme (DTP) website which can be found at http://www.dtp.nci.nih.gov/docs/dtp_search.html.

Most of the compounds labelled as hits for this screen have not been named, with the exception of Antineoplastic-92893 and Buclizine Hydrochloride, as shown in table 5.3. Consequently, there was little information available about these compounds other than their structure and formula.

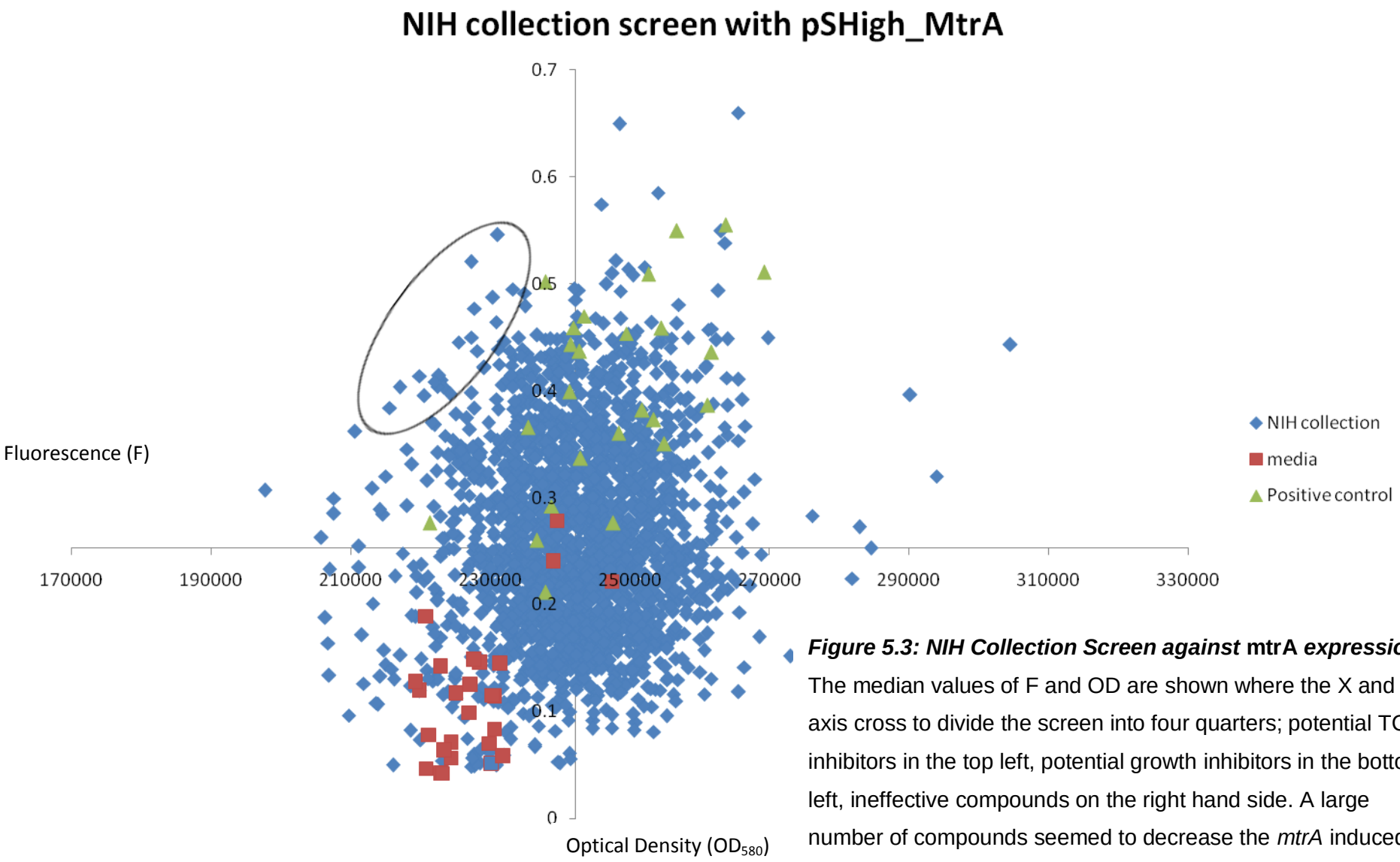


Figure 5.3: NIH Collection Screen against* mtrA *expression. The median values of F and OD are shown where the X and Y axis cross to divide the screen into four quarters; potential TCS inhibitors in the top left, potential growth inhibitors in the bottom left, ineffective compounds on the right hand side. A large number of compounds seemed to decrease the *mtrA* induced expression of GFP, compared with the positive controls shown in green. Those circled were selected and tested in triplicate.

Name	NIH code	Formula	Molecular Weight	F	OD
	NSC 73646	$C_6H_{16}N_4.H_2O_4S$	242.0	210551	0.361
	NSC 96606	$C_{12}H_{14}N_2O_2$	218.0	234878	0.261
	NSC 63786	$C_{10}H_{10}N_4S$	218.0	244330	0.216
	NSC 3535	$C_{13}H_{19}NO_2S$	253.0	215539	0.383
	NSC 133896	$C_{13}H_6N_2O_5$	270.0	227222	0.520
	NSC 130915	$C_{12}H_{21}N_3.2ClH$	280.0	219812	0.413
Antineoplastic-92893	NSC 92893	$C_{13}H_{14}AsNO_3$	307.0	217123	0.403
	NSC 166638	$C1_0H_7C_{l2}F_3N_4O_2$	343.0	228152	0.437
	NSC 97845	$C_{22}H_{27}NO_3$	353.0	231052	0.546
	NSC 357777	$C_{19}H_{15}N_5O.ClH$	366.0	233207	0.495
	NSC 18877	$C_{20}H_{10}N_2O_4S_2$	406.0	227708	0.476
Buclizine Hydrochloride	NSC 25141	$C_{28}H_{33}ClN_2.2ClH$	506.0	225434	0.445
	NSC 115448	$C_{44}H_{20}N_2O_8$	705.0	227322	0.449
	NSC 382796	$C_{31}H_{25}N_3O_7$	552.0	230822	0.464
Positive control (average)				247467	0.385
Negative control (average)				217468	0.056

Table 5.3: NIH Library Candidates. *Names and descriptions of compounds considered for re-testing with their F and OD values from the initial screen*.

5.2.1.4 NIWA collection against *mtrA* expression

A collection of marine extracts was also tested against the *mtrA* promoter. This collection contained a smaller number of samples than any other library and displayed no decrease in F while maintaining the OD of the culture compared with positive controls, as displayed in figure 5.4. The antibiotic controls indicate that a number of extracts had an effect on the growth of cells. All the other libraries used a constant concentration of 20 µM for all compounds but the NIWA collection was composed of crude marine extracts, so the final concentration of active compounds is unknown.

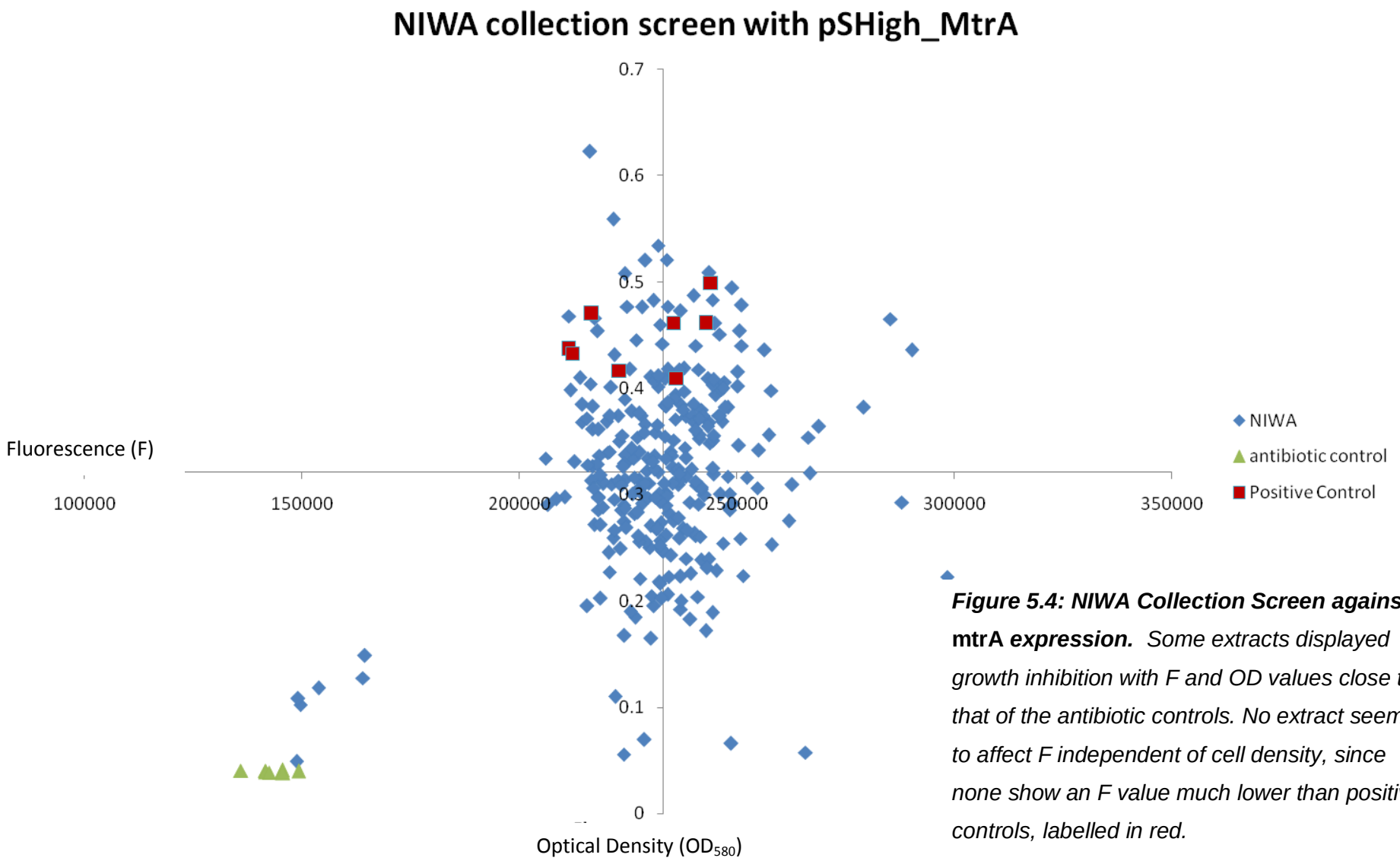


Figure 5.4: NIWA Collection Screen against* mtrA *expression. *Some extracts displayed growth inhibition with F and OD values close to that of the antibiotic controls. No extract seemed to affect F independent of cell density, since none show an F value much lower than positive controls, labelled in red.*

5.2.2 Drug screens with pSHigh_phoA

The *phoA* promoter-driven F of GFP is relatively lower than that of *MtrA*. This presented a problem, as the variance in F between wells was very high. For library screens with *phoA,* an additional negative control was used to indicate F of the promoterless pSHigh plasmid construct in the same EDTA supplemented media. An un-induced *phoA* control was not really practical in this screen, since these cells would grow at a different rate to the cells grown in EDTA supplemented media. There were no significant results seen in this screen, as changes in intensity between positive and negative controls could not be discerned.

5.2.2.1 LOPAC library screen with *phoA*

Figure 5.5 shows the *phoA* activation and OD against the LOPAC library. Although the position of the oleic acid control is in the top left quarter, as expected, a large number of compounds seem to cause a decrease in F to levels lower than the promoterless control. This could indicate that the use of EDTA imposes conditions which make the cells more susceptible to compounds that would not necessarily give the same result in rich media. This construct was also used to screen against the NIWA collection with a similar result (this data is not shown).

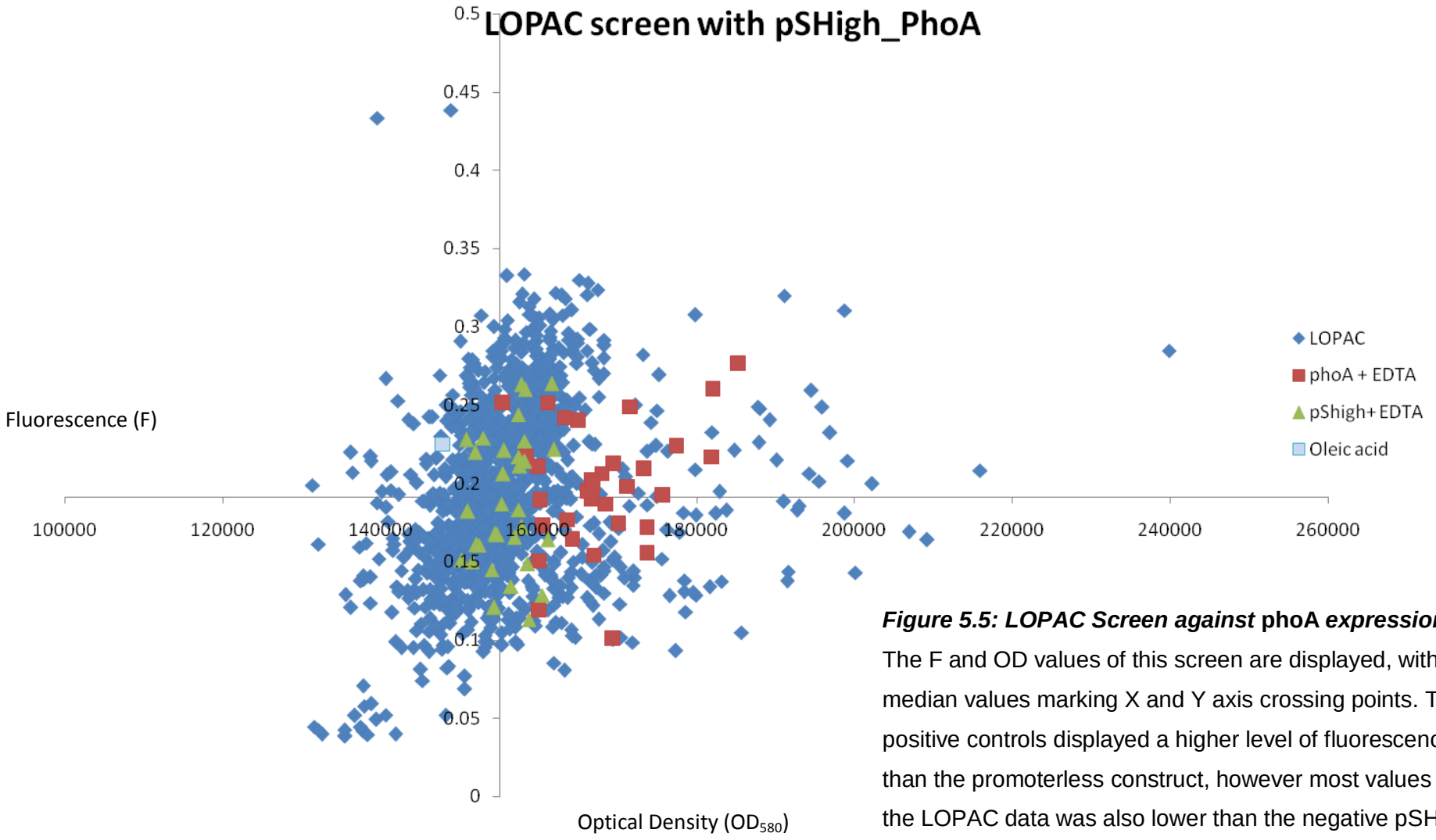


Figure 5.5: LOPAC Screen against* phoA *expression.
The F and OD values of this screen are displayed, with median values marking X and Y axis crossing points. The positive controls displayed a higher level of fluorescence than the promoterless construct, however most values from the LOPAC data was also lower than the negative pSHigh control. Phosphorylation inhibitor oleic acid, shown in light blue is surrounded by a large number of compounds rather than as an outlier.

5.2.2.2 Comparison of *mtrA* and *phoA* LOPAC screens:

Even though the phoA screen was not successful in showing any hits, the oleic acid control was where we expected it to be; the top left hand quarter of the graph. In order to determine whether any of the proposed hits from the *mtrA* LOPAC screen fell in this quadrant during the *phoA* screen they have been represented on the same graph as the previous figure 5.5 in a different colour. If any of the compounds highlighted in the *mtrA* screen were not specific to the MtrB/ MtrA TCS and could inhibit different TCS as well, they might show up in the top left hand corner of the *phoA* screen as well. Figure 5.6 shows the same data as figure 5.5, with the hits from the *mtrA* screen highlighted in red and oleic acid in black and labelled.

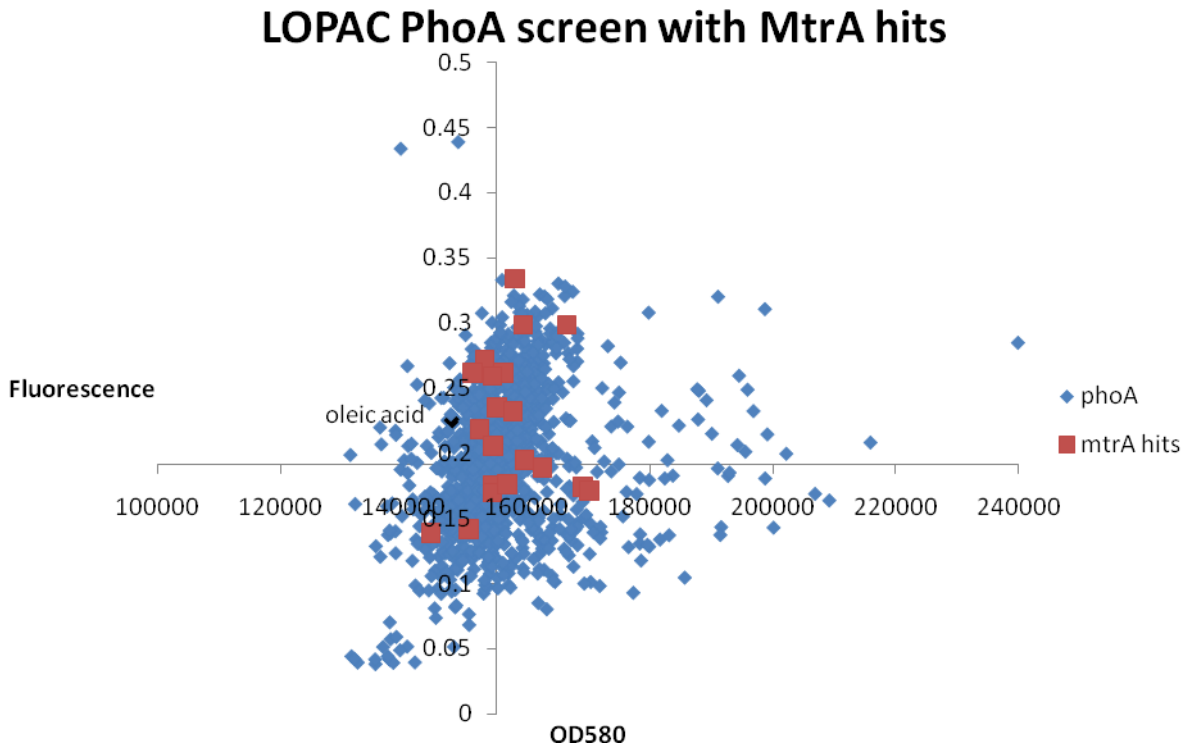


***Figure 5.6: Identification of* mtrA *hits on LOPAC Screen against* phoA.** *The F and OD values of this screen are displayed, with median values marking X and Y axis crossing points. Compounds which gave a lower F reading in the mtrA screen are shown in red. A few values lie in the bottom left hand corner, indicating they may inhibit growth under the conditions of this screen, however none lie far to the left of the top corner which would signify that they are non-specific TCS inhibitors.*

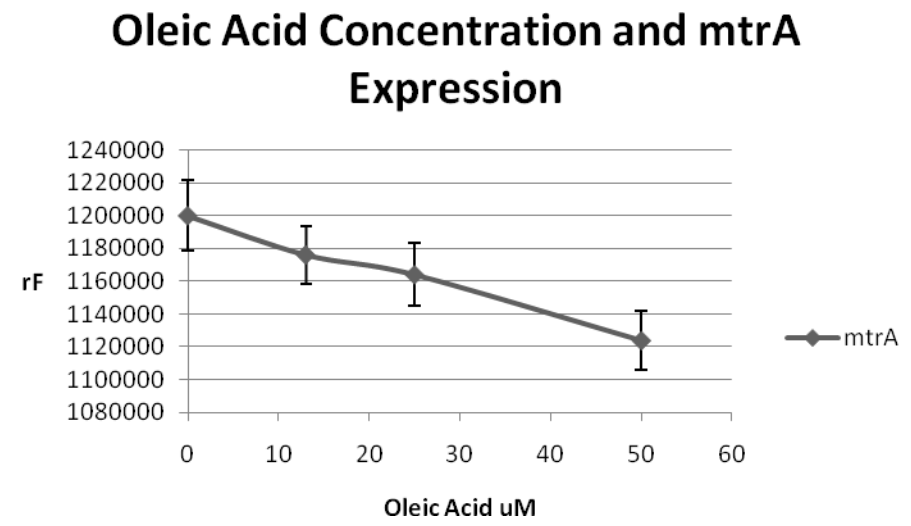
Only one of the compounds from the LOPAC screens showed inhibition of both *mtrA* and *phoA* driven promoter expression. This indicated that any of the other potential inhibitors identified during the *mtrA* screen were specific to this one out of the two signal relay system tested. It is also likely that this method of extrapolating hits may inadvertently overlook compounds with TCS inhibitory properties because only a small number of points were selected from each graph. Another factor is that compounds which conferred a low F reading, as well as a low OD_{580} reading are left out, as it was assumed these compounds are cytotoxic.

5.2.3 Validation of hits from library screens in triplicate

In order to verify that the compounds identified as hits from the library screens inhibited *mtrA* activation, selected compounds from each screen (named in tables 5.1 – 5.4) were re-tested in triplicate. For this validation assay the positive control promoter *hsp60* and the promoterless pSHigh plasmids were included. The positive control would show whether the effect of the compound was a result of a global decrease of gene expression in the cell, or via an *MtrA* specific mechanism. Only one of the compounds tested in triplicate, oleic acid, displayed a reproducible effect on *mtrA* expression. They also displayed no change with respect to *hsp60* expression, indicating that hits selected from each screen had been false positive results and not inhibitors of gene expression.

5.2.4 Validation of whole cell based screen with oleic acid and palmitoleic acid dilution series

None of the compounds picked up as hits in the library screens gave a significant decrease in *mtrA* expression during a validation screen, where each compound was tested in triplicate. In order to show that this screen could potentially identify TCSi, had any been present in the libraries tested, a series of oleic acid and palmitoleic acid concentrations were tested for modulation of *mtrA* activation. Figures 5.7 and 5.8 show the *mtrA* expression in the presence of palmitoleic and oleic respectively at three different concentrations of the fatty acids. These graphs show that the *mtrA* promoter driven GFP expression decreases with the concentration of both oleic and palmitoleic acid added, while the effect on the *hsp60* promoter expression is unchanged.



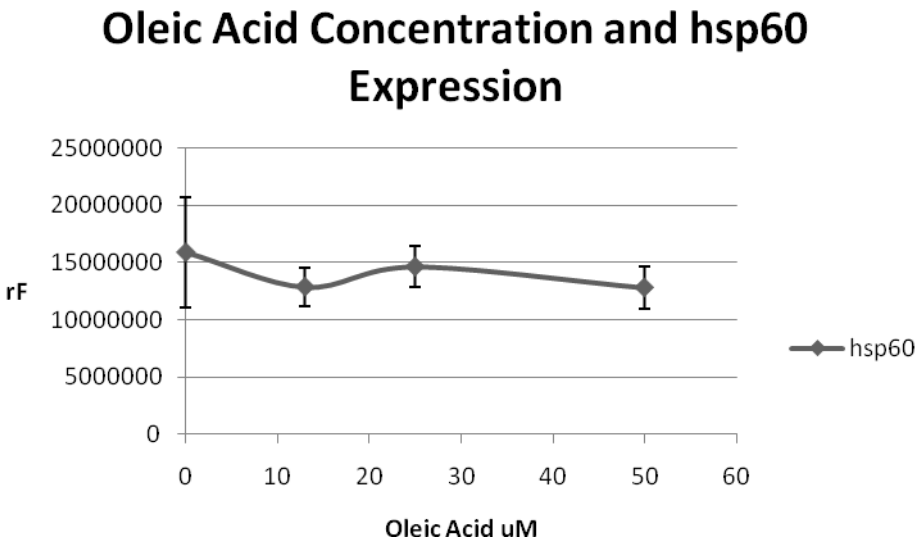
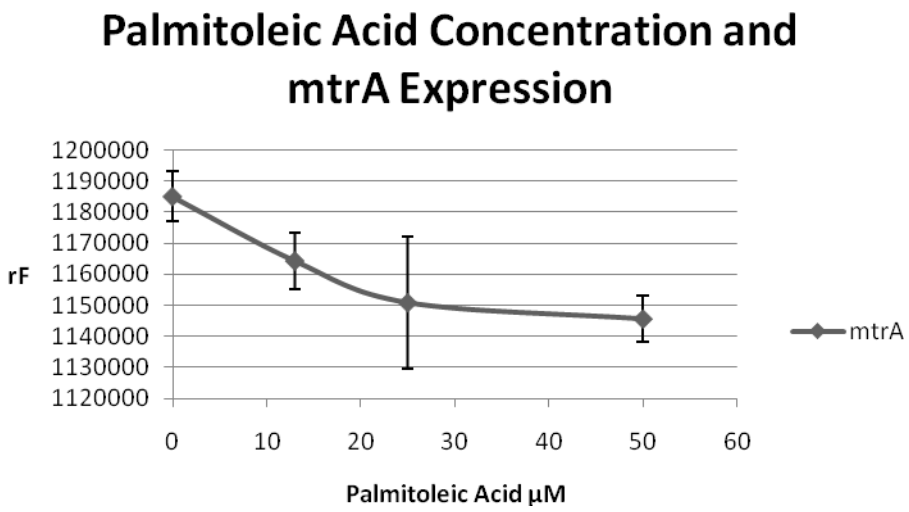


Figure 5.7: Oleic Acid dilution series against* mtrA *expression. *A)* MtrA *and B)* hsp60 *expression under the influence of the oleic acid. The effect of final concentrations of 50, 25 and 12.5 µM of oleic acid on the expression of* mtrA *is dose dependent as the F of the reporter decreases with concentration, while* hsp60 *expression does not change with concentration. Error bars indicate the standard deviation between triplicate cultures.*



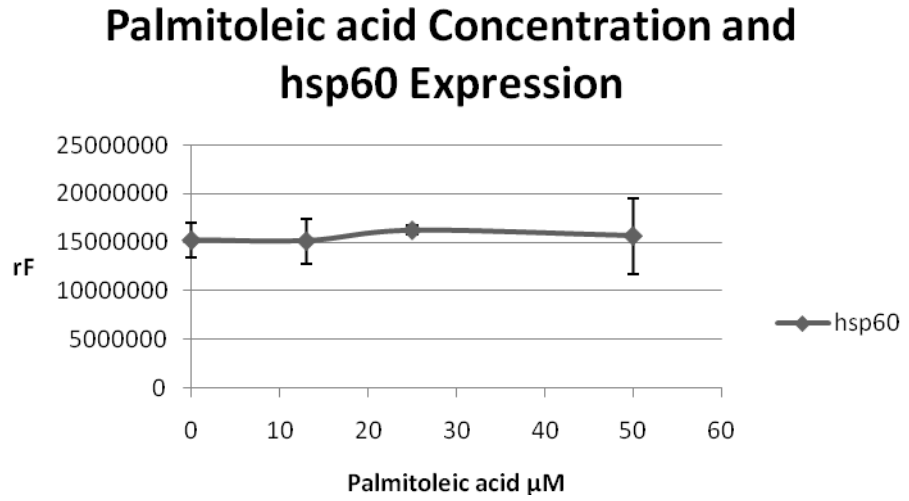


Figure 5.8: Palmitoleic Acid dilution series against* mtrA *expression. *A) MtrA and B)* hsp60 *induction under the influence of the palmitoleic acid. The effect of final concentrations of 50, 25 and 12.5 µM of palmitoleic acid on the expression of* mtrA *is dose dependent as the F of the reporter decreases with concentration, while* hsp60 *expression does not change with concentration. A solvent control of ethanol is shown as 0 µM palmitoleic acid. Error bars indicate the standard deviation between triplicate cultures.*

5.3 Discussion

5.3.1 Compound Library screens and TCS controlled gene expression

5.3.1.1 Spectrum Collection

A total of 2000 compounds from the Spectrum library were tested for activity against the expression of the *mtrA* promoter. Figure 5.1 shows that compounds had an array of effects on both OD and F. The positive controls of *M.smegmatis* containing pSHigh_mtrA remain in the top right hand corner of the graph, indicating no reduction in F or OD, while antibiotic controls shown in the bottom left corner indicate background F and turbidity produced by media without cells. The compounds have a range of effects with a main cluster halfway between these controls. The general trend shows that a number of compounds effect the growth of cells to some extent. The cluster of data points that is lower than the positive controls indicates that the presence of most compounds has some affect on cells either acting on the promoter activity or outright cell growth. Compounds that inhibit cell growth are present near the antibiotic control points. This graph depicts a number of hits which have a low level of F and no change in OD compared with positive controls.

5.3.1.2 NIH Collection

The NIH collection is a free library of compounds available from NCBI. 2240 compounds were tested from this library including a number from the natural diversity set. Unlike the Spectrum screen the positive controls are not all located in the top right hand quarter of the graph; however there is a clear trend between

positive and negative controls. A total of 15 hits were selected for validation from this screen.

5.3.1.3 NIWA samples

A selection of marine samples donated by the National Institute of Water and Atmospheric Research (NIWA) in Wellington were also tested in this manner. This screen contained 290 samples, none of which gave an F reading lower than the positive controls. It did produce a number of data points with F and OD inhibition which will be used to verify future work with this library in a search for anti-mycobacterial drugs.

5.3.1.4 LOPAC library

The LOPAC collection, Library of Pharmacologically Active Compounds, available from Sigma Aldrich includes 1280 compounds. This screen was carried out for both *mtrA* and *phoA* driven GFP expression. Ten compounds were selected and retested with the *mtrA* construct. A major benefit of this library screen was the presence of a phosphorylation inhibitor oleic acid, displayed separately on figures 5.5 and 5.6. This compound was identified as a hit for the *mtrA* screen, conveniently providing an intrinsic control to our assay. The *phoA* screen shows oleic acid in the top left quarter of the graph but not as a hit as in the *mtrA* screen.

The *phoA* screen was less successful in that the difference between EDTA induced pSHigh_phoA and promoterless pSHigh was not well defined. A majority of the data points fell below this negative control, indicating there was some other interaction occurring between compounds and GFP expression.

Interestingly none of the *mtrA* hits identified were common to inhibitors on the *phoA* screen, indicating the library search had not successfully unearthed a non-specific inhibitor of HK to RR phosphotransfer. A recent study has highlighted the relationship between drug efficacy and the conditions in which cells are grown [142]. It has already been established that an anti-TB drug metronidazole is much more effective in hypoxic conditions [159]. These considerations add a level of complexity to drug screening as most *in vitro* drug screening is carried out in a specific media, and results can vary if these parameters are changed.

5.3.2 Discussion on the Screen

There a number of noteworthy limitations to this screen. Firstly the variance observed between samples when dealing with a multitude of compounds in this manner. The presence of outliers in both directions of the graph indicate that there is a significant chance of error that can come from a number of sources, from human handling and data processing error to technological errors such as pinning of compounds or reading of wells, the latter obviously far less likely. False positive "hits" may arise from a number of scenarios

- Contamination of a well in the assay which was not picked up upon inspection of plate, giving a high OD but no F
- If the compound has an effect on the GFP protein processing at any point a misfolded protein may not give off the same F as positive controls
- Coloured compounds can alter the OD reading of its well; a highly coloured compound may give a reading which indicates high cell viability when the chemical has in fact inhibited growth

This data shows that the best results from the HTP screen did not translate to a decrease in F when repeated in triplicate. The high level of variance displayed in control and compound containing wells suggests that there is a loss of uniformity when working in a 96 wp set up in terms of OD and F. The validation results call into question the methods used during the HTP screen, and whether adequate parameters had been set. Given the high level of variance seen in a HTP screen with such a large number of compounds, the hits listed may either be false positives from the initial screens or too variable in the validation to be observed.

One concern with using the *mtrA* promoter in these screens stems from the fact that *mtrA* may be essential in *M. tuberculosis.* It has not been previously described as an essential gene in *M. smegmatis*; however it brings to light the concept that an inhibitor of this system may display an effect on cell growth if it is indeed essential. However, the data from both LOPAC screens and the oleic and palmitoleic acid experiments indicate that at a low concentration this inhibitor does not affect the cell density.

One way that this result may have been improved would have been to use another expression condition for *mtrA*; minimal media with EDTA. The decision to use rich media over minimal media stemmed from an observation that cells grew with much more uniformity in the former. As observed with the *phoA* promoter screens, minimal media gives a lower dynamic range making the difference between induced and uninduced GFP expression less pronounced.

5.3.3 Validation of the screen with *cis* fatty acids

Figures 5.7 and 5.8 show a dose dependent decrease in F in the presence of oleic and palmitoleic acid. This trend is not seen in F which is restricted to the *mtrA* and not the *hsp60* promoter indicating that their mode of action is not on the GFP reporter itself or general global gene expression.

Oleic and palmitoleic acids are thought to inhibit phosphorylation of ATP to ADP in the histidine kinase activation [160]. They occur naturally in human, animal and plant fat and do not inhibit bacterial growth [130]. This activity was discovered in a kinase involved in sporulation named KinA of *B. subtilis.* The mode of action of cis unsaturated fatty acids is not known, except that they inhibit KinA –ATP binding in a non competitive fashion with an IC_{50} of around 20 µM [160]. Oleic acid is a component of mycobacterial media OADC, and must be broken down by the bacteria as an energy source, so its inhibitory activity may only act at a certain concentration, or in a particular arrangement of the fatty acid.

Oleic acid has also been described as an uncoupler of mitochondrial oxidative phosphorylation and an activator of protein kinase C [161, 162]. The full extent of its activity on phosphorylation reactions in mycobacteria is unknown.

This data shows in principal that using a serial dilution setup with three strains containing pSHigh_MtrA, pSHigh_hsp60 and pSHigh each in triplicate is a method which will illuminate TCSi from a library of compounds. The screen in this study aimed to test as many compounds as possible for activity; in other words, one compound per well at one concentration. This data clarifies the need for compounds to be tested in a dilution series and in triplicate. This method is more time consuming

and probably reduces the number of compounds which can be tested per screen, but clearly yields a more reproducible result.

Previous work in HTS for active compounds has developed methods for statistical validation of primary screening [142, 163]. This method determines the validity of hit selection in an assay based on a number of factors. In our assay only one hit was validated, indicating that this screen requires a significant amount of optimisation in order to obtain an acceptable hit rate or z score.

Chapter Six:

General Discussion and Future Directions

6.1 Summary of Findings

The primary accomplishments of this thesis were the optimisation of a high copy number plasmid to report gene expression, the application of this methodology to TCS controlled genes and an analysis of the expression of these genes under stress. This led to a number of chemical library screens, in search of compounds which inhibit the TCS signal transduction. In theory, compounds could be potential TCS inhibitors if they exert a drop-off in TCS controlled gene expression compared with what is normally expected under particular conditions. Since they change the eventual outcome of the signalling mechanism i.e., the induction of a particular gene, these compounds could be affecting any part of the TCS signalling mechanism.

The insertion of the oriM high mycobacterial origin of a replication and the *gfpmut2* gene resulted in the successful optimisation of a reporter plasmid. The results in Chapter Three demonstrate that the presence of the plasmid provides a high level of F under the influence of the *hsp60* promoter without causing any obvious detrimental effects on *M. smegmatis* cells. This plasmid was further equipped with alternative promoters which were selected after a literature search of TCS controlled genes; namely *hspX, phoA* and *mtrA.*

The activation of these genes was explored under a number of stress conditions including oxygen and nutrient starvation, antibiotic stress, heat shock, cold shock, SDS and ethanol. Out of all the stress conditions tested, the most noteworthy effect on the expression of *hspX* was seen during oxygen starvation.

PhoA activation was observed under phosphate starvation and magnesium starvation, by chelation with EDTA. The *mtrA* promoter gave high levels of expression in rich media as well as under the influence of EDTA. The effect on *hspX* during oxygen starvation and *phoA* during phosphate starvation was expected given their proposed function. The induction of both *phoA* and *mtrA* under the influence of magnesium starvation lead to a number of new questions about the influence of magnesium, or at least the chelator EDTA, in relation to the SenX3/ RegX3 and MtrB/ MtrA systems.

A number of assumptions have been made about the conditions experienced by AFB within the macrophage and granuloma which cannot be truly replicated *in vitro*. It is known that *mtrA* is induced after macrophage infection in *M. tuberculosis* and this result brings to light the possibility that magnesium concentration or some other effect of the chelating agent on media may be involved in the MtrB sensor kinase activation.

Chemical library screens were carried out using the *mtrA* reporter expression as an indicator of TCS inhibition, as this promoter produced high levels of F in rich media. A number of compounds decreased the expression of *mtrA* after the incubation period of the library screens. Re-testing of these compounds in triplicate did not confirm a considerable difference in expression. We did, however, unearth a known TCSi in the LOPAC library which was identified as a hit in this screen. Further experiments on this compound and palmitoleic acid, another *cis* fatty acid, indicated that the F induced by *mtrA* was reduced in a dose dependent manner in a serial dilution assay.

Importantly, the screen identified an intrinsic TCSi control, oleic acid, out of thousands of compounds tested. This compound has not previously been shown to inhibit any TCS in mycobacteria.

6.2 Critique of the screen

There are a number of benefits to this screen compared with isolated protein methods. In theory any inhibition of the signal transduction mechanism will be highlighted by this screen. The method can be carried out in a high through put manner to test thousands of compounds at a time in one assay.

This method could be adapted to other targets. As well as finding inhibitors of transcriptional control, the use of GFP translational fusions can be applied to discover inhibitors of proteins which are controlled by other means.

A significant point here is the ability to test for inhibitors of genes that may be essential. Few methods can directly target a gene which the organism cannot live without, but the reduction in *mtrA* promoter activation observed as a result of oleic acid showed that changes in gene expression can be observed in this way without completely inhibiting cell growth. One the other hand, this will depend on the MIC of the inhibitor in question and the concentration of compounds used in a screen. In all likelihood a successful candidate drug which inhibits TCS would also decrease the cell density of a population and thus be missed out in our screen.

However, more potent inhibitors may not have been picked up by our methods. This situation could be remedied by a more relative measure of F, by recording information on a single cell basis with FACs. It comes down to a compromise between high throughput and relativity of gene expression. By using a whole cell

assay thousands more compounds can be screened than in a low throughput single cell counting method such as FACS.

The use of a reporter of gene expression, however accurate, is essentially an indirect method of testing two component signal transduction. We do not know how many other regulators are acting on the genes in this study. Previous work has shown that all of the selected genes contained RR binding sites within their promoters, but this does not exclude the possibility of other factors such as universal sigma factors [100, 108, 150]. The mechanisms controlling gene expression are, in all likelihood, much more complicated than simply one response regulator acting as a transcription factor on a gene. There are probably multiple levels of regulation for every gene in an organism's genome. The constitutively expressed control promoter is one way of showing if a condition or inhibitor is affecting the expression of a specific gene; however in practice the control of these important housekeeping genes could be controlled another way.

Although the TCSi oleic acid was picked up this screen a number of other false positive results were also picked up using this method. Clearly the level of variance in terms of both F and OD observed from screens of this size can lead to false positive results and perhaps screens of this nature should be repeated before selecting hits to re-test. In order to test as many compounds as possible each library was only screened once. This meant that there was no way to test the statistical significance of "hits". In hindsight, this meant that the screen was not an efficient way of finding TCS inhibitors. If each library had been tested in triplicate it is likely that the rate of false positives would have been reduced. Hit selection is an area which requires significant development in order to obtain an acceptable z score. Our

method for manually selecting compounds based on their position on the scatter plot did not render an acceptable hit rate as described in other work on HTS analysis [163].

Inevitably, the number of compounds being screened will affect the degree of accuracy each screen will present, since larger screens give more variable results. Perhaps the parameters of "hit" selection could be optimised.

One aspect of the screen, which was briefly mentioned earlier, is the relativity of results in terms of the number of plasmids per cell. As with all plasmid reporter methods, including *xylE* expression, one must assume that this remains relatively constant between strains and conditions. It is generally accepted that using appropriate controls will balance out any changes in copy number from cell to cell. It is worth noting here that oriM high increased copy number from 5 to anything between 32 and 64 copies per cell raises questions about this assumption of consistency. Large differences in copy number of the plasmid from cell to cell may be the cause of the variance seen in these screens. The benefits of this new origin of replication are convincing (Chapter One), but the resulting variance is something that needs to be considered.

In this study, over 4500 compounds from a number of sources were screened, a fair few less than the 25,000 compounds tested to discover the first TCS inhibitors [127]. A possible future direction in this area could be the study of fewer compounds after a more rational method of compound selection. For example, selecting compounds or classes of compounds identified using an in silico screening approach based on the protein structure of the target. The current work highlighted the potential of *cis* fatty acids, so perhaps this is a class of compounds worth developing.

6.3 Future Direction

The identification of oleic acid in the LOPAC assay and the dose dependent inhibition displayed by oleic and palmitoleic acids indicate that this methodology could be applied to study more compound libraries against the reporter system. Before further libraries are tested, there is a substantial amount of optimisation required in terms of HTS. A number of statistical tests, for example, would help to indicate an improved hit rate as a result of a more refined selection criteria. As well as increasing the number of chemicals tested, there is also the possibility that new TCS controlled genes could be screened in this manner. Other *M. smegmatis, M. bovis* BCG or avirulent *M. tuberculosis* TCS controlled genes could be investigated using this method of screening.

The observation that *cis* fatty acids may inhibit the expression of both *mtrA* and *phoA* is an important finding, as this inhibition has so far only been observed in the *Bacillus subtilis* KinA protein. The method of action has not yet been elucidated, except that fatty acids act in a non competitive manner with respect to ATP during phosphorylation. It would be interesting to solve the crystal structure of MtrB and compare it with that of KinA for homologous sites.

The large number of compounds screened was achieved in this study using a fast growing species of mycobacteria. Slow growers would require lengthy assays, but could also potentially be screened either with entire libraries or a selection of hits discovered by assays with fast growers.

Another application of these methods is the study of genes induced within the macrophage. In particular, *mtrA* expression, which has been previously shown during macrophage expression, could induce GFP at a much higher level during intra-macrophage growth. If the difference between induced and un-induced GFP is more apparent, the level of variance seen in these screens could be reduced.

As well as TCS, other signalling such as Serine Threonine Protein Kinases and one-component regulators could be selected and screened for in this manner. DevS/DevR and other dormancy related targets would be highly appropriate targets to develop in this sort of screen.

PhoP/ PhoR could also be targeted in this manner by analysing the expression of the gene under its control. The benefits of inhibiting this system would be plentiful, as it is so important to the survival and virulence of *M. tuberculosis.* Its significant role in cell wall maintenance would make any inhibitor capable of knocking out this signalling mechanism an excellent drug candidate. Even if it did not inhibit growth outright, its use in combination with other drugs such as cell wall targets could significantly reduce the treatment time required for TB.

Other parts of this thesis underline the need for further studies into the conditions of expression of TCS controlled genes. In particular *mtrA;* how and why this gene is expressed and its protein product's function within the cell are all important questions, due to its significance as an *M. tuberculosis* essential gene. This work has revealed a possible condition of its expression to be magnesium starvation. Further work to validate this may help to understand its function and potential as a drug target.

There are generally two ways to go about discovering drug targets for a particular pathogen. We argue that a targeted approach to significant genes involved in latency, dormancy and survival within a macrophage cell may be a better way to prevent TB than exploring any and all compounds which affect the growth rate of the species. For a number of reasons, *M. tuberculosis* requires a different approach to drug discovery, compared with rapidly growing pathogens. The high incidence of drug resistance, its extended incubation and latency period and its apparent switch to a reductive metabolism make *M. tuberculosis* a difficult pathogen to eradicate. Screening for compounds, and selecting "essential" targets by measuring simply how they affect the level of growth of the organism in rich media is not the best representation of what the pathogens really cannot survive without *in vivo*. The *mtrA* gene has been labelled essential, however, its role in macrophage infection has also been established, making it the sort of target which should be explored further.

Similarly, expression of the *hspX* reporter plasmid represents the switch to a reductive metabolism, something which makes the pathogen extremely successful. As a drug target, or an indicator of the cell dormancy program, this gene is highly useful in the study of latency. Compounds which affect this and other dormancy related targets could be worthy candidates in the effort to stop TB.

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APPENDICES

Appendix 1: PCR reactions

The PCR reactions carried out in this study have been displayed below in A.1. Generally PCRs were carried in out using one of the three protocols depending on the desired fragment length and fidelity of PCR required. High Fidelity polymerase was used to clone promoters, *gfpmut2* and *oriM* high. In the case of screening for fragment insertion into a plasmid, a regular polymerase was used such as Mastermix (Bioline). Modifications to annealing temperatures were made depending on particular primer melting temperatures in some cases.

A.1 PCR reactions in this study

a) High Fidelity PCR: Standard protocol for amplifying promoters and *gfpmut2*

Temperature	Time	Cycles
94^{o}C	10 minutes	
94^{o}C	1 minute	30
56^{o}C	1 minute	
68^{o}C	1 minute	
12^{o}C	hold	

b) High Fidelity PCR: Gradient protocol for amplifying long fragment *oriM* high

Temperature	Time	Cycles
94^{o}C	10 minutes	
94^{o}C	1 minute	30
55 -65^{o}C	1 minute	
68^{o}C	1 minute	
68^{o}C	10 seconds	
12^{o}C	hold	

c) Standard PCR: Screening fragment insertion using pTKmx_F1 primer

Temperature	Time	Cycles
94^{o}C	10 minutes	
94^{o}C	1 minute	30
44^{o}C	1 minute	
72^{o}C	1 minute	
72^{o}C	10 seconds	
12^{o}C	hold	

Appendix 2: Ligation Reactions

A number of ligation reactions were carried out in this screen. The method used was altered depending on whether the reaction involved one or two restriction enzyme sites. Since the pSHigh plasmid had only one cloning site infront of *gfpmut2* the plasmid was first incubated with CIP to reduce the amount of self ligation occurring in the reaction. Consequently a larger volume of plasmid was added to these reactions as the concentration was reduced by the DNA clean up step after CIP incubation. The volume of fragment added was also increase to give a more efficient reaction by increase the fragment to plasmid ratio. In addition the efficiency off each reaction was observed by carrying out a negative control with distilled water, as the number of colonies on the negative control plate were indicative of self ligation of the plasmid. The following tables (A.2 a, b and c) describe the stock concentrations and volumes of reagents used in this study for cloning into a) cloning vectors, b) pOT71 and c) pSHigh.

A.2 Ligation Reactions used in this study

	Stock Concentration	In a 10 µL reaction
Promoter fragment	variable	7 µL
TA cloning vector	1 ng	1 µL
T4 ligase	0.4U/ µL	1 µL
T4 ligase buffer	10 x	1 µL

a) Ligation reaction for cloning vectors

	Stock Concentration	In a 10 µL reaction
Promoter fragment or SDW	variable	7 µL
Reporter plasmid pOT71 (after digestion with *Eco*RI and *Bam*HI)	variable	1 µL
T4 ligase	0.4U/ µL	1 µL
T4 ligase buffer	10 x	1 µL
***Kpn*I**	1U/µL	1µL

b) Ligation reaction for pOT71

	Stock Concentration	In a 10 µL reaction
Promoter fragment or SDW	variable	20- 30 µL
Reporter plasmid pSHigh (after digestion with *Kpn*I and incubation with CIP)	variable	2 µL
T4 ligase	0.4U/ µL	2 µL
T4 ligase buffer	10 x	2 µL

c) Ligation reaction for pSHigh

Appendix 3: Restriction Enzyme Digestion

Restriction enzyme reactions were carried out according to the manufacturer's instruction using appropriate buffers for each reaction. This information available from NEB. Checking for insertion of fragment to cloning vector required only a single enzyme digest desctibed in table A.3 a). Other digestion reactions involving the removal of fragments, or cutting of a plasmid before fragment insertion were carried out with a longer incubation period and appropriate enzymes. Restriction enzyme digestion reactions to remove the fragment from the cloning vector or pre ligation cutting of reporter plasmids are displayed in tables A.2 b) for pOT71 and c) for pSHigh. The reaction volume was altered in some cases to increase the final volume of cut DNA.

A.3 Restriction Enzyme Digestion

	Stock Concentration	**In a 10 µL reaction**
Cloning Vector + insert	Variable	8 µL
***Eco*RI**	1U/ µL	1 µL
***Eco*RI buffer**	10x	1 µL

a) Digestion of cloning vectors to check for insert

	Stock Concentration	**In a 10 µL reaction**
Cloning vector + insert/ pOT71	variable	7 µL
***Eco*RI**	1U/µL	1 µL
***Bam*HI**	1U/µL	1µL
NEB buffer 2	10 x	1µL

b) Digestion of pOT71 plasmid before insertion of promoters

	Stock Concentration	**In a 10 µL reaction**

Cloning vector + insert/ pSHigh	variable	7 µL
***Kpn*I**	1U/µL	1 µL
BSA	10 x	1µL
NEB buffer 3	10 x	1µL

c) Digestion of pSHigh plasmid before insertion of promoters

Appendix 4: Preparation of Calcium Competent DH5α Cells

Calcium competent *Escherichia coli* DH5α cells were prepared for use in cloning procedures by the following protocol, ensuring all solutions and cells were kept on ice during preparation. TFB I and II are solutions used to induce competency as described by Hanahan et al [172] and are made up with the following concentrations of metal salts, glycerol and sterile distilled water before sterile filtration with a 0.22µM Millipore filter:

TFB I:	**for 100mL**	**final concentration**
$MnCl_2$	0.629g	50mM
$CaCl_2$	0.147	10mM
K acetate	0.294	30mM
Glycerol	15mL	15%
SDW	85mL	

TFB II	**for 100mL**	**final concentration**
Na MOPS (pH7)	0.209	10mM
$CaCl_2$	0.629	75mM
KCl	0.075	10mM
Glycerol	15mL	15%

DH5α cells from a freezer stock were incubated with agitation at 37^{o}C overnight in 3ml liquid cultures of LB with 10mM $MgCl_2$ with a negative media control to ensure there was no contamination. Cells were then inoculated into four 50mL cultures of the LB with 10mM $MgCl_2$ in 250mL flasks and grown for around two hours until their OD_{600} was between 0.4 and 1.0. Cells were then transferred to 50mL flacon tubes and stored on ice to inhibit further growth. Cell suspensions were then spun down in a refrigerated centrifuge at 4 ^{o}C for 10 minutes at 5000rpm and resuspended in 40 mLs of competence inducing TFB I solution before further incubation on ice for two hours. Each culture was then spun down again at 4 ^{o}C for 10 minutes at 5000rpm and resuspended in 4mLs of TFB II solution and kept on ice. Cells suspended in TFB II were transferred to eppendorf tubes and snap frozen then stored at -80 ^{o}C until required for transformation.

Appendix 5: Preparation of Electrocompetent *Mycobacterium smegmatis* mc^2155

Electrocompetent cells were prepared by a method revised from Sambrook and Russel Volume 1, 1.12-1.14 [157] .

Mycobacterium smegmatis mc^2155 cells from an LA plate were incubated with agitation at 37 ^oC overnight in 10ml liquid cultures of LB with a negative media control to ensure there was no contamination. 2 – 3 mLs of this cells culture was inoculated into 100mLs of LB and grown for around five hours until their OD_{600} was between 0.5 and 1.0. Cells were then transferred to two 50 mL falcon tubes and stored on ice to inhibit further growth for 20 minutes. Cell suspensions were then spun down in a refrigerated centrifuge at 4 ^oC for 10 minutes at 5000 rpm and re-suspended in 50 mL 10% cold glycerol. This incubation and pellet step was repeated three times before cells were finally re-suspended in 2mLs of cold 10% glycerol before they were dispensed into eppendorf tubes and stored at -80^oC until required for transformation.

Appendix 6: Media used in this study for DH5α cells

DH5α cells used for cloning were grown on solid media Luria Agar (LA) or Luria Broth (LB) both from Sigma. Overnight cultures of TA cloning vector containing cells were grown in LB supplemented with Ampicillin and Kanamycin when using pCR2.1 or just Ampicillin for pLUG vector. For blue/white colony selection, using β-galactosidase gene disruption, tranformed cells were grown on LA containg the same concentration of antibiotics as well as IPTG and Xgal.

Solid Media (LA)

- Kanamycin 50 µg/mL
- Ampicillin 200 µg/mL
- Xgal 40 µg/mL
- IPTG 100 µg/mL

Liquid Media (LB)

- Kanamycin 50 µg/mL
- Ampicillin 200 µg/mL

Appendix 7: Media used in this study for mc^2 155 cells

All plasmids used in this study contained the Kanamycin resistance cassette *ophA.* Accordingly, all experiments using mc^2 155 were supplemented with Kanamycin on both solid and liquied media. In order to obtain planktonic growth of liquid cultures, media was also supplemented with D-arabinose and $Tween_{80}$. All media was autoclaved for sterilisation before use.

Solid Media (LA)

- Kanamycin 50µg/mL

Liquid Rich Media (LB)

- Kanamycin 50µg/mL
- D-arabinose 100µg/mL
- 0.1% $Tween_{80}$

Nutrient starvation experiments were carried out using Hartman deBonts minimal media made from a base of metal salts, $Tween_{80}$ and SDW. Phosphate and Nitrate sources were added to this base from stock solutions names 100X P and N respectively, while glycerol was added as a Carbon source.

Metal Salts	**(in 1L SDW)**	**final concentration**
• $CaCl_2.2H_20$	0.10 g	1 mM
• $CoCl_2.2H_20$	0.04 g	240 µM
• $CuSO_4.5H_20$	0.02 g	80 µM
• EDTA	1.00 g	3.4 mM
• $FeSO_4.7H_20$	0.50 g	1.8 mM
• $MgCl_2.6H_20$	10.00 g	50 mM
• $MnCl_2.2H_20$	0.10 g	621 µM
• $NaMoO_4.2H_20$	0.02 g	90 µM
• $ZnSO_4.7H_20$	0.20 g	732 µM
100 x Nitrogen		
• $(NH_4)_2SO_4$	200 g	1.5 M
100 x Phosphates		
• K_2HPO_4	155 g	1 M
• NaH_2PO_4	85 g	700 mM

HdeB base was made using 945 mL of SDW with 10mL metal salts and 5 mLs 10% $Tween_{80}$. For each sort of media, mimimal, Carbon starvation, Nitrogen starvation and Phosphate starvation, 960mL of HdeB base was used as well as nitrogen, carbon and phosphate sources as shown in the table below. For

nutrient starvation experiments, the 4 levels of starvation were investigated using a range from 0% -100% as highlighted.

	HdeB Base (mL)	100x Nitrogen (mL)	100x Phosphates (mL)	10% Glycerol (mL)	SDW (mL)	5M NaMOPs (mL)
Minimal Media	960	10	10	20	0	
Nitrogen Starvation	960	0.1 - 10	10	20	9.9 – 0.1	
Phosphate starvation	960	10	1 - 10	20		10 - 1
Carbon Starvation	960	10	10	8 - 20	12 - 0	

Table A.3 Alterations to HdeB media for nutrient starvation