DISSERTATION

DRUG DISCOVERY FOR *FRANCISELLA TULARENSIS* AND *MYCOBACTERIUM TUBERCULOSIS*

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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Fort Collins, Colorado

Summer 2009

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May 12, 2009

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KATHEEN ENGLAND ENTITLED DRUG DISCOVERY FOR: FRANCISELLA TULARENSIS AND MYCOBACTERIUM TUBERCULOSIS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

DRUG DISCOVERY FOR
FRANCISELLA TULARENSIS AND *MYCOBACTERIUM TUBERCULOSIS*

Today, we are faced with the challenges of fighting infectious organisms that have either been neglected or have developed ways to resist current treatments. Longstanding public health problems, such as tuberculosis, have evolved into mulitdrug-resistant bacilli, tolerant to current drug regimens. In addition, neglected diseases such as *F. tularensis* have concerns regarding their use in bioterrorism. Current research initiatives search for novel chemotherapeutics to alternative targets in order to resolve the problem of resistance and the continued battle against infectious disease. The research presented utilizes both genomic and structure-based approaches to identify new drug targets for *Mycobacterium tuberculosis* and optimize a series of diphenyl ether compounds with specific inhibitory activity against ftuFabI, the enoyl ACP-reductase enzyme of *Francisella tularensis*.

Structure-based design efforts have provided a lead diphenyl ether compound, 5-hexyl-2-phenoxyphenol (SBPT04), demonstrating high affinity enzyme inhibitory activity, *in vitro* bactericidal activity, and *in vivo* efficacy against *F. tularensis* in the murine model of infection by both intraperitoneal (i.p.) and oral (p.o.) routes. SBPT04

cleared infection by day 4 of treatment (200mg/kg/dose) with 100% survival rates and no relapse of disease for over 30 days by i.p. delivery. Oral delivery exhibited delayed dissemination with significant efficacy. Pharmacokinetic analysis revealed sufficient bioavailability upon i.p. delivery. *In vitro* metabolism studies suggested limited phase I oxidation with phase II O-glucuronide as the primary metabolite. In addition to being a potent lead, this work establishes diphenyl ethers as a platform for the development of broad-spectrum agents to other pathogens in addition to tularemia.

Genomic-based strategies were used to identify novel drug targets involved in cell cycle regulatory events of *Mycobacterium tuberculosis*. Homologues to proteins involved in SOS responses and min-system regulation that direct cell division events were identified and characterized via morphological changes and gene expression profiles induced by gene dosage experiments. Transcriptional responses revealed connections between cells that have stopped dividing with conditions defined for bacilli in a nonreplicating persistent state. Understanding regulatory events of cell cycle progression and identifying specific players will allow the design of compounds, which may either prevents bacteria from entering a nonreplicating persistence or regulate the events of reactivation.

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ACKNOWLEDGEMENTS

Of highest regard, and the person who profoundly impacted my graduate career at CSU, my advisor and mentor, Dr. Richard Slayden, I wish to give my sincerest gratitude. In addition to his technical instruction and drive for excellence in research, he provided me with continued support on many levels and a confidence in my abilities as a scientific researcher and intellectual.

I would like to also offer a sincere thank you to all my committee members who guided me through the processes and directed my graduate studies.

I am grateful to Dr. Herbert Schweizer and members of the interview committee, for allowing me the opportunity to enter this program and fulfill one of my life goals; achieving a doctor of philosophy in an infectious disease related science. This opportunity will hopefully lead me into a path in which I may contribute actively in the fight against emerging and endemic diseases.

A special thanks is given to all the Slayden lab members past and present for their guidance, instruction, intellectual support, and friendship. A sincere thank you is given to Susan Knudson for her moral support and continued guidance.

DEDICATION

I dedicate this dissertation to my children; Miguel, Bradley, and Grace. Thank you for your understanding of your Mom's absence and her desire to pursue her career. Your continued encouragement and strength to deal with the constant challenges we faced during these past six years provided me the conviction and drive to succeed. I hope to be standing beside you all one day, as you embark on your own journeys in life and paths to success.

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LIST OF ABBREVIATIONS

16s rDNA	gene coding for 16s rRNA
16s rRNA	long component 30S ribosomal subunit
23S rRNA	long component 50S ribosomal subunit
3-D	three dimensional
ADME	adsorbtion, diffusion, metabolism, and excretion
Anova	Analysis of variance
ATP	adenosine triphosphate
AUC	area under the curve
B.C.	before christ
BLAST	Basic logical alignment search tool
BLASTP	Basic logical alignment search tool for proteins
BLASTX	Basic logical alignment search tool for nucleotides
C57BL/6	genetically modified mice for use as models of human disease
CaCl2	calcium chloride
CD4	cluster of differentiation 4, specialized white blood cell
CD8	cluster of differentiation 8, specialized white blood cell
CDC	Centers for Disease Control
cDNA	complementary DNA
cfu (CFU)	colony forming unit
Cl	clearance
Cmax	maximum concentration
CMR	Comprehensive Microbial Resource
CR	Complement receptor (CR1,CR3...)
Cy3/Cy5	reactive water-soluble fluorescent dyes of the cyanine
D5W	5% dextrose in water
DNA	deoxyribonucleic acid
DOTS	direct observed therapy and surveillance
E.	*Escherichia*
EHR	enduring hypoxic response
EMB	ethambutol
EMBOSS	The European Molecular Biology Open Software Suite

ERs	Emergency rooms
EtOH	ethanol
F	bioavailability
F.	*Francisella*
FASI	Fatty Acid Biosynthesis Type I pathway
FASII	Fatty Acid Biosynthesis Type II pathway
FBS	fetal bovine serum
FDA	Food and Drug Administration
HIV	human immunodeficient virus
HLM	human liver microsome
HMM(s)	Hidden Markhov Model(s)
hr(s)	hour(s)
HTS	high through-put screening
i. p. (IP)	intraperitoneal
i. v. (IV)	intravenous
IC50	inhibitory concentration that will kill 50%
ICR	Imprinting Control Region
IND	Investigational new drug
INH	isoniazid
K1/K2	kintetic rate constants step 1 and step 2
Kapp	apparent rate constant
Kd	rate dissociation constant
Kg (kg)	kilogram
Ki	inhibitory rate constant
LC/MS/MS	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
LC50	lethal concentration that will kill 50%
LLOQ	lower limit of quantification
logP	octanol/water partition coefficient
LPS	lipopolysachharide
LVS	live vaccine strain
M.	*Mycobacterium*
MABA	molecular alamar blue assay
MAGP	mycolyl-arabinogalactan-peptidoglycan complex
MBC	minimal bactericidal concentration
MDR	multidrug resistant
μg	microgram
mg	milligram

MgCl2	magnesium chloride
MIC	minimal inhibitory concentration
MIC90	minimal inhibitory concentration that will kill 90%
min	minute
mL (ml)	milliliter
μL (μl)	microliter
MLM	mouse liver microsome
μM	micromolar
mM	millimolar
MMC	mitomycin C
MMH	modified Mueller-Hinton broth
MR	Mannose receptor
MRSA	Methicilin resistant *Staphylococcus aureus*
Msm	*Mycobacteria smegmatis*
Mtb	*Mycobacteria tuberculosis*
MUSCLE	multiple sequence comparison by log-expectation
NAD/NADH	nicatinamide adenine dinucleotide
NCCLS	National Committee for Clinical Laboratory Standards
NDA	new drug approval
ng	nanogram
NIAID	National Institute of Allergy and Infectious Diseases
nM	nanomolar
NMR	nuclear magnetic resonance
NO	nitric oxide
O.D.600nm	optical density at a wavelength of 600 nanometers
OADC	oleic acid, albumin, dextrose, catalase
ORF(s)	open reading frame(s)
OsO4	osmium tetroxide
p.o. (PO)	per oral (per os)
P450 (CYP)	diverse superfamily of hemoproteins, cytochrome
PAE	post-antibiotic effect
PAE-SMC	post-antibiotic effect at submicroconcentrations
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PE	proline-glutamine rich family of proteins in *M.tuberculosis*
PE-PGRs	proline-glutamine family with glycine-arginine rich domains
pH	power of hydrogen, acidity scale

pKa	acid-dissociation constant
PKPD	pharmacokinetic and pharmacodynamic parameters
POA	pyrazinoic acid
PPE	proline-proline-glutamine rich proteins in *M.tuberculosis*
pyMOL	open source visualization tool available for structural biology.
PZA	pyrazinamide
Q/RT-PCR	Qunatitative real-time polymerase chain reaction
QPCR	Quatitative polymerase chain reaction
RBH	recipricol best hits
RIF	rifampicin
RNA	ribonucleic acid
ROI(s)	reactive oxygen intermediate(s)
RPMI	Rapid Prototyping and Manufacturing Institute
rRNA	ribosomal RNA
S.	*Staphylococcus*
SAR(s)	structure activity relationships
SBPT04	5-hexyl-2-phenoxyphenol
SCID	severe combined immunodeficiency
SEDDS	self-emulsifying drug delivery system
SEM	scanning electron microscope
Ser	serine
SI	selective index
SMEDDS	self-microemulsifying drug delivery system
SOS	Morse code distress signal
SPA-R	Surfactant protein A receptor
t1/2	half-life
TB	tuberculosis
TBLASTN	Basic logical alignment search tool: nucleotide into protein
Thr	threonine
TIGR	The Institute for Genomic Research
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
Tmax	time of maximum concentration
Tn	transposon
tRNA	transfer RNA
U.S.	United States
UDP	uridine diphosphate

UPEC	Uropathogenic *Escherichia coli*
Vd	volume of distribution
VRE	Vancomycin resistant *Escherichia coli*
WHO	World Health Organization
XDR	extensively drug resistant

CHAPTER 1

Literature Review Part I: Bacterial Pathogens

1.1 *FRANCISELLA TULARENSIS*

1.1.1 A brief introduction

Francisella tularensis is the causative agent for the human disease, tularemia. In 1911, the zoonotic form of the disease was first recognized in ground squirrels suffering from a "plaguelike" disease in Tulare County, California [1]. By 1925, Edward Francis began to correlate human clinical syndromes such as "rabbit fever", "meat-cutter's disease", "deer-fly fever", and "glandular-type tick fever", with the zoonotic transmission of the bacterium, *Bacillus tularense* [2, 3]. Human infections arose from arthropod bites, handling infectious animal materials, and later were identified with the inhalation or the ingestion of the bacterium [3-6]. Over the past century, tularemia has been identified in several parts of the world, primarily in the northern hemisphere with recent emergent cases in Australia (2003) and Thailand (2008) [7-9]. On average there are approximately 120 cases reported annually in the United States primarily in Missouri, Arkansas, South Dakota, and Oklahoma [10]. However, it is thought that this pathogen has been under recognized throughout the world, especially in developing countries.

Originally the bacterium was classified under the genus *Pasteurella*; however, with the use of 16S rDNA analysis it was identified as having a relationship to the γ-subclass *Proteobacteria* [11, 12]. Since this was not a strong relationship, the organism was placed in its own genus, *Francisellacea* [11-13]. *Francisella* species *tularensis* has four primary subspecies: *tularensis, novicida, holarctica,* and *mediaasiatica,* all of which can cause disease [14]. However, the most virulent of these subspecies is *Francisella tularensis tularensis* has a low infectious dose and can have high mortality rates of up to 60 % if untreated [15, 16]. This feature of the bacterium made it of interest for the development as a biowarfare agent.

During the 20th century, Japan, the Soviet Union, and the United States incorporated *F. tularensis* into their bioweapons programs [17-19]. In fact, the use of *F. tularensis* and other agents on humans in various Japanese germ warfare research facilities that began in the 1930s under the direction of Major Ishii Shiro are well documented [17]. These research programs were designed to evaluate the effects that biological agents and toxins had on humans and to perform field tests for the evaluation of their use in combat. Research continued into the cold war as both the Soviet Union and the United States performed bioweapons research on a variety of highly infectious bacterial agents, including *F. tularensis* [18, 19]. These experiments provided insight into the virulent nature and disease pathogenesis of the organism, along with the development of a vaccine and treatment regimen [15, 16, 20, 21]. Initial vaccine studies that were conducted defined the minimal infectious dose as 10 organisms and demonstrated protective immunity from an attenuated vaccine [15, 16]. Early experiments noted no human-to-human transmission, as infected

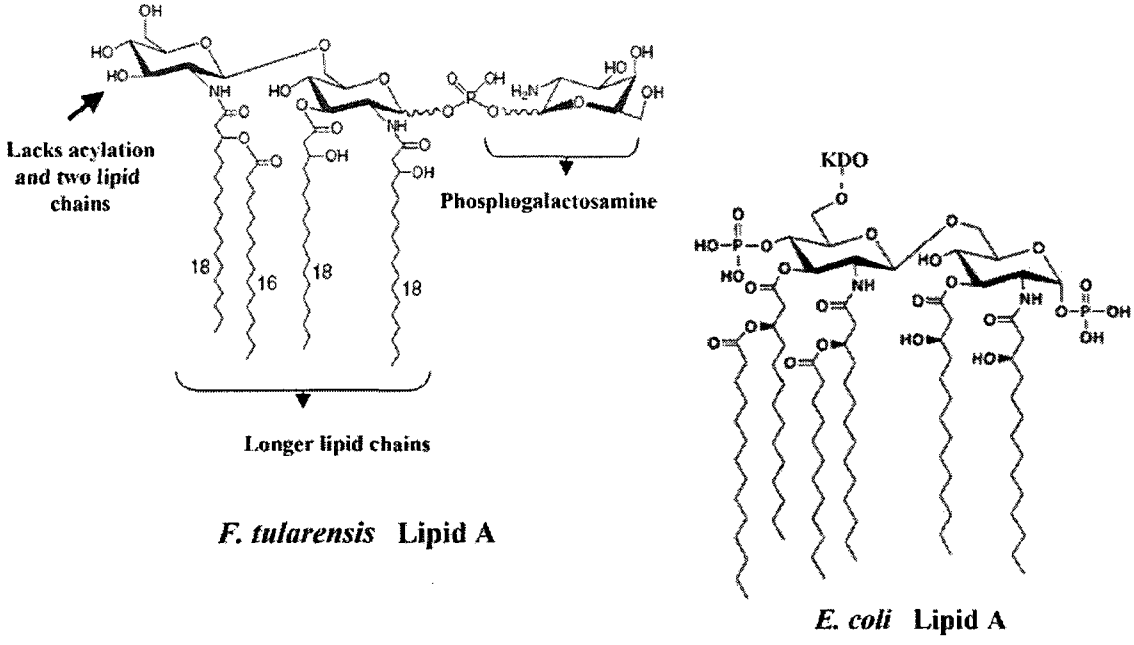
volunteers were not housed in isolation and the disease demonstrated no form of communicable transfer [20]. In addition, it has been established that engineered strains of the bacterium were designed with resistance to recommended antibiotics and vaccines [22, 23]. Later that century, a Biological Weapons Conference Treaty was formulated and signed by both the United States and the Soviet Union (the superpowers at that time) along with other countries, prohibiting the development, production, stockpiling, and transfer of biological or toxin agents [23]. However, it is unclear today as to whether or not other countries have developed or are developing bioweapons containing virulent pathogens such as *F. tularensis*. Today, *F. tularensis* is listed as a Category A priority pathogen by the NIAID, with research being conducted toward the development of new diagnostics, alternative therapies and new vaccines as an act of biodefense and preparedness [24].

1.1.2 The bacterium

Francisella tularensis is a Gram-negative facultative intracellular aerobic bacterium that is found in a diverse range of animal hosts and habitats. Interestingly, the organism has been found living freely in waterborne protozoa, which has been suggested to be an important environmental reservoir [25]. The bacterium has specific nutritional requirements that include iron, essential amino acids, sulfhydryl compounds, thiamine, and a source of phosphorus making it unlikely to be a free-living organism [4].

Francisella tularensis has an outer membrane composed of a unique structure of LPS (lipopolysaccharide) that lacks two fatty acid chains at the O-3 position of the

hexosamine II in a dephosphorylated lipid A backbone [26, 27]. The fatty acids that make up the structure are much longer than those of other Gram-negative bacteria, such as *Escherichia coli* (Figure 1.1) [26-28]. Other differences are found in the core region of the LPS molecule, which contains single 3-deoxy-D-manno-octulosonic acids and lacks heptose [26]. Alterations in the LPS structure may explain its reduced endotoxin activity and the limited induction of initial host responses, which allow the bacterium to enter and replicate efficiently in macrophages [29, 30].



***F. tularensis* Lipid A** **E. coli Lipid A**

Figure 1.1 Lipid A structures for *F. tularensis* and *E. coli.* *Francisella* Lipid A lacks O-acylation and two lipid chains off the second hexosamine, has an added phosphogalactosamine substituent, and longer lipid chains. Alterations in structure are associated with decreased endotoxin activity. (as reported by Phillips et al., 2004 and Holst et al., 1993) [27, 28]

In addition, the O-antigen structure of highly pathogenic subspecies differs from less pathogenic subspecies, identifying a possible virulence component [31-33]. Another structural feature of the bacterium is its exopolysaccharide capsule, which protects the bacterium from compliment during host infection [34]. The capsule is thought to be involved in virulence as experiments using decapsulated bacteria and mutants of genes involved in capsule biogenesis (*capBCA*) showed attenuation in mice [34, 35].

The genome of *F. tularensis* is small and contains more than 1,800 open reading frames (ORFs). Over 300 are unique to *Francisella tularensis tularensis* and are postulated as genes associated with its highly virulent nature [36-38]. In addition, there are approximately 200 pseudogenes or inactivated genes that may explain a loss of specific metabolic processes, which have directed the organism toward an intracellular lifestyle. Gene reduction often occurs in pathogens that, over time, evolve altered lifestyles and become host dependent. This is reasonable, as *F. tularensis* has over 14 nutritional requirements that are supplemented for growth on medium. The most unique feature in the genome is a duplicated 33.9kb region that contains 25 genes with no known bacterial homologues to other γ-*Proteobacteria* genomes [37]. Disruptions of the genes in this region (*iglC and pdpA*) generate bacteria that are no longer able to survive in macrophages and show attenuation in mice [39-41]. This region is referred to as the *Francisella* pathogenicity island (FPI), and is thought to encode many virulence attributes. Duplication of these genes implicates their importance for the organism's survival. Additional bioinformatic analyses of the elements in this region revealed homology to a Type VI secretion system similar to those recently identified in *Pseudomonas aeruginosa* and *Vibrio*

cholerae. [42-44]. The secretion system is postulated to be upregulated during infection and involved in the translocation of effecter molecules that modulate the host cell environment for intracellular survival. Finally, the bacterium encodes several Type IV pili, which have been visualized on the surface of the cell [45]. Type IV pili are typically involved in adhesion, conjugation or the exchange of DNA, and motility. Type IV pili (PilF and PilT) found in *F. tularensis* are proposed to have roles in host cell adhesion and virulence [46]. Many of the virulence attributes of *F. tularensis* subspecies *tularensis* remain unknown and are under current investigation.

1.1.3 Infection and disease

Upon infection, *Francisella tularensis* is capable of invading a variety of host cells, but primarily target macrophages where they are able to replicate in high numbers. Uptake of the organism, similar to most bacteria, is complement mediated, and involves a unique process of entry through the induction of actin organized pseudopod-like loops [47]. Components underlying this unique method of phagocytosis have yet to be defined; however, studies implicate a role for surface carbohydrates, possibly LPS, in modulating conventional phagocytosis of the bacterium [47]. Other surface components, such as the capsule or pili, demonstrated no alterations to the process when inactivated or removed [47]. It is possible that the unique structure of *Francisella* LPS may contribute to a limited or transient affinity to compliment molecules, reducing the number on the surface of the bacterium, promoting a loosely associated interaction with the host cell membrane.

Immediately after entry into the phagosome, the vesicle constricts tightly around the bacterium, the bacterium enters early endosomal stages, prevents phagolysosome fusion, and blocks phagosomal acidification [48]. Shortly after modulation of phagosome maturation, the bacterium escapes into the cytosol, which supports intracellular survival and replication [48, 49]. AcpA, a respiratory burst inhibiting acid phosphatase is reported to promote intracellular survival and phagosomal escape [50]. This protein is thought to have various functions, which include phospholipase activity to hydrolyze the phagosomal membrane allowing escape into the cytosol, disruption of host signaling via dephosphorylation of host signal proteins that induce respiratory burst, or the amplification of proteins (IglC and MglA) required for intracellular survival [50]. Recent work has established that early events in phagosomal maturation are critical for phagosomal escape, and that these events control subsequent activities of the bacterium (i.e. expression of FPI proteins) required for survival within the intracellular macrophage environment [51]. Extensive replication within the macrophage eventually leads to cell apoptosis and further spread of the bacterium for uptake by more host cells. An increase in bacterial burden in affected organs leads to dissemination of the disease and severe illness. Recent experiments conducted in mice after intranasal infection suggest that the primary factors that promote the success of this pathogen are its ability to evade macrophage killing and replicate into high bacterial burdens, its limited early control by host innate immune responses (i.e. delayed neutrophil infiltration and delayed proinflammatory cytokine response), intracellular protection from host proteases,

antibodies and compliment, and finally, the probable induction of a massive cytokine storm provoked later in the infection similar to that associated with severe sepsis [52].

Tularemia can present itself in a variety of clinical forms (ulceroglandular, oculoglandular, oropharyngeal, pneumonic, typhoidal and septicemic) that are dependent upon the route of infection, the dose upon infection, and the virulent nature of the infecting subspecies [4-6]. After incubation of 3-6 days, symptoms are flu-like consisting of fever, chills, malaise, sore throat and headache, with advanced disease presenting chest pain and cough [5, 6, 15, 16, 20, 53]. Infection is found in several organs: liver, lungs, spleen, lymph nodes, and kidneys. The most severe form of disease can arise from inhalation resulting in pneumonia that can lead to systemic disease and death [20]

1.1.4 Antibiotic therapy and concerns

Reports by early physicians before the discovery of antibiotics established tularemia as a debilitating and deadly disease [5, 6]. Often patients were chronically ill for months, some with relapsing disease, while more severe cases lead to death. After assessing the therapeutic effect of several antibiotics, streptomycin became the drug of choice over tetracycline, chloramphenicol, and gentamicin as it had a higher cure rate with no relapse of disease [5, 54]. Today, both streptomycin and gentamicin are the preferred treatments for tularemia, with recommended doses of 1 g IM twice daily and 5mg/kg IM or IV once daily, respectively [22, 54]. Treatment duration should continue for 10 days and patients should be monitored for toxic effects. Drawbacks to these two drugs are in their method of delivery (by injection or

infusion), and that they both elicit ototoxic and nephrotoxic side effects [22, 54, 55]. In addition, gentamicin is less effective than streptomycin, and in some cases, relapse of disease was observed. The fact that neither of these drugs can be administered orally limits their use for a mass exposure setting from an act of bioterrorism. The issuance of these drugs to tens of thousands of infected or exposed people by IV therapy would be extremely difficult and highly expensive.

Other antibiotics such as tetracyclines and chloramphenicol have been previously used in the clinical setting, but these drugs are prone to cause relapse due to their bacteriostatic nature [5, 21, 54]. Limited risk of relapse with these drugs is observed with an extended therapy of 14 days, decreasing the bacterial burden in order to allow host cell-mediated immune responses to develop and eliminate the infection. Chloramphenicols were used in the early 1950s and 60s but are rarely used today due to their severe hematological effects [56].

Other alternative treatments include doxycycline and ciprofloxacin. Treatment with doxycycline involves a 14-21 day regimen of 100 mg twice daily [22]. Treatment with ciprofloxacin requires a 10-day regimen of 500 mg twice daily [22]. Both drugs can be administered orally, which makes them more suitable for distribution to a large population after a mass release from an act of bioterrorism. In studies by Russel et al. (1998), these two drugs were compared in their use as a prophylaxis and treatment for low dose aerosol infections of virulent *F. tularensis* (SCHU S4) in mice [57]. Both drugs offered prophylactic protection and therapeutic efficacy, but in both cases, relapse was observed after treatment [57]. Clinically,

ciprofloxacin has shown promise with less virulent strains of *F. tularensis* [54, 59, 60].

The need for new therapeutics effective against highly virulent forms of *F. tularensis* has arisen due to concerns of its potential use as a bioterrorist agent. Most of the current therapies are effective at treating less virulent strains. In addition, successful treatment of inhaled highly virulent *F. tularensis* has been noted under controlled human experimentation [16, 20]. However, in the event of an aerosol release of this agent, major concerns would include the dose upon exposure and the use of engineered resistant strains or strains with enhanced virulence. Additionally, the need for new broad-spectrum alternatives is relevant for all select agent pathogens, in that many present similar symptoms. Disease presentation for many of them correlates with community-acquired syndromes like influenza and other respiratory illnesses. Therefore, by the time the medical community is aware of the situation, the need for rapid dissemination of orally administered drugs that are highly effective at killing a wide range of pathogens is critical. Extremely high numbers of patients with flu-like syndromes entering the ERs of a densely populated urban community would definitely raise suspicion, as current awareness has been heightened from a relatively recent event involving the deliberate release of anthrax through the use of the U.S. Postal Service in 2001. Regardless, current treatment options for *F. tularensis* have several limitations related to method of delivery, toxicity, and relapse of disease upon treatment cessation. With all of this understood, there is an undeniable need for the development of novel chemotherapeutics with unique mechanisms of actions for the treatment of pulmonary tularemia.

1.2 *MYCOBACTERIUM TUBERCULOSIS*

1.2.1 A brief history

Tuberculosis has been a source of human disease for thousands of years. One of the earliest reports of presumptive tuberculosis was identified in the archeological remains of a Neolithic skeleton exhumed near Heidelberg, Germany [61]. The disease has been well documented in ancient Egypt and Nubia through art forms and skeletal remains [61-63]. Spinal tuberculosis, or Pott's disease, is most often noted in archeological remains and depicted in artifacts [61, 63]. Through the use of molecular technology, DNA extracted from bone samples of ancient Egyptian mummies marked the occurrence of tuberculosis as far back as 3000 B.C. [61]. Other archeological findings suggest that tuberculosis was a global disease infecting Chinese, Indo-European, and pre-Columbian civilizations [63].

For centuries, tuberculosis plagued the world with suspicion of its infectious nature. However, it was not until the late nineteenth century that Jean-Antione Villemin demonstrated the disease as infectious by inoculating various animals with liquid from a tuberculous cavity of a cadaver [63, 64]. Months later, the animals were necropsied and showed extensive disease. Additional experiments by Koch demonstrated a clear correlation between the disease and a small microorganism now known as *Mycobacterium tuberculosis* [64, 65].

From ancient medical papyrus writings on the treatment of tuberculosis in Egypt up to the 19th century, most of the therapies involved surgical removal of cysts, confinement to temples dedicated to health and worship, chest plasters, gargles, and many other homeopathic remedies [66]. By the late 19^{th} century, climatotherapy,

designed by Hermann Brehmer in 1854, was the best remedy for patients with tuberculosis [66, 67]. High altitude therapy with rest, sunshine, and an abundance of fresh-clean-crisp air, a special diet, and confinement to sanatoriums was considered the best cure for those who suffered from tuberculosis. The isolation of streptomycin in the early 1940s by Selman Waksman, led the way to the first effective antituberculosis therapies [68-70]. Later, other drug compounds were developed that demonstrated efficacy in clinical trials, such as para-aminosalicyclic acid (PAS), isoniazid (INH), and rifampicin (RIF) [66, 71-73]. Over time, resistance to these drugs developed, and the principle of multi-drug therapy became the cornerstone of any effective regimen against tuberculosis, as it prevented the development of drug resistance [74, 75]. Despite early drug developments, tuberculosis remains a common disease with a heavy global impact on public health. The emergence of HIV in 1981 and its effects on the resurgence of tuberculosis, together with newly developed resistance to multi-drug therapies, prevents global eradication and demands new developments in prevention, treatment, and control for this disease.

1.2.2 The bacilli

Mycobacterium tuberculosis is classified in the genus of *Mycobacterium*, which belongs to the family of *Mycobacteriaceae* of the order of *Actinomycetales* [76]. The organism is a non-motile, aerobic, slow-growing, facultative intracellular bacillus. The bacilli are considered Gram-variable with a cell envelope that is only slightly stained with traditional Gram-staining methods due to its lipidy impermeable nature. The cell envelope is one of the organism's most unique structural features.

The structural design of the cell envelope of *M. tuberculosis* consists of a plasma membrane, a cell wall, and a capsule-like surface layer. The envelope's inner membrane barrier is comprised of a mycolyl-arabinogalactan-petidoglycan complex (mAGP) that surrounds the plasma membrane (Figure 1.2) [77]. Its outer barrier consists of covalently bound mycolic acids (C_{70}-C_{90}) and a variety of free lipids comprised of mycolic acid derived acyl glycolipids such as glucose monomycolates, trehalose monomycolates, trehalose dimycolates, and additional free lipids such as sulfolipids, pthiocerol dimycoserates, phenolic glycolipids, as well as phosphatidylinositol derived lipids; phosphatidylinositol mannoside, lipomannan, and lipoarabinomannan [77]. The unique components of the cell envelope are recognized as primary factors for the bacterium's survival and pathogenicity. The core provides an impermeable barrier to harmful agents such as antibiotics, and the extractable free lipids are effecter molecules involved in disease pathogenesis.

Much research has been conducted in order to understand the structure and synthesis of the mycobacterial cell wall, since it is the most characteristic feature of the bacterium. This knowledge has been used to identify drug targets, and many of the chemotherapeutics used today for the treatment of tuberculosis are, in fact, cell wall inhibitors, the most primary example being isoniazid, which targets mycolic acid synthesis [78-80].

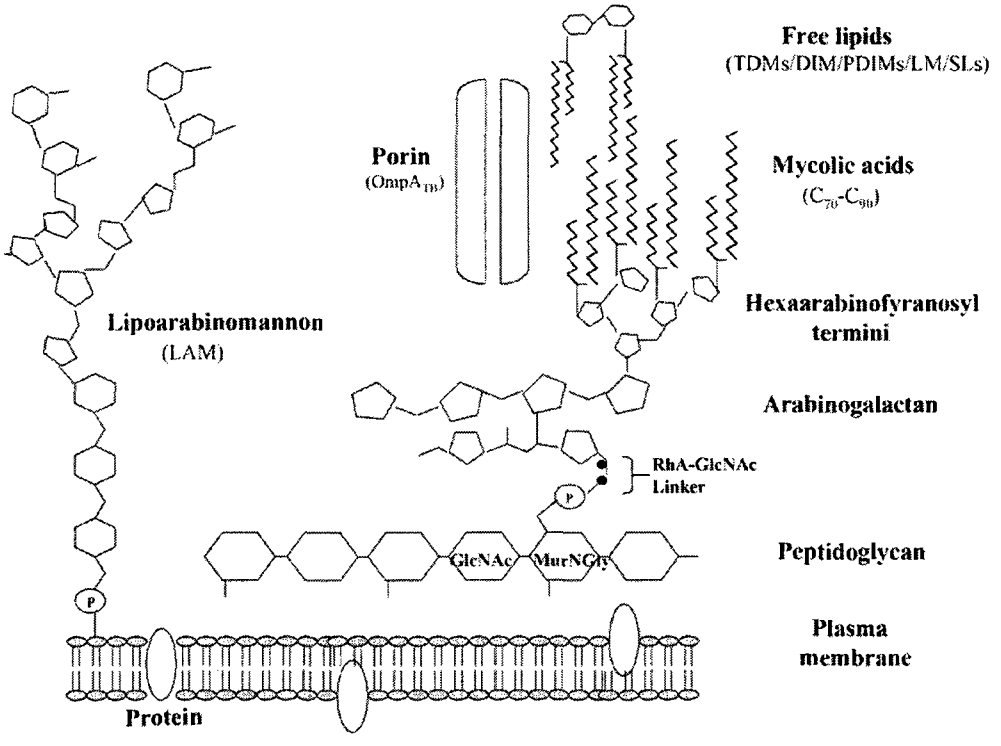


Figure 1.2 Structural components of the mycobacterial cell wall. (modified from Basso et al., 2005) [81] The cell envelope consists of a plasma membrane with a thin layer of peptidoglycan similar to other bacteria. Unique features are found associated to the outer layer consisting of a composition of arabinogalactans linked to mycolic acids that provide a waxy impermeable barrier. Other novel features are virulence attributes, which include lipoarabinomannon structures covalently bound to the plasma membrane and surface glycolipids (free lipids).

The *M. tuberculosis* genome contains over 4,000 genes (Table 1.1) [82]. The bacterium has adapted to an intracellular lifestyle and carries some unique features. The genome encodes a variety of insertion elements, mostly found in noncoding regions. These sequences provide evidence for early events of horizontal gene transfer of a free-living ancestor prior to evolving into an intracellular endosymbiont. Interestingly, there are two prophage elements identified in the genome with similarity to bacteriophages found in *Streptomyces* and saprophytic mycobacteria. The presence of these elements offers another possible mode of early gene acquisition as the organism evolved. Evidence of phage-like activity for cell lysis has not been well documented, but their existence offers an explanation for a persistent low-level lysis of cells in stationary culture [83]. Phage activity may be triggered during moments of nutrient starvation, where altruistic cells are lysed to provide necessary nutrients for the rest of the population. In addition, there are over 200 genes annotated as encoding enzymes involved in lipid metabolism, with half of these genes dedicated to the of β-oxidation of fatty acids [82]. This large number suggests an adaptive lifestyle in which lipids may be utilized as a primary carbon source under conditions where traditional carbon sources are limited. In addition, there are two fatty acid synthesis systems (FASI and FASII) along with other genes that provide the genetic program for the synthesis of the unique lipid structures that construct the bacterium's signature cell wall. Another large group of genes belong to unique PE and PPE families of acid, glycine-rich proteins and are thought to provide antigenic variation during infection [62, 82]. In addition, the genome encodes 13 sigma factors involved in global gene regulation and nearly 200 regulatory proteins required for the

bacilli to adapt and survive under a variety of conditions [82, 84]. It is likely that these regulatory elements are used during various stages of disease pathogenesis as the bacterium adjusts to different environmental pressures, evades host responses, and establishes a program for long-term survival in a chronic asymptomatic persistence. A particularly interesting regulon in the genome that has been identified as important for the initial induction of the bacilli into a dormant, nonreplicating state is the *dosR* regulon. DosR regulates the expression of over 50 genes of which most (60%) have no annotated function [85, 86]. Many of the annotated genes represent proteins involved in alternative metabolic functions, supporting survival under conditions of

Table 1.1 Functional classifications of *M. tuberculosis* genes.
(as reported by Smith, 2003 and Cole, 1998) [62, 82]

Function	No. genes	% Total
Lipid metabolism	225	5.7
Information pathways	207	5.2
Cell wall and cell wall processes	517	13.0
Stable RNAs	50	1.3
IS elements and phages	137	3.4
PE and PPE proteins	167	4.2
Intermediary metabolism and respiration	877	22.0
Regulatory proteins	188	4.7
Virulence, detoxification, & adaptation	91	2.3
Conserved hypothetical proteins	911	22.9
Proteins with unknown functions	607	15.3

low oxygen and limited nutrients [85, 86]. Several genes upregulated in the regulon are involved in fatty acid and carbohydrate metabolism, altered pathways for biosynthesis and the formation of oxidizable metabolites, along with mechanisms to use alternative terminal electron acceptors. Recent evidence has demonstrated the importance of the *dosR/dosS* regulatory system for full virulence in 3 different animal models: mouse, guinea pig, and rabbit [87]. Regulators such as DosR provide evidence supporting the global changes that the organism requires to establish the phenotypic state of nonreplicating persistence.

Virulence attributes still being assessed involve proteins that promote cell surface modulations required for adaptation and survival, proteins involved in secretion of antigen-like molecules that promote intracellular uptake, and proteins that can modulate host-cell environments. Esat-6 and CFP-10 are secreted proteins present in TB patient sera that are under great focus. Their actual functions remain unclear. These proteins are secreted simultaneously by the Esx-1 secretion system and are thought to modulate the innate immune response [88, 89]. Another recent study demonstrates that these proteins affect ROS (reactive oxygen species) production, thus altering macrophage killing of the bacterium [90]. Additionally, a secreted transcriptional regulator has been identified (EspR) as a feedback control mechanism that monitors Esx-1 expression, which would regulate secretion of antigenic substrates (Esat-6 and CFP10) and perhaps tightly control immune system stimulation during the course of infection [91]. It is understood that *M. tuberculosis* has mechanisms that modulate the host immune system in order to establish a latent

persistent existence. However, identifying and defining such mechanisms has been a serious challenge.

The ability of *M. tuberculosis* to establish a latent chronic infection in a symbiotic relationship with its host is one of its most successful survival strategies. It is well understood that *M. tuberculosis* can stop replicating under adverse conditions and lie dormant or inactive for years, with the ability to reactivate and cause disease at a later time under more favorable conditions. Studies evaluating the dynamics associated with latent disease have been conducted for years, and the mechanisms of bacillary survival are still being elucidated. Factors that have been associated with the shutdown of the bacilli include environmental changes that occur within the confinement of the granuloma, adaptations required to evade the host immune activities, and control mechanisms induced by the bacterium to regulate its surroundings or shift into an alternative mode of life. Early research by Lawrence Wayne established that, *in vitro,* the bacterium has a program established to enter dormancy under limited oxygen conditions. [92, 93] These studies identified the importance of a slow, regulated reduction of oxygen for the survival of the bacilli and its adaptation into a nonreplicating persistence. Research continues to evaluate the environmental conditions within the confinement of the granuloma, and to assess metabolic mechanisms the bacilli may utilize for maintaining their viable persistence [85, 86, 94-107]. Existence within confinement of the granuloma, along with altered metabolism under dormant state conditions present great challenges in treating the disease.

1.2.3 Global epidemiology

Historically, tuberculosis has been present in the lives of humans for centuries. Over 60 years after the introduction of antituberculosis drug therapy, at least one third of the world's population is infected with *M. tuberculosis,* and active disease remains a global public health problem. In fact, in 2006 WHO reported over 9 million new cases and nearly 2 million deaths, of which nearly 0.7 million cases and 0.2 million deaths were in association with HIV co-infection [108]. India, China, Indonesia, South Africa and Nigeria are the top afflicted countries, with increases in case identifications found in Africa, South-East Asia and the Western Pacific regions [108, 109]. On a positive note, a WHO report in 2007 indicated that the number of new cases per capita has declined globally since 2003 [108]. This is credited primarily to the worldwide establishment of WHO's "Stop TB Strategy", which incorporates DOTS (Direct Observed Therapy Short Course) and new surveillance programs. Through these initiatives, the rate of case detection for new smear-positive cases reached over 60% in 2006 and the treatment success rate has reached nearly 85% in 2005 [108]. In addition, WHO is developing new HIV/TB intervention programs to address guidance on advocacy, communication, social mobilization, and to implement a strong attack on the treatment of MDR-TB [108]. However, budgets have fallen in all but 5 of the 22 countries with a high burden of disease [108]. Unfortunately, insufficient funding is a primary obstacle for continued success of these programs and the global control of tuberculosis.

The prevalence of *M. tuberculosis* infection within a population varies with regard to demographic (age, sex, location) and socioeconomic (crowding, poverty,

confinement) factors. Today, tuberculosis in the developed regions of the world (Western Europe, Canada, Japan, and the United States) is found primarily in selected groups of individuals: immigrants, homeless, IV drug users, and those that are immunosuppressed [109-111]. However, in less developed regions of the world, the prevalence of the disease continues to rise [108, 109]. Today, HIV has the highest influence on tuberculosis mortality, with other chronic health problems such as diabetes, malnutrition, and respiratory syndromes having an additional impact [108, 109, 112-115]. Other important factors involved in increasing rates of disease include poor access to treatment due to insufficient pubic health infrastructures, improper diagnosis and treatment leading to the development of resistance, lack of patient compliance, and the new development and spread of resistant strains in endemic regions [116-119].

It is important to realize that tuberculosis has been adapting and thriving for thousands of years. The bacterium has evolved and continues to evolve and develop ways to resist current treatment, which is a part of well-understood natural selection processes. Knowing this, it is a given that tuberculosis will continue to have an impact on future civilizations, and therefore, the need for new chemotherapeutic treatments along with the implementation of strict public health managed care for both susceptible and resistant tuberculosis is crucial in the control and continued fight against this well-established pathogen. Efforts to contain disease in endemic regions and limit the spread of resistant strains to other regions of the world are critical at this time. Control efforts should focus on effective diagnosis through drug susceptibility testing in order to provide the appropriate treatment for each individual patient,

limiting the expansion of resistant bacilli that can be spread throughout a population. Furthermore, governments in endemic regions may need to step up their efforts and establish specialized facilities that will enable patients to reside under direct observed care until they are no longer deemed infectious. This would be a costly solution, but it may be necessary to prevent the potential of a worldwide pandemic. Obviously, the solutions to controlling this disease are not simple or inexpensive, and will require combined efforts from government officials, world public health organizations, and established medical communities.

1.2.4 Infection and disease

Tuberculosis is a human disease that can present itself in either an acute or chronic state. An active tuberculosis infection is highly contagious, and transmission occurs when there is prolonged contact between a susceptible person and one with active disease. Most often, the immune system can effectively clear the bacterium upon initial infection, with only approximately 10% of infected individuals developing the disease. Tuberculosis infection is caused by the inhalation of airborne particles (primarily through coughing) containing live bacteria that find residence in lung alveoli. Once established, bacilli are taken up primarily by alveolar macrophages through phagocytosis. Other cells, such as dendritic cells, have been shown to be important for dissemination and activation of T-cells, priming cell-mediated immune responses [120-122]. Contact with surface macrophage receptors such as complement receptors (CR1, CR3, CR4), mannose receptor (MR) and surfactant protein A receptor (SPA-R) facilitate uptake [123-126].

After uptake, the bacilli reside in a tightly fit endocytic vacuole called the phagosome. [127, 128]. Under normal infection conditions, the phagosome fuses with a lysosome, and bacteria are killed due to the actions of hydrolytic enzymes, acid pH, toxic peptides, and reactive oxygen and nitrogen intermediates. However, *Mycobacterium tuberculosis* has evolved ways to evade this hostile confinement by preventing phagosome maturation and then controlling the environment so it can safely hide from host immune molecules and cells and replicate [129-134]. Infected macrophages can either stay in the lung or migrate to other areas of the body causing miliary TB. However, not all bacilli survive and some are killed by activated macrophages or other immune cells.

Over the next several weeks, bacilli residing in inactivated macrophages are able to replicate to high numbers, cause cell apoptosis, and are released to infect more cells [135-137]. At this stage, more proinflammatory cells are signaled from cytokine and chemokine release, and eventually antigen specific T-cells (CD4+/CD8+) and B-cells arrive and proliferate, signaling the activation of macrophages in order to kill the bacilli [137-139]. The slow growth of the organism allows for the onset of the granulomatous response and granuloma formation. The accumulation of cells, bactericidal activity, and continued release of cytokines (TNF-α) and chemokines modulate the formation of granulomas, which is the host's mechanism of containing the infection, localizing the immune response, and preventing dissemination [140, 141]. The ability of the host's immune system to contain the infection determines the course of the disease into either an acute or chronic infection. Typically, several weeks into the infection, granulomas form and consist of a necrotic region of cellular

debris surrounded by layers of differentiated macrophages, multinucleated giant cells, and a zone of blood derived-monocytes, lymphocytes, and fibroblasts [107, 136]. At this point, surviving bacteria are housed in a toxic environment with limited nutrients, and are postulated to be able to shutdown into a nonreplicating persistent state in which they can lay dormant for years. This stage of disease is known as "latent tuberculosis", and is found in over one third of the global population. Latent disease can reactivate into an active infection if the host's immune system becomes compromised, as with co-infected HIV individuals, patients under immunosuppressive therapy, or with age.

The fact that this pathogen has various phenotypes associated with its pathogenesis and disease is the main problem in its eradication and poses several issues for treatment. Antibiotics are effective against actively growing populations of the bacilli, but limited in the eradication of nongrowing persisters. It is thought that the nongrowing population may have a variety of phenotypes including: 1) stationary-state bacilli that have reduced replication rates but are able to resume growth under optimal conditions, 2) drug tolerant bacilli that have developed ways to maintain their existence under drug treatment, and 3) dormant nonreplicating bacilli that have adapted an alternative metabolic state that makes them untouchable to host responses, toxic environmental conditions, and drug treatment [142]. Understanding the modes of success for these phenotypes is the focus of continued research.

1.2.5 Current therapy and resistance issues

There are currently four recommended DOTS regimens outlined by the CDC for drug susceptible TB. These consist of six-months of therapy that begin with a two-month phase of treatment with isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) followed with a second four month-phase of INH and RIF. Variations in the second phase have been set for HIV infected individuals with alterations in weekly drug dosages and alternative drugs to avoid problematic drug interactions with anti-retrovirals [143]. INH is known to have a high level of activity against actively growing bacilli and no activity against resting bacilli. INH is a prodrug that is activated by the *M. tuberculosis* enzyme KatG, a catalase peroxidase, and is broken down into a variety of reactive intermediates that can attack multiple targets, e.g. mycolic acid synthesis, DNA damage, lipid peroxidation, and NAD metabolism in the cell [78, 80]. Rifamycins are known to be effective on both growing and stationary phase bacilli, and demonstrate killing throughout the course of therapy [144]. These compounds are considered the most potent sterilizers in the combination cocktail. The primary target of action is the DNA-dependent RNA polymerase involved in RNA synthesis [144]. Pyrazinamid (PZA) is the third most important drug in the regimen, as it is highly effective on the dormant populations of cells [145]. The compound is well absorbed and undergoes a conversion into pyrazinoic acid (POA) by pyrazinamidase (*pcnA*), affecting the mycobacterial enzyme fatty acid synthetase-1 (FASI) and also disrupting the membrane potential, which is thought to interrupt ATP production [145, 146]. Ethambutol (EMB) is thought to have pleiotropic effects involving the inhibition of various synthesis

pathways (RNA, phospholipids, cell wall, spermidine, and arabinogalactan synthesis) [145]. It is the least effective of the four compounds in the regimen, and is used primarily to decrease the risk of developing resistance to INH and RIF [145]. These frontline drugs are effective at clearing actively replicating TB (over 99% of the population) within the first two months [74, 75, 147, 148]. Extended therapy for an additional four months is designed to attack the persistent phenotype. The outlined combination therapy, when carried out under strict DOTS managed care, is highly successful with relapse rates of <5% [149]. Despite this, differences in susceptibilities between actively replicating and nonreplicating bacteria are significant, and the dependence on frontline drugs for complete clearance can be limiting. Also, the existence of single drug resistant strains being spread within a population, undetected for over 50 years of the same drug exposures, may be partly to blame for the currently established problems. Years ago, patients were treated without drug susceptibility testing, which may have provoked the development of multiply-mutated bacilli leading to established populations of MDR-TB found today. However, it is accepted that the primary factors of patient compliance, treatment duration, and limited resources in the developing world are the main driving forces.

The continued development and spread of resistant bacilli has been most problematic in controlling the disease. Factors associated with the development of drug resistant TB include natural selection due to selective pressures from the duration of treatment, patient compliance due to ineffectively managed or nonexistent DOTS programs, drug toxicity issues, complications associated with HIV co-infection, poor drug quality, lack of drug susceptibility testing to define treatment

regimens on an individual basis, and limited access or poor public health infrastructures in poor endemic regions of disease. Currently, resistance to both INH and RIF are found to be the most prominent forms of resistance, and when in combination are considered multi-drug resistant tuberculosis (MDR-TB) [148, 150]. Failure to identify and treat these resistant genotypes has led to their spread and the use of alternative drug therapy. In situations where MDR-TB is highest in prevalence, WHO recommends the DOTS-Plus regimen involving a second line of TB drugs which are administered over two years under direct supervision [150, 151]. The second-line drug combination can include a variety of cocktails depending on the results of drug susceptibility testing. The new combinations can include injectable aminoglycosides (kanamycin, amikacin, capreomycin), fluoroquinolones (ciprofloxacin, ofloxacine, levofloxacin, moxifloxacin, gatifloxacin), and other bacteriostatic options such as cycloserine, ethionamide, prothionamide, and p-aminosalicyclic (Table 1.2) [148, 150]. Use of second-line therapy is expensive and has significant toxicity issues, along with a high probability for lack of patient compliance if not properly managed. In recent years, a subset of MDR-TB strains have evolved that are resistant to both INH and RIF along with fluoroquinolones and at least one of the injectable drugs in the second-line defense regimen. This subset is known as "extensively" drug resistant tuberculosis, XDR-TB [151]. In some cases, XDR-TB has been a particularly aggressive form of TB, having high mortality rates, with death ensuing in an average of 25 days [152, 153].

Table 1.2 Currently used TB drugs
(as reported in Leanerts et al., 2008) [154]

Drug compound	Mode of action
Isoniazid	Prevents mycolic acid biosynthesis in the cell wall
Rifampicin	Binds bacterial RNA polymerase
Pyrazinamide	Affects pH and possibly NAD biosynthesis
Ethambutol	Disrupts arabinogalactan layer biosynthesis
Aminoglycosides	Disrupts protein synthesis
Fluoroquinolones	Interferes with DNA gyrase/topioisomerase
Ethionamide	Interferes with mycolic acid biosynthesis
Cycloserine	Prevents D-serine insertions into peptidoglycan
P-aminosalicyclic acid	Folic acid biosynthesis
Clofazimine	Unknown
Thiacetazone	Unknown

The rise in MDR and XDR cases has set off an alarm to public health officials and governments in severely problematic countries as well as the developed world. WHO's "Stop TB strategy 2008-2009" has outlined increased efforts to scale up treatment management, strengthen diagnostics, expand surveillance measures, improve infection control measures, increase awareness and advocacy measures with community programs, and promote research and development in the areas of vaccines, antituberculosis chemotherapeutics and improved diagnostics [155]. Increased efforts may prove successful, but there is a definite need to develop new effective drug therapies to deal with existing and future problems. One of the primary challenges in drug design efforts is to develop new compounds with alternative mechanisms of action that can either act alone or support current treatment regimens

to effectively clear all TB phenotypes (drug tolerant, active replicating, and latent persisting). In addition, these compounds should be limited in toxicity, and should not interfere with other compounds in the regimen. Finally, alternatives are needed that will shorten treatment duration, reducing the risk for the development of newly resistant strains. Obviously, the challenges for global control are many in a world where poverty is on the rise, the global economy is in crisis, and there is increased movement of people across the globe.

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CHAPTER 2

Literature Review Part II: Antimicrobial Drug Discovery

2.1 DRUG DISCOVERY

2.1.1 An introduction to the process

The discovery and development of new drug compounds for effective therapy can be extremely challenging, time consuming, and costly. In the late 20^{th} century, it was estimated that the development of a drug from the time of inception to the market place costs several billion dollars and takes approximately 15-20 years of research and development [1, 2]. Statistical evidence indicates that on average only one in ten drugs entering clinical trials becomes marketable [1]. However, the discovery and development of new and effective chemotherapeutics is critical for improving health, combating newly emerging diseases, and fighting evolving drug resistance. Additionally, finding new drugs to combat longstanding endemic diseases such as persistent tuberculosis are imperative to reducing their global public health burden. Recently, efforts in drug discovery have added a new focus directed toward the design of active compounds with new modes of action for highly infectious agents considered for bioterrorism, as engineered resistant strains to current therapies are of concern.

Rational drug design is based on a comprehensive study of important biochemical pathways that are involved in pathogen survival and pathogenesis. These studies identify key elements in these pathways, and set the stage for the design of small molecules to modify or manipulate their function. Most of the approaches involved in the process are target oriented and hence structure based. The identification of new and clinically relevant targets with novel modes of action are of highest priority and can lead to the innovated design of compounds that are effective for combating current and future infectious disease problems.

The process of drug discovery is a multi-disciplined endeavor, interfacing chemical, biological, and computational research. The use of computer technology along with bioinformatics can utilize databases in search of "best-fit" compounds to specific enzyme or protein structures through *in silico* approaches, model specific active site interactions providing key information for optimal drug design, or identify targets through homology based studies in related species[1-4]. Chemical research focuses on providing the creative thought necessary to design a blockbuster drug. Medicinal chemistry efforts involve the synthesis of new compounds, the characterization of their properties (pKa, logP, melting point, and solubility), and validation of their structures *via* NMR and Mass Spectroscopy[1, 2]. These compounds are then introduced into preliminary screens against validated targets or whole cell bacterial assays. Biological strategies included in the process help to correlate a specific enzyme activity to disease pathogenesis, and assess drug potency, activity, and efficacy[1, 2]. Additionally, pharmacologists assess effective compounds for *in vivo* bioavailability and ADME properties (absorption,

distribution, metabolism, and excretion), which can optimize lead compounds for increased therapeutic effect. The successful design and development of a new drug compound involves cooperative efforts with the common goal of providing an effective treatment to combat infectious disease.

Successful compounds demonstrate specific properties such as potency, selectivity, bioavailability, efficacy, and limited toxicity [3]. A potent drug is one that provides the desired effect, in the desired time frame, at a low dosage. A selective drug will produce the desired activity without side effects to the host. Bioavailable compounds get to the site of infection at effective concentrations. Presumably, the best drug compounds are those that can be orally delivered with minimal frequency at low dosages as to allow for outpatient therapy. This means that the drug should be soluble under physiologic conditions, survive the acidic conditions of the stomach and the attack of enzymes, be absorbed through the intestinal wall, transport across membranes and get to infected cells, and lastly cross the bacterial cell wall to elicit action against the targeted protein. Finally, drug compounds should not be toxic to host tissues or organs. The fact is that most drug compounds are not void of side effects, although with optimizations in design and formulation these effects can be minimized. Once a compound has fulfilled all the above criteria, it is ready for advanced stages of development, and preclinical and clinical trials.

The basic strategy involved in developing a drug from fundamental research has been diagrammed in Figure 2.1. The initial stages of discovery involve the identification of an essential target and a promising lead compound with inhibitory

activity against that target *in vitro* and *in vivo*. The development process optimizes this compound for druggable features such as limited toxicity and sufficient ADME properties preparing it for preclinical and clinical evaluation.

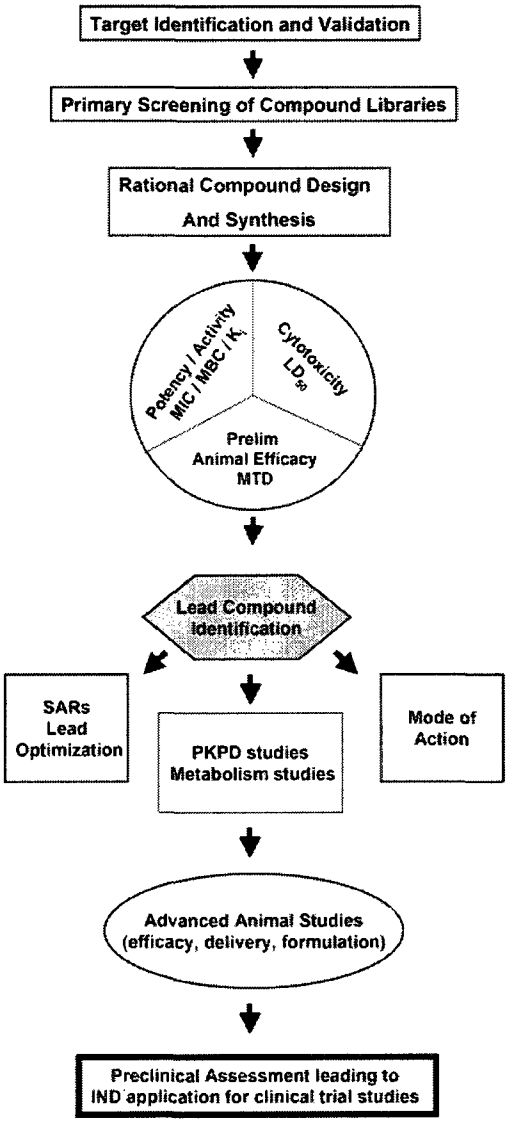


Figure 2.1 Flow diagram of the drug discovery process. Steps outlined represent elements involved in the design and development of lead molecules that can be advanced into human clinical trials to assess safety and efficacy for future use in humans against infectious disease.

2.1.2 Target identification and validation

Identification of a good target sets the stage for compound design. In general, a "good" drug target is identified as one that has specific or selective function in the microbe with limited or no activity to human homologues. Ideally, the best targets are those that have no human counterpart and are specific to the pathogen of study. Targets should demonstrate essential function for growth and viability or provide a unique function in disease pathogenesis.

Current antimicrobials target a small group of cellular functions encompassing cell wall, DNA, RNA, and protein synthesis. However, many organisms have developed resistance to these strategies, and thus the primary focus today is on the design of new compounds with inhibitory activity to alternative targets with novel modes of action. Alternatives can be either essential or nonessential as long as they demonstrate an effect in eliminating disease. Advances in technology have made it possible to dissect and understand the physiology of any disease process at a molecular level, opening new areas for target-based discovery [3, 5]. Methods in cellular and molecular biology, along with genomics and proteomics, have become increasingly important in target identification. These methods can be used to identify unique genes or proteins expressed during infection or at a particular disease state. Examples of unique processes that may offer new avenues for drug discovery include elements involved in mechanisms of secretion, cell division, unique areas of biosynthesis, alternative metabolic pathways, and elements of virulence or pathogenesis.

Through various genomic initiatives there has been an accumulation of large amounts of DNA sequence information, which are now available in comprehensive databases. These databases contain sequence information for genes and proteins found in thousands of organisms. Bioinformatic research currently provides strategies for comparative analyses of sequence information for related microbial species as well as various isolates from the same species with different features, i.e. pathogenic and nonpathogenic. This type of analysis is useful to identify unique targets involved in metabolism, biosynthesis, virulence and pathogenesis [2, 4]. In addition, primary sequence similarities can be used to predict potential function and target specific pathways. These analyses can extend into human homology studies and provide information in regard to similarities with human proteins to examine the selectivity of the potential target.

Other genomic approaches utilize gain or loss of function studies through gene knock-in or gene knockout strategies to characterize essentiality, or assess the importance of the gene product in growth, survival and pathogenesis [6-9]. However, these methods may not always mimic the effects of a drug since many organisms have additional genes or pathways to balance out the overall change. In addition, the use of mutagenesis experiments can also be useful for the identification of genes required for cell viability [4, 10, 11]. Often many of these genes are not annotated and thus have no predicted function or similarity to other known proteins. The role these gene products may have in cell growth and viability may not be easily defined. However, if they belong to a group of genes that define a specific pathway, then that pathway may be considered necessary for cell survival

[6]. Identifying a pathway can provide a number of target opportunities for further investigation.

Another way to elucidate gene function is through gene dosage experiments where gene expression is induced *in vitro*. For example, cells producing altered levels of protein other than wild type can demonstrate differences in resistance or susceptibility to drug treatment known to affect a specific enzyme activity. Other techniques can use microarray whole genome expression technology or proteomics to compare genes transcribed or proteins expressed under healthy and disease conditions. For instance, microarray transcriptional profiling can be helpful in assessing global effects of drug treatment by identifying changes in gene expression for specific pathways or validating drug-to-target responses. These tools are used to identify and validate new chemotherapeutic targets that can then move forward in the discovery process.

Once a target has been validated, it is important to establish details of its structure and function. Primarily, a good understanding of the 3-D structure of the target is critical for drug design, and the most precise technique for detailed 3-D structure is X-ray crystallography [1, 2]. In structure-based drug design, the three-dimensional structure of a drug target interacting with its substrate can provide direction for modeling activity and is helpful in the design of inhibitor compounds [1, 12, 13]. Additionally, inhibitor-bound crystal structures can provide detailed information regarding favorable interactions that may provide insight for further structural modifications that will enhance inhibitor affinity and specificity. Enhancing these features will increase the inhibitor's ability to compete for the

active site or binding site of the protein or enzyme. In addition, details of active site chemistry can be used to modulate physiochemical properties of the inhibitor to enhance its druggability [1, 12, 13]. Computer programs using algorithms to predict free energies of interaction, binding geometries, solvation properties, and binding affinities for lead molecules can guide design motifs [1, 12]. Once a target is identified and its structure and function well characterized, it is essential to design and optimize assays that measure the biological activity of the target *in vitro*. After these *in vitro* assays are in place, compounds are screened to identify lead inhibitors.

2.1.3 Lead compound identification and optimization

Lead compounds can be identified using whole cell or enzyme inhibition screening strategies. Identifying lead compounds is accomplished through biological assays that rely on the use of detection methods to quantify activity, or whole cell assays that determine the relative inhibition of cell growth [2]. The use of *in vitro* assays can provide additional information regarding structure-activity relationships (SARs) used to optimize small molecules through changes in molecular structure that demonstrate significant changes in biological activity. Once a molecule has been identified to provide inhibitory properties that reduce not only enzyme function but also demonstrate antibacterial activity, it can be moved into preliminary animal screening to assess its overall efficacy. Identified lead compounds with demonstrated efficacy *in vitro* and *in vivo* can then proceed into areas of development to enhance druggability.

The optimization of lead compounds into drug-like substances involves pharmacological characterization. Turning an active compound into a safe and effective drug requires *in vitro* and *in vivo* assays to assess toxicity and pharmacokinetic properties. Structural design can be guided through information obtained from current drugs that exhibit druggable properties. In 1997, Christoper Lipinski established a set of rules to assess drug-like properties of molecules based on evaluations of structural components found in currently used medications [14, 15]. He noted that most medications are small molecules of lipophilic nature. His rules identify key molecular properties that promote ADME properties in the human body (Table 2.1). Daniel Veber provided additional information from animal experiments on factors that influence oral bioavailability [15, 16]. His work established that molecules with reduced flexibility (number of rotatable bonds) and low polar surface area were important features to producing a drug with good oral bioavailability. In addition, certain functional groups were identified as reactive, prone to decomposition, or were readily metabolized and therefore unstable in the body [15, 17]. Utilizing these guidelines for structural limitations, compounds can be optimized according to druggability and prioritized for further development.

Table 2.1 Rules for rapid physiochemical profiling (as reported by Lipinski & Veber) [14-16]. The assessment physical properties of currently used drugs and candidate compounds by Lipinski and Veber established a set of guidelines for designing drug-like molecules.These rules guide medicinal chemist's compound design for ultimate physiochemical traits to enhance *in vivo* bioavailability.

Lipinski's Rules

1) A compound should have no more than 5 hydrogen bond donors (N and O atoms with one or more H atoms)

2) A compound should have no more than 10 hydrogen bond acceptors (N and O atoms)

3) The molecular weight of the compound should be under 500 daltons

4) The compound should have an octanol-water partition coefficient (logP) less than 5.0

Veber's Rules

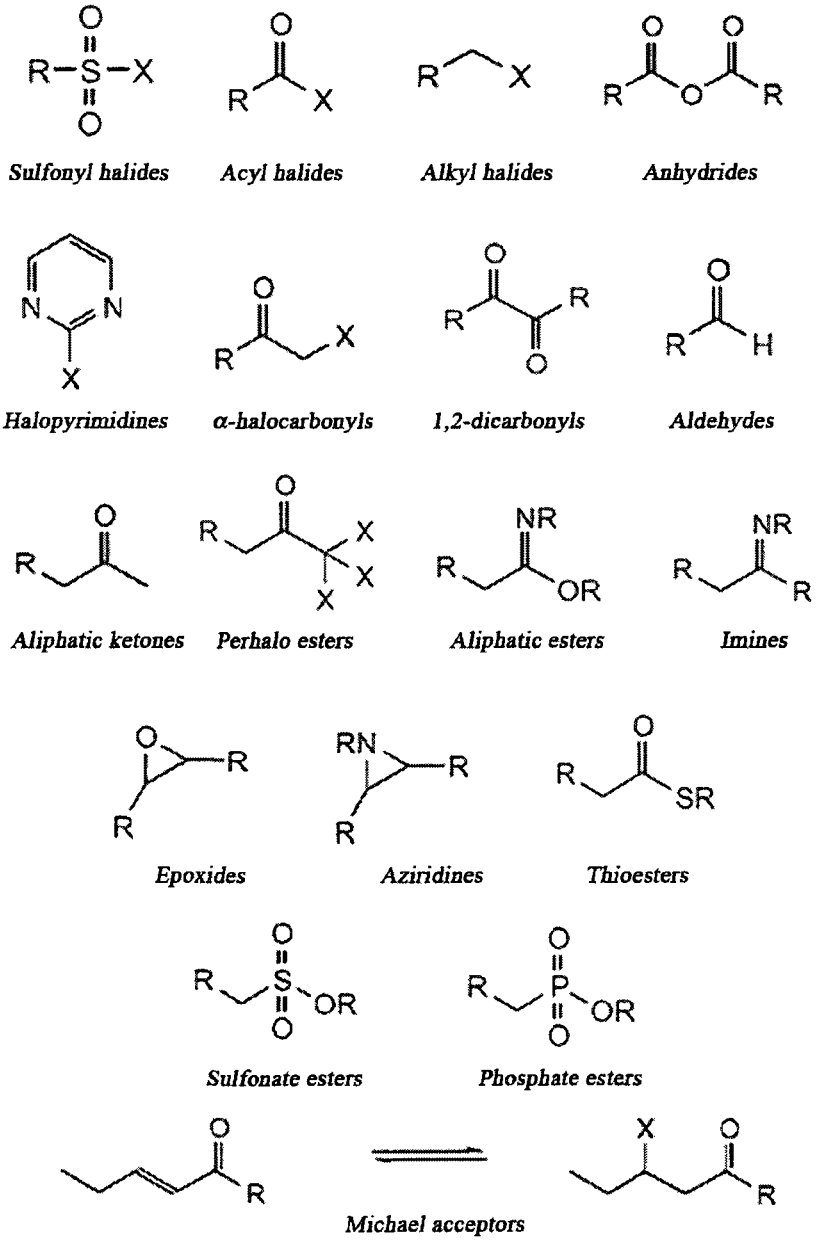
1) A compound should have $\leq$ 10 rotatable bonds

2) A compound should have a polar surface area of ≤ 140 $(Å)^2$, or < 12 H-bonds

Other constraints

- A compound should have $\leq$ 6 ring structures
- A compound should have $\leq$ 7 C chain length
- A compound should have $\leq$ 7 C ring size
- A compound should have $\leq$ 7 halogen atoms
- A compound should have < 15 N & O atoms

Table 2.2 Undesirable functional groups. (as reported by Rishton, 2003) [17] Several functional groups are known to have electrophilic protein reactivity and are considered "false positives" for biological inhibitory activity. Electrophiles can decompose either by hydrolysis or solvolysis, and react to biological substrates. Reactions by acylation or alkylation are covalently binding and not biologically inhibitory of a specific enzyme function. Functional groups listed below are useful as guidelines but are not all-inclusive. (X= halide groups, R= acyl, alkyl, or aryl groups)



2.1.4 Preclinical development and clinical trials

Researchers evaluate the pharmacokinetic and pharmacodynamic (PKPD) parameters and toxicological properties of optimized lead compounds through advanced *in vivo* (animal model) assays [1, 18]. Bioanalytical studies identify and quantify the compound and its metabolites in order to develop dosing regimens and optimal formularies. The drug compound is tested for ADME properties along with its duration of action. Pharmacokinetic parameters determined from blood serum studies include 1) area under the curve values (AUC), which predict the level of drug exposure by determining serum levels over a period of time, 2) volume of distribution (Vd), which represents the volume of the compound which is dissolved and distributed throughout the body, 3) clearance (Cl), which measures the rate at which the compound is removed from circulation, 4) elimination rate (k), which determines how rapidly the compound is excreted from the body, 5) half-life ($t_{1/2}$), which refers to the time it takes for the compound's concentration in the blood to be reduced by half, 6) bioavailability (%F), which is the percentage of the active compound in circulation that remains unchanged, 7) maximum concentration (Cmax), which is the highest level of concentration of the compound in the blood, and 8) tmax, which represents the point in time that Cmax is reached [12-14]. This information is used to predict therapeutic efficacy, define susceptibility breakpoints, and correlate dose-efficacy relationships. [19, 20]

Antimicrobial activity of a compound can be assessed *in vivo* to provide evidence for either concentration-dependent killing or time-dependent killing effects [19, 20]. Compounds that exhibit concentration-dependent killing have a higher power of killing at higher concentrations. A correlation with clinical outcome from these compounds can be derived from ratios AUC/MIC or Cmax/MIC. Typically, a single daily dose is sufficient, limiting the concern for toxicity. Compounds that exhibit time-dependent killing have the best clinical outcomes when the concentration remains above MIC for 40-50% of the dosing interval [20]. These compounds typically require multiple daily doses that are time-dependent and can present toxicity issues depending on the nature of the compound.

Pharmacodynamic properties of a compound are represented by a relationship between the drug and the pathogen regarding the rate of killing vs. rate of growth [20]. Thus, maintaining an effective serum concentration (T>MIC) for an appropriate killing time is key to establishing an overall therapeutic effect. Interestingly, some antibiotics can suppress bacterial growth even after the drug concentration falls below MIC, which is described as a post-antibiotic effect (PAE) [19, 20]. The exact mechanisms by which antimicrobials induce PAE have not been clearly defined. The extent of these effects can vary between compounds and correlates with AUC/MIC or Cmax/MIC ratios [19, 20]. For Gram-positive bacteria, AUC/MIC >25 are often associated with clinical cure [20]. Gram-negative bacteria require higher ratios (>100), as they have a double membrane system to penetrate and many of these organisms have efflux systems that efficiently pump out drug compounds, making it difficult to reach effective MIC levels inside the cell

[20]. Furthermore, intracellular pathogens have additional issues, as the concentration within the infected cell is different than that presented in the serum. Drugs against intracellular pathogens must clear several hurdles before they can be effective, which include diffusion through tissues and elements of pathology (i.e. granulomas), entering the infected cell (macrophage), and surviving harsh oxidative and acidic conditions prior to reaching the targeted bacterium. Ineffective dosing at sub-MIC exposure can promote resistance, or have a bacteriostatic action, which can lead to disease relapse.

In addition to PKPD parameters, the metabolic stability of the compound is also assessed during early stages of development. Drugs are metabolized in the human body in two phases. Phase I metabolism involves the oxidation, reduction, or hydrolysis of the drug compound. Phase II metabolism involves conjugation reactions that add polar groups such as a glucuronic acid to a hydroxyl moiety. Both of these phases increase the polarity and solubility of the molecule, making the drug compound and its metabolites more readily excreted [21]. Several enzymes catalyze these reactions, with the most prominent being monoxygenases such as cytochrome P450 family and flavin monoxygenase family enzymes [21]. Metabolism of drug compounds can vary between species, providing various metabolite profiles and rates of production [22, 23]. Since the liver is the primary source for detoxification mechanisms involving these enzymes, *in vitro* liver microsome assays are used to assess metabolite formation, along with their levels in circulation and rates of production. Liver microsomes are subcellular fractions of liver homogenates that contain the drug-metabolizing enzymes previously

described, and can provide insight into *in vivo* bioavailability and efficacy of a compound by identifying its overall stability [21]. Information gained from these studies can help to remodel drug structures in order to bypass metabolism events, design a "pro-drug" that utilizes key metabolic events to make an active compound *in vivo*, or direct alternative drug formulation.

The development of a strong candidate drug molecule depends on its capacity to be administered to humans and show therapeutic effectiveness with limited side effects. Before testing candidate molecules on humans, scientists must show that the candidate compound does not present an unacceptable level of risk, given its therapeutic benefit. Toxicology studies address the potential toxicities of a drug and determine how therapies can be managed to minimize side effects. Toxic mechanisms include reactive metabolites, gene induction, mutagenicity, induced oxidative stress due to the formation of free radicals and peroxides, and the induction of autoimmune responses [21]. Often, toxic effects follow a dose response relationship. Most of the studies used to address toxicity occur in early preclinical animal models and involve dose escalation, safety and tolerance studies. However, these preliminary studies are not foolproof in humans, and any adverse effects are possible in Phase I human trials.

The development of a compound into a usable drug takes several years and involves several phases of human clinical study. Phase I clinical trials begin the initial introduction of an investigational new drug (IND) approved by the FDA into humans, and focus on assessing safety. A small number of healthy volunteers, usually less than 100, are given the compound to determine what happens to the

drug in the human body [1, 18]. These studies are designed to determine the metabolic and PKPD parameters of the drug in humans, and to assess the side effects associated with increasing doses. Phase II clinical trials utilize placebos in randomized trials involving several hundred volunteers affected by the disease or illness [1, 18]. These trials gather information regarding effective dose, effective method of delivery, necessary dosing interval to provide optimal activity, and reconfirm safety. In Phase III trials, the drug is tested in hundreds to thousands of patients, and provide a thorough understanding of the risk-benefit relationship [1, 18]. These studies are the most time consuming, taking up to 10 years, and are the final stage before FDA approval [1, 18]. If successful, an NDA (new drug application) is submitted to the FDA for review.

2.1.5 Evaluating the process

It is well noted that the drug discovery process is time intensive, involves combined efforts from a variety of disciplines, and utilizes a vast amount of resources. The early stages involving target identification and validation along with lead compound identification are relatively rapid steps in the process. Most of the difficulties arise during the optimization of a lead compound into druggable molecules for human use. Clinical trials used to determine safety, consume the largest amount of time as required by federal regulatory agencies. Critical stages of the process involve enhancing bioavailability for limited dosing to avoid toxicity issues. One of the most effective ways to address bioavailability is through formulation. Formulations may vary depending on the route of delivery.

Formulation for an orally delivered drug must be able to withstand degradation as it moves through the gastrointestinal tract, be readily absorbed by intestinal cells, and bypass metabolism or detoxification processes. When delivered intravenously, formularies must be physiologically sound and not induce hypersensitive effects or hemolysis. The key is to produce a formulated compound that is stable and readily absorbed that can get to the site of infection at effective levels.

In general, the oral route of drug delivery is preferred, as it is most accepted by the patient and promotes outpatient therapy. Since most drugs are not water-soluble and thus present issues with poor bioavailability, new approaches to enhance bioavailability are being met through the formulary design. Recent advances in technology offer alternative formulations that include microencapsulation of compounds in digestible polymers, carrier delivery systems involving liposomes, microspheres, and nanoparticles, and finally lipid or surfactant based solutions (oils, emulsions, SEDDS, SMEDDS) [24 , 25]. The properties that enhance bioavailability in these systems is due to their ability to protect the active compound from gastrointestinal processes maximizing the amount of free drug available for absorption. One of the primary concerns is developing relatively inexpensive formulations that will keep the cost of treatment down and allow distribution to a global market, including developing countries.

2.2 TUBERCULOSIS: DRUG DISCOVERY

In the 21st century, the spread of multidrug-resistant TB (MDR-TB), the appearance of extensively drug-resistant TB (XDR-TB), co-infection with HIV, and

the long-term battle with latent tuberculosis are issues that pose daunting challenges in the treatment and control of *M. tuberculosis*. Current treatment regimens utilize drug compounds designed more than 40 years ago. Extensive use of several of these drugs, poor patient compliance, and the fact that these compounds are not effective against latent bacilli, have spawned drug resistant strains with few alternatives for treatment. TB treatment regimens are extremely lengthy, ranging from 6-9 months for drug susceptible and MDR patients, while treatment for XDR patients can extend into two years. Reactivation of latent TB due to immunosuppression caused by co-infection with HIV (human immunodeficiency virus) is also a serious challenge because of the inability to co-administer anti-TB and anti-HIV drugs, due to drug-to-drug interactions [26]. The most problematic issue for the treatment of TB is the limitations in effective eradication of latent TB bacilli. Currently, RIF is the best drug available to treat latent TB infections, but the increase in drug resistance has limited its effectiveness. Thus, there are various reasons for the need to develop new TB drugs that will not only provide less complicated and shortened treatment regimens, but will also be effective against MDR/XDR-TB, allow for co-administration with antiretroviral agents, and target latent bacilli.

2.2.1 Compounds in the pipeline

Through the research efforts of various institutions, the TB drug pipeline has several promising candidates. Recent efforts have compiled a database containing information on approved drugs used for treatment, drugs in clinical trials, and

potential new drugs in early stages of development [27]. These compounds have been listed in Table 2.3 with information regarding mode of action and drug development status.

The two compounds furthest advanced in clinical trials are fluoroquinolones, gatifloxacin and moxifloxacin [28-35]. Fluoroquinolones target DNA gyrase and interfere with DNA replication. Both drugs are currently being evaluated in combination therapies with current first-line drugs under phase 3 trials in Africa [28, 31, 35]. Eighty-five percent of patients who received moxifloxacin combinations had cleared active tuberculosis from the lungs within 2 months with favorable safety profiles [35, 36]. Both compounds show promise to shorten duration of therapy to four months, and will be evaluated in both HIV positive and HIV negative patients. The primary concern with these drugs is their high use for treatments of community-acquired infections, which may pose problems for the development of resistance. People that have latent TB infections that get treated for other infections with these compounds may stimulate selective pressures that can enhance the populations of resistant phenotypes. Recent studies have demonstrated a high prevalence of fluoroquinolone resistance associated with the Beijing phenotype [37]. In addition, XDR strains have demonstrated resistance to currently used fluoroquinolones as defined [38 , 39, 40].

The next advanced compound, diarylquinoline TMC207, is now in phase 2 clinical trials [29, 31, 32, 34, 41-43]. This compound has a novel target, mycobacterial ATP synthase, and inhibits the ability of the bacterium to synthesize ATP, depleting the bacterium's energy stores. This mode of action can affect all

cells replicating and resting, and provide a powerful potential as a sterilizing agent. *In vitro*, it has demonstrated bactericidal activity to both susceptible and drug-resistant *M. tuberculosis* with an MIC of 0.06 μg/ml. It has an extended half-life of over two days in mice (173h in man), and its bactericidal activity is maintained for one week in mice [41]. TMC207 demonstrated a significantly greater killing power under monotherapy than INH or RIF, and thus is presumed to act on an additional subset of bacteria (nonreplicating persisters) not susceptible to current drugs in the frontline regimen [42]. Thus, its use in combination with compounds in the current regimen is presumed to show increased activity and may reduce the duration of current therapy. The most impressive data recently established TMC207's combinatorial activity with rifapentine and pyrazinamide given once a week to a decreased bacterial burden (7 $\log_{10}$) with negative lung cultures in mice after only two months of therapy [41]. This not only exhibits the potential for a once weekly dosing regimen, but also suggests a greater efficacy with shortened treatment duration. The primary concern would be that the effects demonstrated in mice do not reflect similar activity in humans. Also, TMC207 is metabolized by cytochrome P450 3A4 enzyme, and its serum concentration is reduced by rifampin, one of the first-line drugs in current treatment [31, 41]. Thus there is question as to whether rifapentine has a similar effect. Promisingly, TMC207 has demonstrated good safety and tolerability in early human trials [43].

Another top class of compounds currently in human clinical trials is nitroimidazoles: PA-824 and OPC-67683 [29, 31, 34, 44-48]. The mechanism of killing with these compounds is complex. Both are known to disrupt mycolic acid

synthesis, and thus are involved in inhibiting cell wall biogenesis of actively replicating bacilli. Interestingly, both are shown to act on nonreplicating bacilli through a novel mode of action that involves the metabolism of the compound into a reactive Des-nitro metabolite that induces the formation of reactive nitrogen intermediates, mimicking the killing activity of the innate host immune response [46, 49]. This activity is effective on aerobically growing and intracellular anaerobically resting bacilli making the compound a most interesting alternative for TB treatment. PA-824 has shown significant activity *in vitro* with drug-sensitive, drug-resistant, and persistent phenotypes [44, 45]. Its activity against nonreplicating bacteria has been reported comparable to metronidazole and RIF [29, 44, 50]. Most significantly, PA-824 was recently shown to act on residual persisting bacilli remaining after a 100-day microaerophilic culture after rifampicin treatment, thus acting on drug tolerant bacilli are the most difficult to eliminate [47]. These results indicate that PA-824 has substantial sterilizing power, and offer another alternative for human therapy. OPC-67683 also demonstrates *in vitro* and *in vivo* activity against drug-sensitive and drug-resistant strains [31, 48, 49]. In addition, this compound demonstrated activity against H37Rv in immunosuppressed SCID mice, and was not affected by or induced the activity of liver microsome enzymes (CYPs), making it a candidate for treatment of patients co-infected with HIV [49]. In chronic mouse models, its activity was 6-7 fold higher than firstline drugs and was an effective addition in combination therapy with the possibility of shortening treatment [49]. The effective action of both nitroimidazole compounds for various TB phenotypes along with their novel

mechanism of action make these molecules extremely promising candidates for further development as a treatment for tuberculosis.

In January 2009, SQ-109, a new diamine compound entered a Phase 1B study to assess safety and pharmacokinetic parameters in healthy subjects. SQ-109 has demonstrated *in vitro* activity against H37Rv and Erdman *M. tuberculosis* strains with MICs of 1.56 μM and 0.7μM, respectively [51]. The compound also demonstrated significant activity against drug-resistant strains and in macrophage assays was able to reduce intracellular burden by 99% at its MIC [51]. *In vivo* efficacy testing at 1mg/kg was as effective as ethambutol at 100 mg/kg with no visible toxicity at concentrations as high as 600 mg/kg [51]. The compound demonstrated cooperative killing in a drug combination of INH, RIF, and PZA that was more efficient and effective than the current frontline regimen [52]. Phase 1a (single-dose) clinical studies found that doses of SQ-109 up to 300 mg were safe and well tolerated, with no serious side effects [52].

Several other compounds are in early stages of discovery and development, providing hope for new effective therapies against tuberculosis that have begun to address current treatment issues. The fact that there are new compounds being designed to attack TB bacilli through novel alternative mechanisms suggests that, with proper control and treatment measures, the problems that are being faced today may be resolved in the future.

Table 2.3 Current TB drugs and those in the pipeline of development [27]

(as reported in Tuberculosis (2008) 88 (2) 85-170) *Grey= in clinical trials*

Compound	Mechanism of Action	Target	Status
Amikacin	protein synthesis	30S ribosomal subunit	second line drug
Capreomycin	protein synthesis	30S ribosomal subunit	second line drug
Clarithromycin	protein synthesis	50S ribosomal subunit	current use published
Clofazimine	membrane perturbtations	unknown	second line drug
Cycloserine	cell wall biogenesis	alanine racemace	second line drug
Ethambutol	cell wall biogenesis	arabinosyl transferases	first line drug
Ethionamide	cell wall biogenesis	inhA, enoylACP reductase	second line drug
Gatifloxacin	DNA synthesis	DNA gyrase/Topoisomerases	phase 3 clinical trials
Isoniazid	cell wall biogenesis	inhA, enoylACP reductase	first line drug
Kanamycin	protein synthesis	30S ribosomal subunit	second line drug
Levofloxacin	DNA synthesis	DNA gyrase/Topoisomerases	second line drug
Linzolid	protein synthesis	23S ribosomal subunit (fm-tRNA)	some clinical use reported
LL3858	unknown	unknown	early clinical trials
Moxifloxacin	DNA synthesis	DNA gyrase/Topoisomerases	phase 2,3 clinical trials
OPC-67683	cell wall biogenesis/RNIs	mycolic acid synthesis/RNIs	phase 2 clinical trials
PA-824	cell wall biogenesis/RNIs	mycolic acid synthesis/RNIs	phase 2 clinical trials
PAS	folate synthese	Dihydropteroate synthase	second line drug
Prothionamide	cell wall biogenesis	inhA, enoylACP reductase	second line drug
Pyrazinamide	membrane potential	alters pH in the cell/	first line drug
Rifabutin	RNA synthesis	DNA dep-RNA polymerase	cotreatment HIV patients
Rifalazil	RNA synthesis	DNA dep-RNA polymerase	phase 2 clinical trials
Rifampin	RNA synthesis	DNA dep-RNA polymerase	first line drug
Rifapentine	RNA synthesis	DNA dep-RNA polymerase	cotreatment HIV patients
SQ109	unknown (EMB analog)	unknown	phase 1 clinical trials
Streptomycin	protein synthesis	30S ribosomal subunit	limited use first line drug
Thioridazine	respiration	NADH oxidoreductase	compassion therapy
TMC-207	ATP synthesis	ATP synthase	phase 1,2 clinical trials

2.2.2 The search for novel targets

Today, even with several new prospective drug candidates in clinical evaluation, researchers are working hard to identify alternative drug targets with novel modes of action to address the various issues posed by current TB therapy. The premise that only 1 in 10 drug compounds successfully make it to market and become available as a useful therapeutic, lingers in the background casting a shadow of doubt on the prospective success of drugs currently in clinical trials. Thus, the search for alternatives continues and provokes new ideas as to what could be considered essential for a viable drug target.

Typically, good drug targets are associated with "essential genes", or genes whose inactivation leads to the death of the bacterium. In the past, various studies have focused on targets essential for growth and viability, and through the use of mutagenesis strategies, many "essential" candidates have been identified over the past several years [4, 6-9, 11]. However, new research is looking at candidates that promote pathogenesis and survival mechanisms that are more representative of all TB phenotypes under *in vivo* conditions. For example, virulence associated factors, intracellular signaling components, and elements involved in regulation of cell activities are becoming areas of interest for identifying lead targets [53-58]. Potential drawbacks for these candidates are that the inhibition of these factors may not be lethal to the bacteria, and that drugs targeting these modes of action may have little effect on established infections [13, 53-55]. The premise behind these targets is that drugs acting on them could be used in conjunction with current regimens to enhance treatment by limiting bacterial pathogenesis and allowing

natural host immune system responses to cooperatively clear infections [54, 55, 59]. In addition, since these genes are not "essential" for growth, the development of resistance is less likely.

In the past, most TB drugs have targeted cell wall biogenesis, as the cell wall of TB has a variety of unique features, and targeting cell wall proteins is very selective for the bacterium. A continued focus of cell wall synthesis involves targeting enzymes associated with lipid synthesis in the FASII system [60-65]. INH is the best-known inhibitor of the FASII pathway and is currently one of the most effective drugs against actively replicating TB. Drug candidates such as Thiolactomycin, Cerulenin, and N-ocanesulfonylacetamide are also inhibitors of enzymes in this system, and demonstrate activity against *M. tuberculosis* [66]. Recent work by Sullivan et al., used structure-based design methods to develop a series of diphenyl ether compounds with activity against InhA, the enoyl reductase enzyme of the FASII pathway in *M. tuberculosis* [64]. These compounds not only demonstrate nanomolar inhibition against InhA, but also have shown activity against INH-resistant strains. However, this system is likely to be effective primarily against actively growing bacilli and less effective on nonreplicating persistent bacilli.

Unique physiologic features of *M. tuberculosis* offer additional opportunities for drug design. Studies have alluded to some surprising weaknesses of the bacilli, which could be exploited. First, it is known that *M. tuberculosis* has a deficiency to efflux pyrazinoic acid (POA), and is susceptible to pyrazinamide (PZA) [67]. POA accumulates under acidic pH, a condition known to be found

within the granuloma, and disrupts membrane potential altering the flow of H+ ions important for ATP synthesis [67 , 68]. This, along with the fact that the new compound TMC207 inhibits ATPase activity, offers a possible synergism for these two drugs in combination. Identifying other important targets involved in respiration under aerobic or anaerobic metabolism could pose other synergic mechanisms with PZA that prove highly effective. Additionally, the bacterium is known to have a difficult time defending itself against endogenous oxygen radicals like those generated by KatG-mediated INH activity [67, 69]. There is evidence that *M. tuberculosis* is highly susceptible to endogenously reactive nitrogen intermediates. Several compounds (e.g. PA-824) that generate these reactive nitrogen species have demonstrated activity against *M. tuberculosis*, even nongrowing bacilli [46, 49]. These compounds act as a prodrug that requires intracellular activation by a nitroreductase encoded by *rv3547* (Ddn). The enzyme Ddn metabolizes the compound into derivatives that generate reactive nitrogen species. Therefore, designing "prodrugs" like PA-824 to target the action of TB enzymes that promote killing mechanisms innate to host defenses is another avenue for finding novel areas to target.

The primary focus in target identification for *M. tuberculosis* has been directed toward finding targets unique to latent TB bacilli. For tuberculosis, the ideal drug would have bactericidal activity for all phenotypes found in both extracellular and intracellular niches. New drug candidates for tuberculosis are currently focused on intracellular bactericidal activity against nonreplicating persisters. Attacking this population of cells would reduce the duration of therapy and the development of

resistance. New compounds that are effective at killing persisting bacilli used in combination with drugs effective on actively replicating bacilli may provide a faster cure rate.

To date, only two drugs, RIF and PZA have been shown to be effective on dormant bacilli, but do not kill all of the phenotypes found in the persistent population. Tubercle bacilli in lesions are thought to consist of several distinct subpopulations: the active growers that can be killed with primarily firstline cocktails, those that have spurts of metabolism that can be eliminated with RIF, those in acidic environments with low metabolism that are knocked out with PZA, and those that are "dormant" which remain untouched by any therapy to date [70]. The nonreplicating (dormant) persisters are a major problem in therapy and are the primary initiators of relapse of the disease.

A number of studies, originating with Wayne's studies, have attempted to develop models of nonreplicating persistent *M. tuberculosis* in order to understand elements required for a persistent lifestyle [71, 72]. These studies have provided information about the bacilli under altered conditions of hypoxia, nutrient starvation, oxidative stress, and survival in macrophage or murine models [68, 71-95]. However, each of these studies mimics some, but not all of the clinical aspects of the disease in humans. Hypoxia studies may capture one aspect of nature in the granuloma, but it does not address effects of the immune response, macrophage phagocytosis, or release into the extracellular milieu. Starvation models are not hypoxic, and do not address the unique diet associated with the bacilli inside the granuloma. Macrophage experiments address early adaptations to the immune

response, but do not address long-term metabolic changes. Murine models of infection pose many similarities to human infection in relation to the immune responses; however, mice do not typically form granulomas with caseous, necrotic centers characteristic of human infection. Other animals such as the guinea pig model, the rabbit model, and the non-human primate model more closely resemble human disease, but studies are often costly and more restrictive. Recent experiments by Via et al. (2008), used several alternative animal models (mouse, guinea pig, rabbit, and non human primates) to assess the level of oxygen in TB granulomas using an oncology probe, pimonidazole hydrochloride, a hypoxia-imaging agent [68]. These studies found that all models, except the murine model, had hypoxic granuloma lesions. Important work like this can help define the unique metabolism associated with nonreplicating persisters, or identify interesting facets of *M. tuberculosis* infection, which may lead to the identification potential drug targets of latent-persistent tuberculosis.

Studies by Murphy and Brown [96] identified gene targets associated with dormant phase tuberculosis by compiling information from genome-wide microarray analyses and genome-wide mutagenesis analyses performed under the various models previously described [8, 10, 97-99]. Common trends associated with conditions of nutrient starvation, hypoxia, and mouse infection include upregulation of genes controlled by *devR (dosR)*, down-regulation of protein and ATP synthesis, and adaptations of two-carbon metabolism and anaerobic respiration. Promising targets for drug discovery defined by this study include several regulatory elements (*devr/devS, relA, and mprAB*), enzymes involved in

alternative metabolic processes that include redox balance and anaerobic respiration mechanisms, enzymes involved in sulfur transport and fixation, those involved in pantothenate, isoprene, and NAD biosynthesis, enzymes associated with C_2 metabolism, and those involved in regulating stress, (Appendix A) [96].

As noted previously, systems of regulation are also being considered as possible targets for drug action. Transcriptional regulators offer another set of unique targets in *M. tuberculosis*. Many of these are associated with adaptive strategies to altered environmental conditions associated with pathogenesis and survival. Plausible drug targets include alternative sigma factors that bind to RNA polymerase and initiate transcription under stress-induced conditions that allow the bacilli to adapt and survive. *M. tuberculosis* has 13 sigma factors in the genome, most of which are only activated upon various stressful conditions [100]. Several of these factors have either undergone knockout studies or over expression studies to demonstrate their significance *in vitro* and *in vivo*. SigA is primarily the housekeeping factor normally expressed under standard growth conditions. SigB is networked with SigEH and is involved in global gene expression under heat shock, oxidative stress, chemical stress, survival in macrophages, and virulence [100, 101]. SigC is thought to play a role in immunopathogenesis as a mutant resulted in attenuation in mice and a lack of necrotic lesions in guinea pigs [102, 103]. SigD demonstrates a role in virulence as a mutant also resulted in attenuation in mice [104]. Recent work establishes SigE as a master regulator of a regulon involved in modulating the host inflammatory response [105]. It was also previously noted to control genes required for intracellular survival, and a *sigE* mutant provided

delayed death in mice [106, 107]. SigF is induced under stationary growth and stress conditions involving nitrogen depletion, oxidative stress, cold shock, hypoxia, and antibiotic treatment [100, 101]. It was also found to directly regulate the transcription of PhoY1 a regulator of phosphate uptake [108]. SigG was recently found to be associated with SOS stress responses and SigH is induced by oxidative stress and heat shock [109, 110]. Very little is known about sigma factors SigI, SigJ, SigK, SigL, and SigM. Recent overexpression studies of SigL and SigM have shown increases in expression of genes involved in modulating cell surface molecules and secretion functions [111, 112]. Because sigma factors are important in global gene transcription, their requirement for pathogenesis and adaptive survival, along with their absence in the host, these genes hold promise as possible drug candidates for *M. tuberculosis*.

Finally, unique targets involved in cell cycle regulatory events offer another arena for drug discovery efforts in *M. tuberculosis*. Current efforts involve targeting FtsZ, a tubulin homologue involved in bacterial cell division [113-116]. It is known that FtsZ polymerization and ring formation initiates cell division [117-126]. Inhibitors of this event would prevent cell proliferation and thus kill the cell. FtsZ is a promising target, as its function and structure are well defined. In studies by Slayden et al. (2006), compounds thiabendazole and albendazole demonstrated inhibitory activity against *M. tuberculosis* [127]. Both compounds provided evidence for an inhibition of cell division through ultrastructure analyses and drug treatment transcriptional profiling. These results provide compelling data that suggests FtsZ as a novel target for drug discovery efforts. Other efforts describe the

effects of noncytotoxic taxanes as novel antituberculosis agents that target FtsZ [113]. In addition, there are several proteins that regulate cell cycle events through the regulation of FtsZ. Most of these candidates function to stabilize or destabilize FtsZ ring formation. Stabilizing factors include ZapA, ZipA, and FtsA, while destabilizing factors include SulA, EzrA, and MinCD [117-126, 128]. Two of these regulatory elements under investigation in this research (Chapters 4 and 5) are min-system proteins (MinCD) that regulate septum placement prior to cell division, and stress-induced proteins (SulA/YneA) involved in DNA damage repair systems that stall cell division while DNA is under repair. Many of these genes, however, are not annotated in *M. tuberculosis*, and thus identification of these targets requires the use of bioinformatic strategies. However, the interest in identifying cell cycle regulators involved in preventing cell division is due to the enticing possibility that they may have a connection with a transitional event leading to the induction of dormant state nonreplicating bacilli and latent tuberculosis. Research described in chapters 4 and 5 describe initial efforts toward identifying cell cycle regulatory mechanisms in *M. tuberculosis*, by identifying two proteins involved in septum regulation through bioinformatic and other genomic strategies.

In summary, primary targets of interest for exploration involve altered metabolism and adaptive survival mechanisms associated with latent disease and the tubercle bacilli. Promising candidates are those involved in respiration and energy metabolism, those that respond to oxygen limitation, enzymes involved in fatty acid degradation/utilization, elements induced under nutrient starvation that utilize alternative carbons sources, enzymes involved in the synthesis of unique cell

wall components that establish virulence and persistence, transcriptional regulators required for adaptive responses, elements that promote intracellular life and inhibit phagosome maturation, and those components resistant to oxidative stress. Additional candidates include proteins involved in cell cycle regulation associated with cell division events and septum formation that could provide a link between a halt in cell division and nonreplicating persisting bacilli.

Steps taken to identify novel drug targets associated with the nonreplicating latent phenotype will provide additional arsenals for attacking dormant persisters that have been troublesome with current TB therapy. Establishing drug alternatives to these bacilli will undoubtedly reduce treatment duration and thus alleviate issues concerning compliance and the development of resistant phenotypes. Continued efforts in attacking acute replicating bacilli are also needed to support currently effective compounds and deal with problematic drug resistant strains. Thus, areas identifying novel targets of cell wall synthesis, cell division, or unique physiologic traits will offer alternatives to fighting resistant strains. In addition, prodrugs mimicking enzyme substrates that activate innate killing mechanisms may bypass detoxification strategies involving CYPs, and provide alternatives for HIV co-infected patients needing co-infective therapies. Therefore, the areas of focus for target identification are all justifiable for enhancing the currently available TB drug regimens.

2.3 TULARENSIS: DRUG DISCOVERY

Current firstline therapies against all clinical forms of tularensis are effective. However, it has been established that many of these compounds have various toxicity issues, and relapse of disease upon treatment cessation is problematic. Additional concerns owe to the possibility of engineered resistant phenotypes that have been developed under bioweapons programs that may pose future problems if placed in the hands of individuals who would release them in a bioterrorist event. Top candidates that are most effective are delivered by IV/IM and thus are not suitable for either prophylactic therapy measures or mass distribution in the event of large outbreaks from an act of bioterrorism.

2.3.1 Searching for alternatives

In the scientific research community, a large amount of government funding has been directed toward designing new diagnostic tests, preventative vaccines, and alternative chemotherapeutics for category-A select agents like *F. tularensis*. Most of the research so far has focused on understanding the biology of the organism, its associated virulence, and the development of new vaccines and diagnostic tests. Few researchers are working to design alternative drug therapies upon release of a resistant strain or for prophylactic treatments. An important goal is to find a novel drug therapy that targets a conserved function in all category-A and B select agents that could be used as a broad-spectrum alternative. This would allow soldiers to carry with them one drug with complete effectiveness for any bioagent exposure, and provide a broad-spectrum treatment for exposed populations after a mass

release. Most of the select agents are fast acting and develop disease symptoms that are indistinguishable from one another. Initial symptoms may resemble a common cold or flu: sore throat, mild fever, muscle aches and malaise. Release in a densely populated area would presumably result in an abrupt onset of a large number of acute, nonspecific febrile illnesses, typically 3–6 days after initial exposure. Once recognized that a mass release of an aerosolized agent has occurred, it would be critical to distribute an effective therapy as swiftly as possible to the affected population, one that will work against all suspected organisms.

Very little research in the area of alternative therapies for *F. tularensis* has occurred in the past due to its low incidence rate and the fact that current effective therapies exist. Most efforts have focused on designing an alternative vaccine, a more preventative therapy. However, recent concern of its use as a bioterrorist agent has led to a resurgence of interest in drug discovery.

2.3.1.1 Fluoroquinolones

In 2006, the newest fluoroquinolones, gatifloxacin and moxifloxacin were assessed in murine models for treatment of pneumonic tularemia [129]. Both prevented disease during treatment, although significant failure rates occurred after cessation of therapy as a result of relapse. Higher mortality rates were seen for ciprofloxacin (100%) within 7 days of treatment cessation than with either gatafloxacin or moxifloxacin. It was suggested that longer treatment regimens would provide better efficacy, however partial bactericidal activity was observed in these studies and elimination of *F. tularensis* was not complete. A recent

evaluation of 145 cases of oropharyngeal tularemia caused by drinking spring water contaminated with *F. tularensis holarctica* (Type B) in a small district of Turkey assessed the roles of various antibiotics, including the fluoroquinolones ciprofloxacin and moxifloxacin [130]. The majority of the cases received traditional aminoglycosides or tetracycline treatment, which are proven effective therapies. Forty-eight patients received fluoroquinolone treatment; 37 were treated with ciprofloxacin and 11 were treated with moxifloxacin. Treatment success was similar to recommended therapies with streptomycin or gentamicin. This was the first published report of human treatment with moxifloxacin demonstrating success. However, it must be noted that this case study involved a less virulent strain of *F. tularensis* and presented a less severe form of the disease compared to that of inhaled virulent *F. tularensis tularensis.*

2.3.1.2 Targeting two-component systems

Strategies targeting virulence features of pathogens are at the forefront of drug research. Targeting elements of secretion, signaling pathways, factors involved in intracellular survival, or components involved in adhesion, invasion, or biofilm formation are of current interest [54-56, 59, 131]. Recently, Rasko et al. (2008), identified a lead compound, LED209, that interferes with QseC signaling, the sensor histidine kinase of a two-component system involved in initiating a cascade of regulatory factors that upregulate key virulence genes in a variety of pathogens including *F. tularensis* [58]. The compound demonstrated significant attenuated virulence *in vivo,* but did not inhibit the growth of several screened pathogens.

Importantly, LED209 appeared to slow growth of *F. tularensis* (SCHU S4) in macrophages and reduce the expression of a number of recognized virulence genes. When administered in a single dose to mice three hours after aerosol infection, 80% of the mice survived up to 9 days, much longer than control mice. Bacterial two-component systems are attractive drug targets for several reasons. First, they are not found in humans: however, homologues are found in a variety of pathogens making effective compounds potential for broad-spectrum therapy. Second, inhibition of signaling does not alter growth, thus there is a decreased potential for the development of resistance. Finally, altering virulence and pathogenesis allows the host immune response to battle the infection, develop memory, and establish immunity.

2.3.1.3 Targeting FASII lipid synthesis

The FASII system (Figure 2.2) has been heavily studied in several pathogens and validated as an attractive target for novel antibacterials [61-66, 111]. The most common FASII enzymes targeted are the enoyl reductase FabI and ketoacyl-ACP synthases FabH, FabB, and FabF [62, 66, 132]. Thiolactomycin and cerulenin are natural compounds that target ketosynthases while diazaborines inhibit FabI [62, 66, 132]. In addition, the frontline TB drug INH inhibits InhA, the enoyl reductase enzyme in *M. tuberculosis* [63-65, 111]. Compounds targeting enzymes in this pathway offer a conserved mechanism of actions leading to a broad-spectrum therapy.

The fatty acid synthesis pathway in *F. tularensis* is a type II (FASII) dissociated synthesis in which individual reactions are carried out by separate

enzymes. Importantly, eukaryotes utilize the type I fatty acid biosynthesis multienzyme complex (FAS I), which is different from the enzymes in FAS II pathway [133]. The NADH-dependent enoyl reductase (FabI), which catalyzes the last reaction in the elongation cycle, is known to be an essential component in the

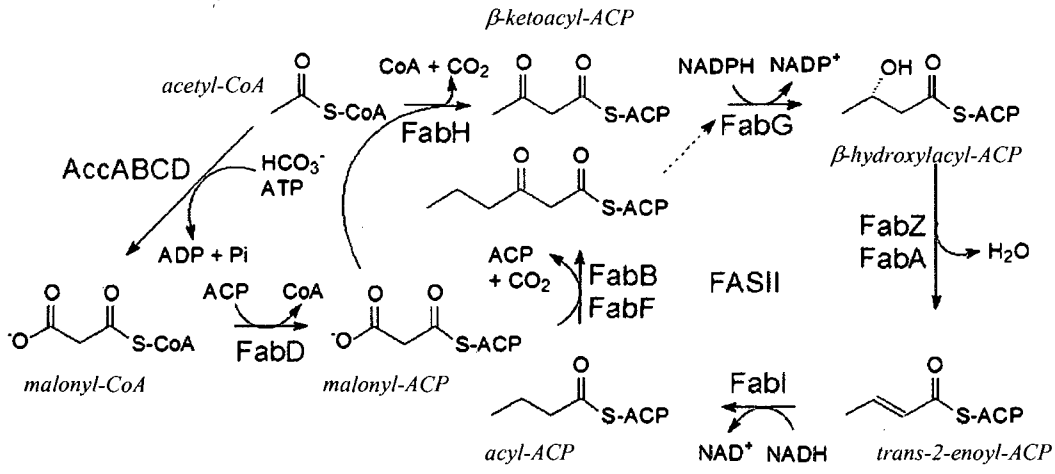


Figure 2.2 FASII fatty acid biosynthesis pathway. The FASII pathway consists of several enzymes that are required to perform two carbon additions to acetyl-CoA in a stepwise process to synthesize lipids needed for bacterial membranes and cell wall structures. The growing fatty acid is carried by an acyl carrier protein, ACP, and the elongation process uses a malonyl-ACP formed from the initial transfer of the malonyl moiety of malonyl-CoA to the ACP protein via FabD, and acyltransferase enzyme. The cycle initiates with a condensation reaction, adding two carbons from acetyl-CoA to the growing molecule attached to the ACP carrier protein. This reaction is facilitated by FabH, a β-keotacyl synthase enzyme, and involves the loss of CO_2. The molecule goes through a reduction and a dehydration carried out by FabG (3-ketoacyl reductase) and FabA/FabZ (3-hyroxyacyl dehydratase) to form 2-enoyl-ACP. FabI, the enoyl reductase in the final step of the cycle reduces this molecule before another round of addition occurs, extending the chain. Several compounds are known to inhibit various stages of this process with triclosan involved in the inhibition of FabI, the enoyl reductase enzyme.

FAS-II system [134]. Genetic knockout and knockdown experiments together with studies utilizing small molecule FabI inhibitors have demonstrated that FabI is essential for bacterial cell growth, thus making it an attractive target for drug discovery [60, 135-137]. Several classes of chemicals have been identified that are picomolar inhibitors of FabI [138-140]. Notably, the diphenyl ether triclosan has broad-spectrum chemotherapeutic activity against a variety of pathogens including *E. coli*, methicillin-resistant *S. aureus* and *M. tuberculosis* [65, 141].

Currently, there are two groups investigating small molecule inhibitors of enzymes found in the FASII fatty acid biosynthesis pathway for *F. tularensis*. Wen et al. (2009), initiated the screening of small molecule inhibitors against *F. tularensis* FabH (3-oxoacyl synthase III) and FabI (enoyl reductase) enzymes found in the FASII system of *F. tularensis* [142]. These preliminary enzyme inhibition screens have identified two indole derivatives with activity against each enzyme: WIUAKP-001 (40μM) against ftuFabH and WIUAKP-031 (37.5μM) against ftuFabI.

More advanced work on ftuFabI inhibitors by Lu et al. (2009) identified a potent lead compound with significant *in vitro* bactericidal activity that demonstrates 100% *in vivo* efficacy in the murine mouse model of infection against aerosolized *F. tularensis* [143]. This research identified and characterized ftuFabI, the enoyl ACP-reductase of *F. tularensis*. These studies provided crystallographic analysis that revealed a high similarity between the structure of the ecFabI:NAD+ complex and the 2.9Å resolution structure of the binary ftuFabI:NAD+ complex. Kinetic studies confirmed the mechanism of inhibition as a slow-onset inhibition

through a loop ordering conformational change similar to that of ecFabI. Further *in vitro* whole cell assays identified several compounds (Figure 2.3) with significant antibacterial more favorable than current clinically used drugs. Importantly, a positive linear correlation between K_i and MIC_{90} exists for their diphenyl ether derivatives, indicating that ftuFabI is the primary cellular target.

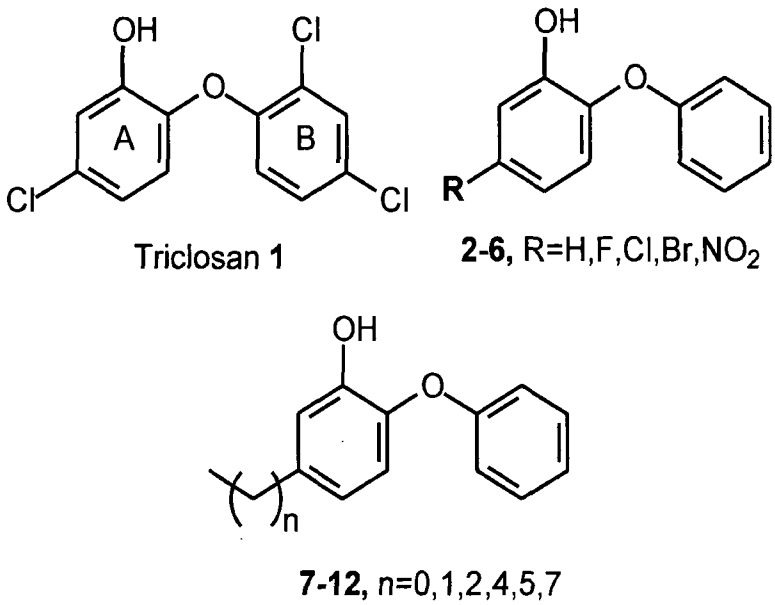


Figure 2.3 Structures of diphenyl ether ftuFabI inhibitors. Several analog molecules were designed from the parent molecule triclosan and assessed for their inhibitory properties against ftuFabI and bacterial growth.

Lu et al., continued their work with *in vivo* animal testing that showed animals treated with diphenyl ether compounds survived longer than untreated control animals, thus indicating that diphenyl ethers are effective at killing the

bacteria *in vivo*. Importantly, compound 11, 5-hexyl-2-phenoxyphenol (SBPT04) had the greatest efficacy, preventing the death of all infected animals up to 10 days with no sign of relapse of the disease. Furthermore, analyses demonstrated a positive linear correlation between the residence times of each compound and *in vivo* antibacterial activity, suggesting that the enhanced *in vivo* antibacterial activity of compound **11** is due to the longer residence time of this compound in the enzyme drug target.

These studies indicate an effective alternative therapy with potential broad-spectrum activity that demonstrates enzyme specificity, potency, and efficacy against virulent *F. tularensis* (SCHU S4). Optimization of compound **11** for ADME properties, bioavailability, oral delivery formularies, and preclinical metabolism studies are needed to advance this compound. Continued work in the optimization and the development of SBPT04 (Compound **11**) is presented in Chapter 3.

Research conducted in the area of new drug candidates for the highly virulent pathogen *F. tularensis* are presently limited to areas in which broad-spectrum therapies may evolve as research studying its mechanisms of virulence and pathogenesis are currently unfolding. Future developments may involve specific virulent features associated with elements found in its pathogenicity island that are responsible for intracellular survival and virulence. Other areas may involve mechanisms associated with its ability to delay immune activation of innate responses. Finally, since one of the primary factors associated with *Francisella's* pathogenesis is the ability to replicate into high numbers and disseminate, drugs that prevent the bacterium from entering cells and replicating, or that prevent

dissemination may be key additions to current treatment enhancing their effectiveness and reducing the risk of relapse. These drugs may be useful for prophylactic therapy and for prevention of recurrent disease upon a mass release scenario.

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CHAPTER 3

Substituted Diphenyl Ethers as a Broad-spectrum Platform for the Development of Chemotherapeutics for the Treatment of Tularemia

The work presented in this chapter consists of a collaborative effort between the laboratories of Dr. Richard Slayden and Dr. Peter J. Tonge with contributions from the RCE Animal Models Core and Discovery Elucidations LLC. Diphenyl ether compounds were developed in the laboratory of Dr. Tonge. Pharmacokinetic parameters along with microsome metabolisms studies were done in collaboration with the laboratory of Dr. Lee Arnold. Animal testing was facilitated in the RCE Animal Models Core with assistance from Dr. Nicole Marlene. My role involved the biological characterization of compounds for their antibacterial activity (MIC/MBC), cytotoxicity screening, assessment of *in vivo* efficacy in the rapid mouse model of infection, and to examine the mode of action via the transcriptional response of *F. tularensis* (LVS) treated with compound SBPT04 using DNA microarray analysis.

3.1 INTRODUCTION

Francisella tularensis is an obligate intracellular bacterium that can cause severe, acute and often fatal respiratory and systemic infection in humans. The ability of this highly infectious and virulent bacterial pathogen to be readily aerosolized along with its history as a potential biological weapon has led to the possibility that it could be used deliberately in an act of bioterrorism [1-4]. Consequently, NIAID has classified *F. tularensis* as a Category A priority pathogen and has stated the need for the development of novel chemotherapeutics that can be used rapidly and effectively to treat infections caused by this organism. While some broad spectrum antibiotics are currently indicated for selected Category A and B pathogens, concern is growing that the widespread and

aggressive use of key antibiotics and potential engineering of specifically weaponized strains will result in the development of drug resistant organisms. Thus, there is an important need for new broad-spectrum compounds with enhanced therapeutic effects compared to existing frontline treatments.

Current treatment regimens for *F. tularensis* infections utilize streptomycin, gentamicin, tetracyclines, chloramphenicols along with alternative treatments including doxycycline and ciprofloxacin [4-7]. However, the efficacy of these regimens is limited because streptomycin and gentamicin have toxic side effects and neither can be orally administered, and tetracyclines and chloramphenicols are associated with relapse rates as high as 10%. Fluoroquinolones have also shown promise in a limited number of cases; however clinical experience is limited and their role in treating severe disease is unknown [8-10]. In animal studies gatifloxacin, moxifloxacin, and ciprofloxacin prevented disease during the treatment period, but significant failure rates occurred after the cessation of therapy [11]. Since existing drugs have limited potency and efficacy, and it is unknown whether drug-resistant organisms might be used in an intentional release, it is prudent that novel chemotherapeutics with reduced side effects compared to existing front-line drugs be developed.

Historically, clinically relevant drugs target DNA, RNA, protein, and cell wall biogenesis. However, emerging alternative targets for novel antibacterial drug discovery include the enzymes of Type II fatty acid biosynthesis. In particular, FabI has been demonstrated as a promising broad-spectrum target for chemotherapeutic action [12-24]. The utility of diphenyl ethers to target FabI in *F. tularensis* has been enhanced by our own drug discovery program which is founded on the observation that the diphenyl ether

triclosan is a potent inhibitor of the FabI enzyme in other organisms [13-15, 17, 21, 25-30]. Using structure-based drug design strategies we have developed a series of substituted-diphenyl ethers that are potent inhibitors of the *M. tuberculosis* FabI and that are active against multidrug resistant strains of *M. tuberculosis* [22, 31]. In addition, selected compounds are also nanomolar inhibitors of the FabI from *S. aureus* and have excellent *in vitro* activity against methicillin-resistant *S. aureus* [31].

Recently, through crystallographic modeling and enzyme inhibition studies of a directed library of substituted diphenyl ethers we identified lead compounds that demonstrated inhibitory activity of the enoyl reductase enzyme, ftuFabI, of *F. tularensis* [32]. The high potency of these compounds against *F. tularensis* substantiates the diphenyl ethers as drugs targeting the under exploited fatty acid biosynthesis pathway. The research presented in this chapter expands on the work reported by Lu et al. (2009) in Chapter 2 which identified lead compound SBPT04 as a high affinity inhibitor that demonstrated *in vitro* activity against ftuFabI, the enoyl reductase enzyme in the *F. tularensis*, and *in vivo* efficacy in the murine model of infection. Accordingly, the work presented here extends enzymatic and whole cell bacterial studies along with preliminary efficacy studies by demonstrating efficacy via oral delivery and assessing bioavailability through PKPD analysis and *in vitro* metabolism studies of the most potent preclinical lead, SBPT04. In addition, this research further investigates the mode of action of SBPT04 through DNA microarray transcriptional profiling of *F. tularensis* under drug treatment conditions. These studies provide critical information for lead optimization that will be used to advance substituted diphenyl ether based compounds into preclinical and clinical trials. Here we demonstrate *in vivo* efficacy against *F.*

tularensis in the mouse model of infection, with enhanced bioavailability, and decreased toxicity to that of the parent compound and other analogs. Importantly, this work substantiates diphenyl ethers as a platform for the development novel chemotherapeutics for the treatment of tularemia and other bacterial agents.

3.2 METHODS

3.2.1 Bacterial strains, culture conditions and mice

SCHU S4 and LVS were cultured in modified Mueller-Hinton broth (MMH) (0.025% ferric pyrophosphate, 2% IsoVitaleX, 0.1% glucose) at 37°C with constant shaking overnight, aliquoted into 1 ml-samples, frozen at -80°C, and thawed just before use as previously described [33]. Frozen stocks were titered by enumerating viable bacteria from serial dilutions plated on modified Mueller-Hinton agar (0.025% ferric pyrophosphate, 2% IsoVitaleX, 0.1% glucose, and 0.025% FBS) as previously described [33]. The number of viable bacteria in frozen stock vials varied <5% over a 10-month period. These stocks were used to start cultures for all bacterial assays.

Six week-old female ICR mice were purchased from Charles River Laboratories. All mice were housed in sterile microisolater cages in the laboratory animal resources facility or in the Biohazard Research Building BSL-3 facility at Colorado State University (Ft. Collins, CO) and provided sterile water and food ad libitum. All research involving animals was conducted in accordance with animal care and use guidelines and animal protocols were approved by the Animal Care and Use Committee at Colorado State University.

3.2.2 MIC and MBC determination

Minimal inhibitory concentrations (MIC) were determined via NCCLS protocols as previously described [34-37]. *F. tularensis* (LVS) was grown to mid-log and diluted to provide an inoculum of 10^5 cells per well when dispensed into a 96-well microtiter drug plate. Compounds were tested at 2-fold serial dilutions from approximately 128 to 0.000015 μg/ml in triplicate. Visual readings (via pellet density) were obtained after 18-20 hours of incubation at 37°C. Microplate Alamar blue assay (MABA), a colorimetric drug-susceptibility testing method that indicates bacterial viability, was also used as described with minor modifications for *Francisella* [38]. MIC_{90} values were determined by graphing %-inhibition calculated from spectrophotometric plate readings (570nm and 600nm) using GraFit software. Minimal bactericidal concentration (MBC) was determined by plating onto MMH agar plates 10-fold serial dilutions from wells indicating a %-inhibition of 90% or greater *via* MABA analysis. The concentration demonstrating a no growth (99.9% killing) was considered the MBC.

3.2.3 Cytotoxicity testing

African green monkey kidney cells (Vero cells) were grown in RPMI 1640 medium supplemented with 1.5 g/L sodium bicarbonate, 10 mL/L 100 mM sodium pyruvate, 140 mL/L100x nonessential amino acids, 100 mL/L penicillin-streptomycin solution (10,000 IU/10,000 μg/ml), and 10% bovine calf serum at 37°C in a 5% CO_2 incubator with 75% humidity. Compounds were evaluated at 2-fold serial diluted concentrations starting at 100.0 μg/ml. Following compound addition, cells were incubated for 72 h at 37°C in a 5%CO_2 incubator. Cells were washed with PBS, CellTiter

96 AQueous One solution was added to each well, and plates were incubated for 4 h at 37°C. Plates were read at 490 nm using a spectrophotometric plate reader, and the absorbance readings were used to calculate the 50% lethal concentration (LC_{50}) using GraFit software as previously described [39]

3.2.4 Mouse infection, drug dosing, and CFU determination

Mice were infected with *F. tularensis* (SCHU S4) via a whole body aerosol. Low-dose aerosol infections were performed as previously described with minor modifications [40, 41]. Conscious mice within a stainless steel basket were exposed to the SCHU S4 strain of *Francisella tularensis* by aerosol exposure in a Glascol Inhalation Exposure System (Glas-Col, Inc, Terre Haute, IN). Prior to exposure, the nebulizer was loaded with bacteria diluted in PBS to a concentration of approximately 5 x 10^6 cfu/mL. Mice were exposed to a total of approximately 4 x 10^7 bacteria, aerosolized into a volume of 5 cubic feet over a period of 30 min, followed by a 20 minute period of cloud decay. When airflow was maintained, bacteria were no longer introduced. Mice were monitored for morbidity and mortality twice daily for a period of 14 days, at which time survivors were euthanized. This inoculum routinely results in 100% mortality and a mean time to death of 5-6 days following infection. Mice were treated by intraperitoneal injection (i.p.) or gavage (p.o.) once daily with SBPT04 or triclosan at 50 mg/kg, 100 mg/kg or 200 mg/kg/ beginning day 1 of infection and continuing daily for four additional days. SBPT04 was formulated in 5% ethanol while triclosan was formulated in 8% Solutol (BASF) and 5% ethanol. Control mice were inoculated with saline. Mice were sacrificed at 2 and 4 days post infection for CFU determination in lungs and spleens. Mouse organs were

homogenized in sterile PBS and 100μl of homogenate was serial diluted (10^{-1}-10^{-8}) and plated on enriched MMH agar plates, which were then incubated at 37°C for 48hrs at which time CFUs were enumerated.

3.2.5 Transcriptional profiling under SBPT04 drug treatment of LVS

Transcriptional profiling experiments consisting of three independent biological replicates were performed to study the response of genes to drug treated conditions over a 2-hour period. Bacterial cultures were grown to an O.D. $_{600nm}$ of 0.4, diluted 1:2, and distributed into 150 mL aliquots. The cultures were incubated for 1 hr at 37°C prior to addition of 2 x MIC concentrations of SBPT04. After 2 hours of treatment, total RNA was isolated as previously described [39]. Competitive hybridizations were performed on *M. tuberculosis* whole genome microarrays (Rocky Mountain Regional Center of Excellence Genomics Proteomics Core, U54 AI-065357) with fluorescent labeled cDNAs generated using direct labeling from 5 μg of total RNA as described [39]. Data reduction and analysis was performed using Genepix software (Axon). Open reading frames considered statistical significant were differentially regulated great than 1.5-fold, with a *p*-value of ≤ 0.05.

3.2.6 Bioavailability, PKPD and *in vivo* metabolites for SBPT04

A pharmacokinetic study was conducted in male C57BL/6 mice via intravenous (i.v.), oral (p.o), or intraperitoneal (i.p) administration of the compound SBPT04. The dose levels were 20 mg/kg (5%EtOH/5%D5W) for i.v., 50 and 200 mg/kg (%5EtOH) for p.o, and 200mg/kg (5%EtOH/5%D5W) for i.p. Blood samples for each route of

administration were collected from retro-orbital puncture at eight time points, with three mice being sacrificed at each time-point. SBPT004 and its major Phase I and Phase II metabolites were monitored in each sample with LC/MS/MS methodology (LLOQ was 1 ng/ml in plasma). Liver microsome assays using mouse (MLMs) and human (HLMs) liver microsomes were performed to identify and quantify of Phase I and Phase II metabolites of compound SBPT04 as previously described [42].

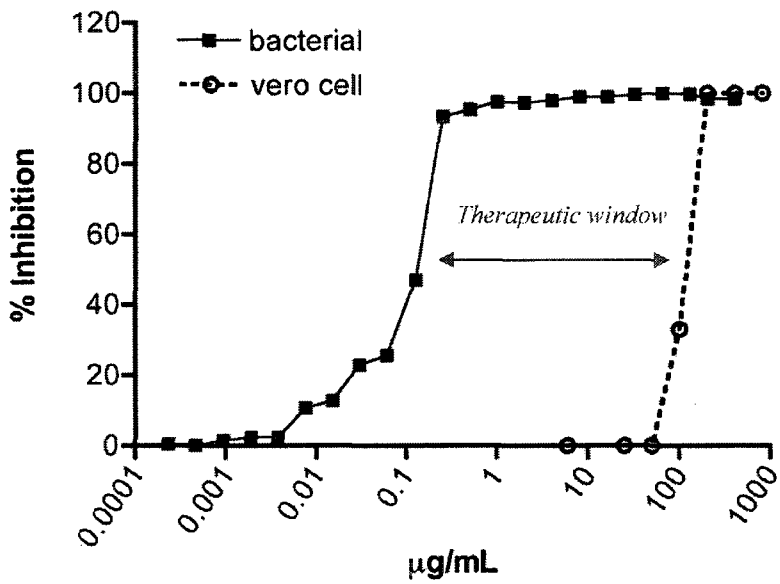
3.3 RESULTS

3.3.1 SBPT04 is a potent inhibitor of *F. tularensis* with low cytotoxicity

Recently, we created and screened a diverse library of alkyl-substituted diphenyl ethers derived from the lead pharmacophore triclosan for activity against the FASII FabI of *F. tularensis* (ftuFabI), and bactericidal activity against *F. tularensis* SCHU S4 and LVS strains. This screening revealed an alkyl-substituted analog, 5-hexyl-2-phenoxyphenol (SBPT04) that had high binding affinity for ftuFabI and demonstrates bactericidal activity *in vitro*. SBPT04 has a minimal inhibitory concentration 90% (MIC_{90}) of 0.16±0.06 μg/mL and a minimum bactericidal concentration (MBC) of 0.25 μg/mL. A 2-fold increase or less in drug concentration in MBC to MIC_{90} is a key characteristic of potent bactericidal drugs and is an important property for a drug to have enduring efficacy. Most significantly, SBPT04 is more potent *in vitro* than current clinically used drugs to treat *F. tularensis* infections. For example, the MICs determined for various clinical strains of *F. tularensis* with streptomycin and gentamicin fall into a range of 2-4 μg/mL and 1-2 μg/mL, respectively as compared to SBPT04 with an MIC

range of 0.12-0.25 μg/mL [34]. To assess the potential safety window of SBPT04, cytotoxicity was evaluated in African green monkey kidney cells (Vero cells). The LC_{50} for SBPT04 was determined to be 101 ± 2 μg/mL which was comparable or better than current drugs that were also evaluated in our assay and resulted in a measured 3 log concentration difference between MIC_{90} and LC_{50} value (Figure 3.1,A and B). This represents a significant difference in the selective index (SI) between tissue toxicity and bactericidal dose.

A



B

Drug	MIC_{90} (µg/mL)	LC_{50} (µg/mL)	SI
SBPT04	0.2	100	500
streptomycin	4.0	>100	25
gentamicin	2.0	>100	50
doxycycline	1.0	3	3

Figure 3.1. Minimal inhibitory concentration and cytotoxicity of SBPT04. (A) Percent inhibition of growth of *F. tularensis* and vero cells over different concentrations of SBPT04. (B) MIC, LC_{50} and selective index (SI) of SBPT04 compared to clinically used drugs to treat *F. tularensis*.

3.3.2 *F. tularensis* transcriptional response to SBPT04 treatment

To assess the mode of action and response of *F. tularensis* to treatment with SBPT04, whole genome transcriptional profiling was performed. Treatment with SBPT04 for 2 hours at 2 x MIC resulted in the differential regulation of 734 open reading frames (ORFs) (p-values ≤ 0.05), with 207 ORFs differentially regulated 1.5 fold or greater (Appendix B). The general trend in the transcriptional response caused by SBPT04 treatment included an overall reduction in genes encoding products involved in cell wall and lipid synthesis, intermediary metabolism and respiration, and RNA and protein synthesis (Table 3.1). Repressed genes include those encoding fatty acid biosynthetic genes (*acc, fabI, fabH, fabZ*), ATP synthesis (*atpA, atpB, atpD, atpG, purK*) NADH utilization (*nuoA, nuoH, nuoI, nuoK, nuoM, nuoN, nadE*) and the menaquinone biosynthesis methyltransferase (*ubiE*), RNA synthesis (*rpoA1, rpoA2, rpoC*), and ribosomal proteins (*rpmA, rpmD, rpsL, rpsI, rpsA, rpsS, rpsD, rpsN, rpsM, rpsK, rpsE, rpsH, rplT, rplQ, rplC, rplX, rplF, rplJ, rplA*) that encode the 30S and 50S multi-subunit ribosomal complex responsible for protein synthesis and a ATP-dependent protease (*lon*). The down regulation of these functions is consistent with reduction in replication potential, metabolic function and overall cell viability. In addition, genes associated with stress responses where induced during treatment. These genes included the *spoT*, *gro* and *sos* genes. Of the functional categories, those assigned to insertion sequences and phages (*n=71*), regulation (*n=30*), unknown (*n=21*), and virulence, detoxification and adaptation (*n=28*) were the highest percentage induced. Thus, the transcriptional response of *F. tularensis* treated with SBPT04 demonstrates that overall metabolic activity was reduced within the treatment period of 2 hours despite the elucidated stress responses.

Table 3.1. Differentially regulated genes by functional classification after 2hr exposure to SBPT04. *F. tularensis* (LVS) was treated at 2 times the minimal inhibitory concentration for 2 hours. Total genes in analysis = 1820 with 734 genes having p-values ≤ 0.05.

Functional Classification	Total	$\geq$ 1.5-fold	$\leq$ -1.5-fold	% up	% dn
Cell wall and cell wall processes	95	8	16	8	17
Conserved hypothetical/ hypothetical	189	34	9	18	5
Information pathways	90	4	27	4	30
Insertion sequences and phages	38	27	0	71	0
Intermediate metabolism/respiration	165	12	23	7	14
Lipid metabolism	18	1	3	6	17
Unknown	100	21	6	21	6
Regulatory proteins	10	3	1	30	10
Virulence/detoxification/adaptation	29	8	1	28	3

3.3.3 SBPT04 demonstrates efficacy against SCHU S4

The efficacy of SBPT04 and the parent compound triclosan was determined in the *F. tularensis* murine model of infection when delivered i.p. and p.o. beginning on day 1 of infection. For these studies, mice were infected with *F. tularensis* SCHU S4 (n=15/group) *via* low dose aerosol. Drugs were administered once a day for 5 days beginning at day one, and monitored for a total of 14 days for morbidity, mortality, and drug relapse. Untreated control mice in the i.p. study had a 0% survival rate with a median survival of 5 days, whereas treatment with 200 mg/kg triclosan delivered i.p. resulted in a 44% median survival. Significantly, treatment with 200 mg/kg SBPT04 delivered i.p. prevented death of all infected animals resulting in a 100% survival rate ($P<0.001$) (Figure 3.2, A). Importantly, mice did not show any signs of relapse of disease for 30 days following 5 days of treatment. This observation is consistent with the lack of colony forming units in the lungs and spleens following treatment (Figure 3.3). To

assess whether SBPT04 had efficacy when delivered orally, mice were again infected with a low dose aerosol of *F. tularensis* (SCHU S4) and dosed once a day for five days as in the previous experiment. Untreated control animals in the p.o. study had a 0% survival and a median survival of 5 days similar to that observed for i.p. delivery. In contrast, SBPT04 when administered orally at 200 mg/kg significantly ($P<0.01$) extended the median survival to 9 days (Figure 3.2, B).

To assess *in vivo* activity and efficacy of SBPT04 delivered by i.p. or orally, bacterial growth in the lungs and spleen was determined at varying doses on days 2 and 4 of infection. Three mice from each treatment group and control group were selected, euthanized, and the bacterial load in lung and spleen was determined by plating and enumeration of colony forming units (CFU). Untreated mice generally displayed 5 $\log_{10}$ and 6 $\log_{10}$ colony forming units in the lung at day 2 and day 4, respectively (Figure 3.3, A and C). While the bacteria in the spleen at day 2 were minimal, dissemination to the spleen was observed at day 4 with 6 $\log_{10}$ colony forming units (Figure 3.3, B and D). Doses less than 100 mg/kg of SBPT04 delivered either i.p. or p.o. did not significantly affect bacterial growth in the lungs or spleens at day 4 of infection when compared to control animals. SBPT04 when delivered i.p. at 200 mg/kg reduced bacterial growth in the lungs at day 2 and significantly cleared bacteria by day 4 ($p<0.01$), (Figure 3.3, A). Similar trends were observed in the spleen with SBPT04 delivered i.p. at 200 mg/kg were no bacteria were detected ($p<0.01$), (Figure 3.3, B).

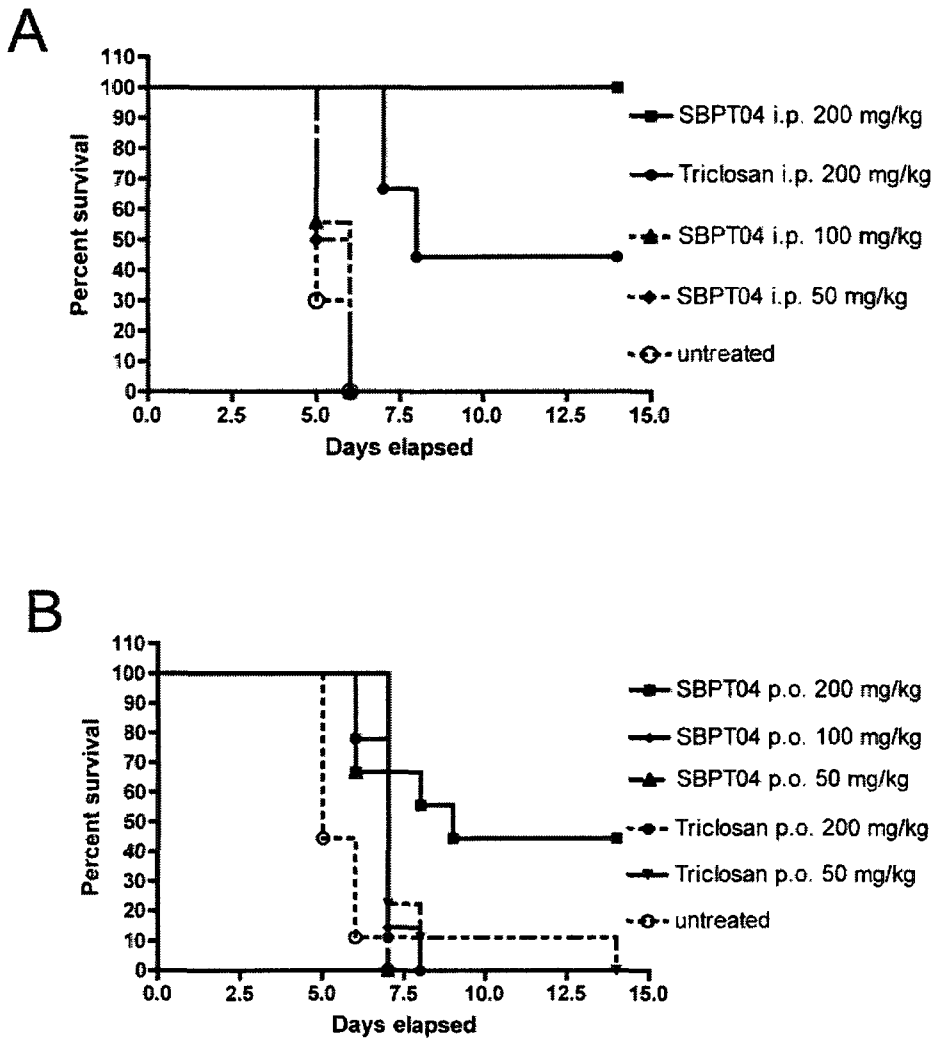


Figure 3.2. Efficacy testing of SBPT04 in the *F. tularensis* mouse model of infection. Survival of mice treated with SBPT04 or the lead pharmacophore vs. triclosan delivered at different doses delivered (A) i.p. or (B) p.o. Mice were dosed once daily for 5 days following a low dose aerosol (SCHU S4) infection and observed until day 14. SBPT04 demonstrated 100% survival (P=0.001) when delivered i.p. 200mg/kg/day and 44% survival (P=0.01) when delivered p.o. 200mg/kg/day.

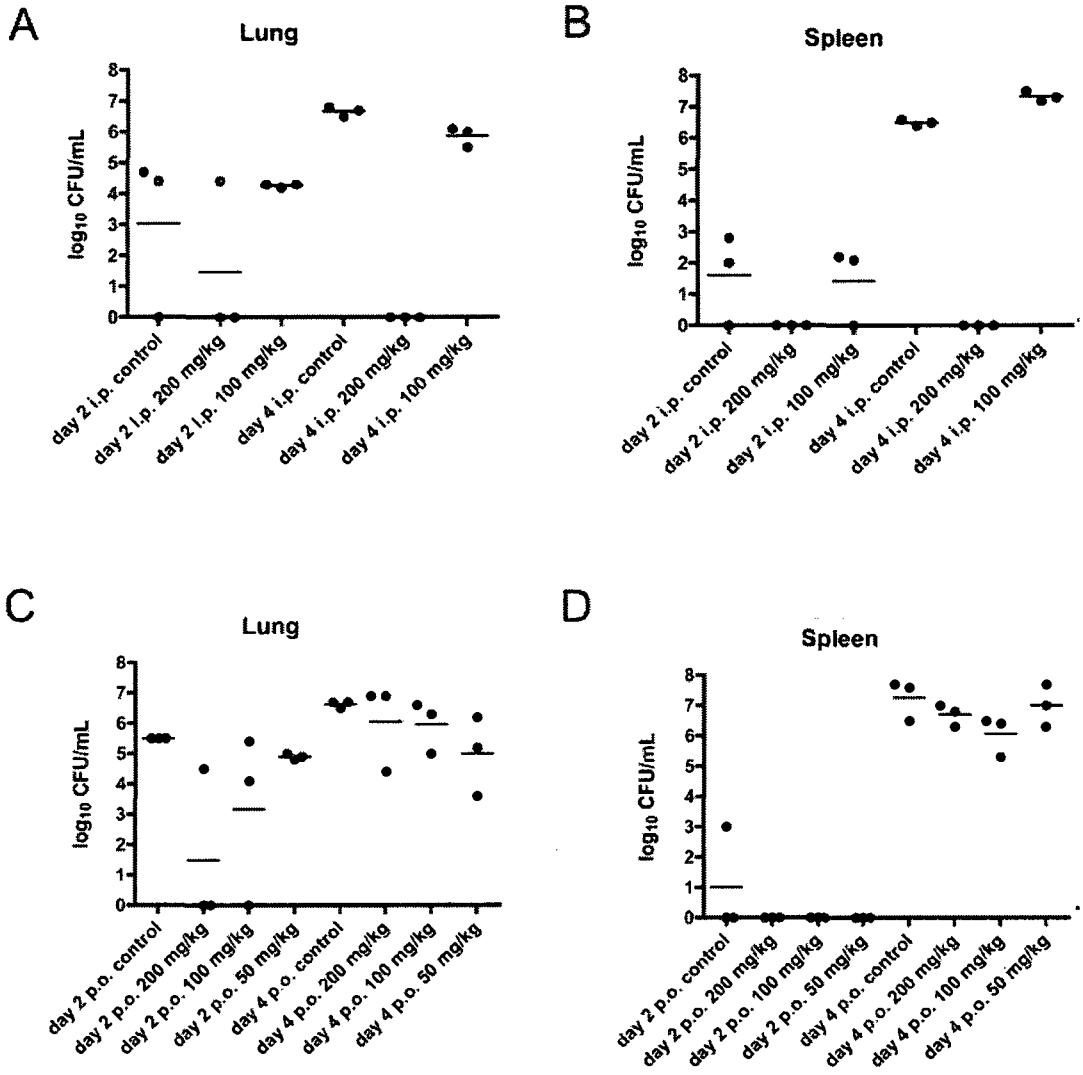


Figure 3.3 Bacterial burdens in the lung and spleen during treatment with SBPT04. *F. tularensis* colony forming units recovered from the lungs (A, C) and spleen (B, D) following treatment with different doses of SBPT04 delivered i.p. (A, B) or p.o. (C, D). Bacterial burden was determined by colony forming units (Log_{10} CFU/mL) recovered from organs at day 2 and day 4 of treatment with SBPT04.

When delivered orally at 200 mg/kg, SBPT04 was able to reduce bacterial growth in the lungs ($p<0.05$) but was unable to control the extent of burden in the lung (Figure 3.3,C) or spleen (Figure 3.3, D) at day 4 of infection. This is consistent with the observed differences in efficacy (Figure 3.2, A and B) and demonstrates correlations with median survival, bacterial burden in the lungs, control of dissemination, and growth in the spleen during treatment.

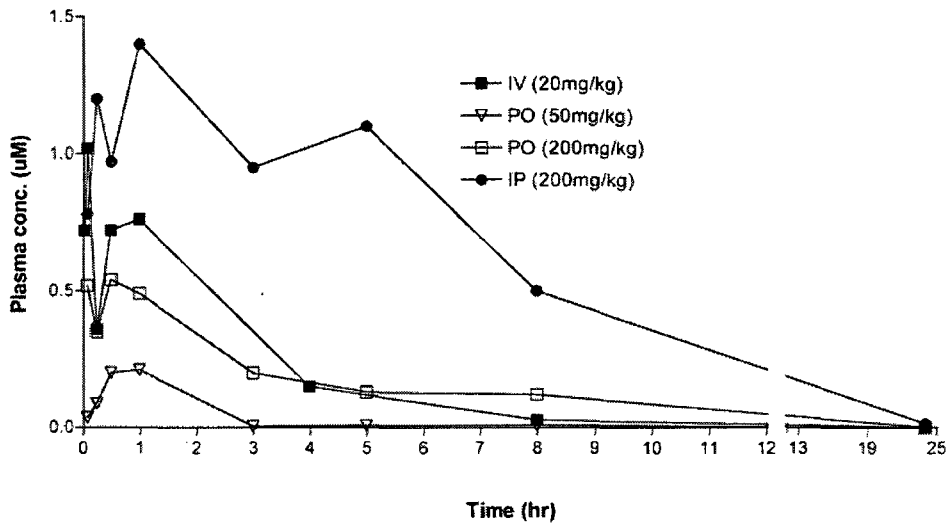
3.3.4 Pharmacokinetics and metabolism of SBPT04

A two-pronged approach consisting of PK analysis and metabolite studies was employed to identify the physiochemical properties of SBPT04 that influence oral bioavailability and the specific structural liabilities of SBPT04. The PK parameters that were determined included area under the curve (AUC), C_{max}, T_{max}, F (bioavailability) and $t_{1/2}$ for SBPT04 following dosing at 50 mg/kg and 200 mg/kg p.o. and 200 mg/kg i.p. in order to estimate the therapeutic dose and pharmacokinetic characteristics associated with efficacy (Figure 3.4). The AUC of SBPT04, which evaluates drug exposure, was 0.61 or 3.3 μg•hr/ml when delivered p.o. at 200 mg/kg or i.p. at 200 mg/kg, respectively. Delivery of SBPT04 at 200 mg/kg p.o. resulted in a C_{max} value of 0.15 μg/ml with a T_{max} of 0.5 hr. A 200 mg/kg dose delivered by i.p. yielded a C_{max} value of 0.38 μg/ml with a T_{max} of 1.0 hr. Comparison of the AUC and C_{max} values from 200 mg/kg p.o or 200 mg/kg i.p. provides an estimate of the minimal values needed for a compound to demonstrate efficacy. Delivery of SBPT04 i.p. at 200 mg/kg afforded a therapeutic effect index (AUC/MIC) of 20 and a C_{max}/MIC of 2.3. When delivered p.o. at 200 mg/kg or i.p. at 200 mg/kg, SBPT04 had $t_{1/2}$ values of 2.1 and 3.4 hrs, respectively. Together, these

values reveal that a compound that exceeds 0.6 μg•hr/mL, a maximum serum concentration greater than the MIC_{90} of the compound, and a $t_{1/2}$ of more than 3 hours demonstrates efficacy in the *F. tularensis* mouse model of infection. The bioavailability (F) of SBPT04 was assessed to determine the fraction of the dose reaching systemic circulation unchanged after administration. SBPT04 delivered p.o. at 200 mg/kg had an F value of 8.94, and SBPT04 delivered i.p. at 200 mg/kg had an F value of 48.56. The observed greater than 5 fold difference in F values for the two routes of administration represents the fraction of therapeutically active drug that reaches the systemic circulation after intestinal absorption or potential first-pass metabolism and is available at the site of action.

Phase I and Phase II metabolism of SBPT04 was assessed using mouse liver microsomes (MLM) and human liver microsomes (HLM) with LC/MS/MS methodology (Figure 5A). Studies, conducted with MLMs and HLMs revealed similar oxidative metabolites with the primary difference being a less extensive profile and an extended half-life in HLMs (Figure 5BC). In MLMs the $t_{1/2}$ of SBPT04 was determined to be 4.1 min while HLMs exhibited a $t_{1/2}$ of 46 min. Phase I analysis revealed the formation of mono and bis-hydroxy derivatives of SBPT04 as well as a ketone metabolite (Figure 5A). Phase II studies revealed that SBPT04 was also susceptible to conjugation with the O-glucuronide metabolite being most abundant. Based on the Phase I and Phase II analyses, Phase I metabolism is a minimal contributor to the depletion of SBPT04 and Phase II O-glucuronidation is the primary eliminator of active SBPT04 from circulation *in vivo*. Together these results are consistent with the notion that metabolism of SBPT04 is less

A



B

In Vivo PK parameters for SBPT04 in male C57BL6 Mice

	IV (20mg/Kg)	*PO (50mg/Kg)*	*PO (200mg/Kg)*	*IP (200mg/Kg)*
MRT (hr)	1.9			
Vss (L/kg)	56.25			
Vd (L/kg)	69.06			
CL (mL/min/kg)	493.09			
AUC (μM-hr)	2.5	0.17	2.24	12.14
AUC(hr.μg/mL)	0.675	0.046	0.61	3.3
AUC(hr.μg/mL)/MIC (μg/mL)	4.22	0.29	3.81	20.63
$t_{1/2}$ (hr)	1.62	0.54	2.08	3.04
$t_{1/2}$ (hr) (0-glucuronide)	3.38	3.24	2.57	5.48
Tmax (hr)	1	0.5	0.5	1
Cmax (μmol/L)	0.75	0.2	0.54	1.41
F(Bioavailability) (%)		2.69	8.94	48.56

Figure 3.4. Bioavailability and *in vivo* PK of SBPT004 (A) Bioavailability was assessed by administering SBPT04 to C57BL/6 mice *via* i.v., p.o. or i.p. routes and determining the amount of compound in blood over 24 hr. (B) *in vivo* PK parameters for SBPT04 delivered i.v., p.o. or i.p. in male C57BL/6 mice

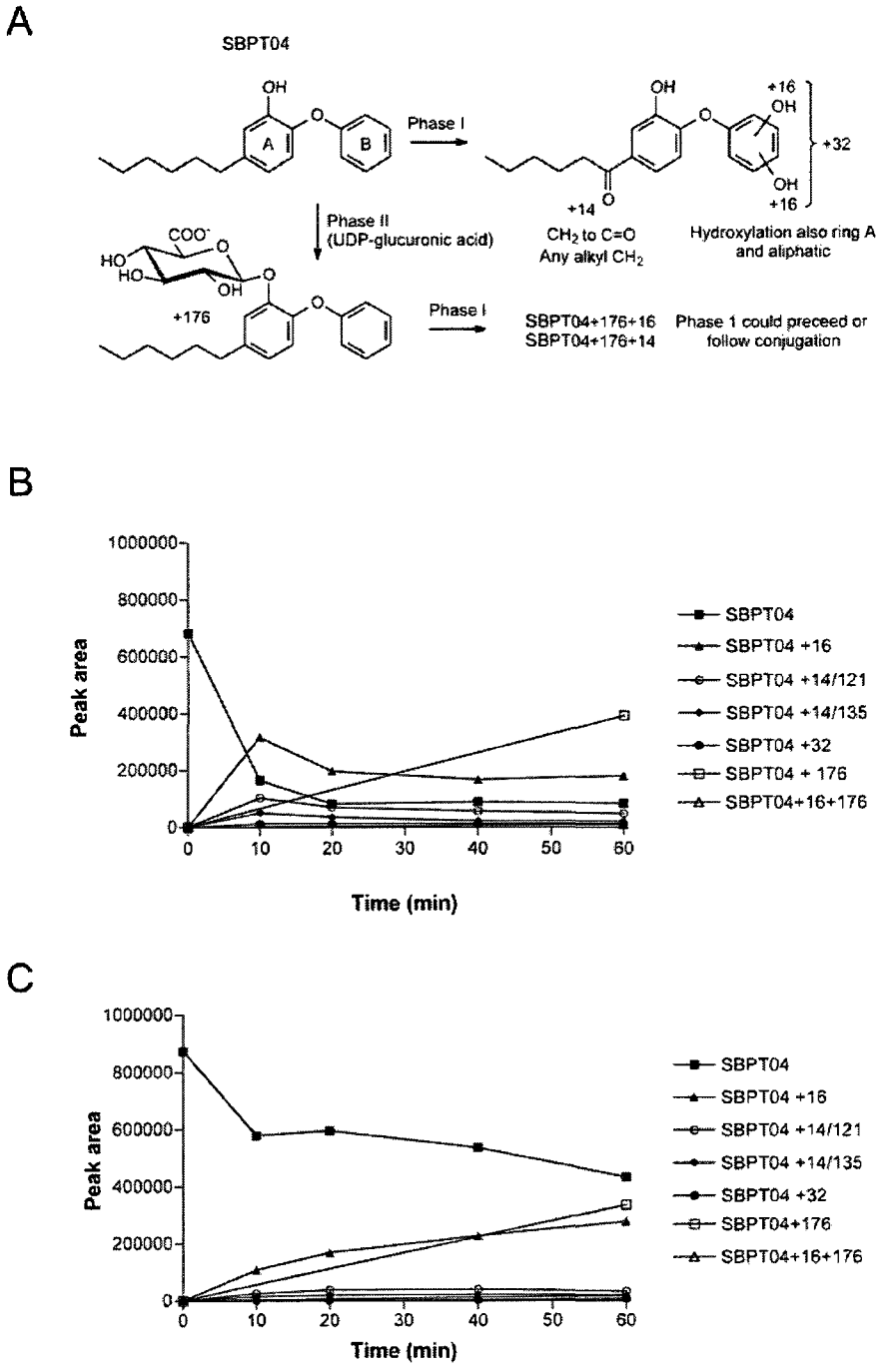


Figure 3.5 Phase I and Phase II metabolism of SBPT04. (A) Phase I and Phase II metabolites of SBPT04. (B) Phase I and Phase II metabolism of SBPT04 in mouse live microsome over 60 minutes. (C) Phase I and Phase II metabolism of SBPT04 in human live microsome over 60 minutes.

extensive in human microsomes. It is known that there are differences in drug metabolism between human and rodent species [43, 44]. Therefore, from this information a significantly longer half-life of SBPT04 is anticipated in humans, which would allow for a reduction in the efficacious dose of SBPT04 and increased duration between dosing in the treatment regimen.

3.4 DISCUSSION

The microbial fatty acid biosynthesis pathway (FASII) is a validated target for novel antibacterial drug discovery [12, 13, 15] and it has been demonstrated that the enoyl reductase enzyme FabI is a sensitive target for chemotherapeutic intervention [31]. The utility of FabI as a broad-spectrum target in *F. tularensis* has been substantiated by our own antibacterial discovery program, which is founded on the observation that the diphenyl ether triclosan inhibits the FabI enzyme from *F. tularensis* (K_i = 7 pM) [31]. Using structure-based design we have developed a series of substituted diphenyl ethers that are potent inhibitors of ftuFabI the *F. tularensis* FabI and that are active against other bacterial pathogens [22, 31]. The development of a novel chemotherapeutic against *F. tularensis* that prevents relapse of disease after treatment requires identification of a therapeutically relevant molecular target, favorable physicochemical characteristics and rapid efficacy.

Visualization of the transcriptionally active genes revealed that SBPT04 treatment resulted in an overall reduction in intermediary metabolism and respiration, RNA and protein synthesis, and macromolecular synthesis. The down regulation of these functions is consistent with a reduction in replication potential, metabolic function and overall cell

viability due to treatment. Stress, detoxification and adaptation processes were also observed in the transcriptional response to treatment as revealed by the up regulation of *spoT, groS, recG, visC, sodC,* and *pilQ*. However, despite the increase in these processes, SBPT04 is a potent inhibitor of cell growth and viability. The induction of detoxification processes in *F. tularensis* is similar to the responses in other organisms to diphenyl ethers, and like those bacteria, the response was not associated with increased resistance to treatment [22, 39].

SBPT04 demonstrated potency against bacteria at concentrations 1,000-fold below concentrations that demonstrated vero cell toxicity. When assessed over a range of concentrations, SBPT04 demonstrated a less than a 2-fold increase in drug concentration in MBC to MIC_{90}, a key characteristic of potent bactericidal drugs and an important property for a drug to have enduring efficacy. In contrast to clinically used drugs the increased potency of SBPT04 allowed for a 5-day treatment period. In studies assessing efficacy of doxycycline and ciprofloxacin along with alternative fluoroquinolones; moxifloxacin and gatafloxacin in the murine model of infection for tularemia, relapse of disease was observed [11, 45]. In these studies there was a relapse of disease even with treatments extending over 14 days. This indicates that these clinically used antimicrobial agents are poor at eradicating *F. tularensis*. Thus, there is an important concern for relapse in humans following treatment with currently recommended therapies. Significantly, there was no observed relapse in disease following a 5-day treatment with SBPT04 when the animals were observed for 30 days. Notably, SBPT04 is more potent *in vitro* than several current clinically used drugs and demonstrates efficacy in mice

without the associated toxicity and relapse for the treatment of pulmonary tularemia infections.

The factors of absorption, distribution, metabolism and elimination in conjunction with the relative susceptibility of the bacteria to drug affect the extent of killing *in vivo*. It has been established that the duration of the drug concentration relative to the MIC of the pathogen (i.e. AUC/MIC or T>MIC) is directly related to bacterial killing in animal models of infection [46]. SBPT04 demonstrates a concentration-dependent killing mechanism as our experiments illustrate a dose dependent efficacy under a short duration of treatment. For drugs that exhibit concentration-dependent killing, the higher the drug concentration the greater the extent of bacterial killing. Pharmacodynamic features of a compound are associated with the relationship between the target pathogen and the antimicrobial agent over time, taking into account the variations in drug concentration on killing and the organism's growth dynamics [46, 47]. Thus, maintenance of serum drug concentration above MIC over an appropriate time is critical to establish effective killing and overall therapeutic effect. Despite the fact that the $t_{1/2}$ of 3.3 hr was modest, SBPT04 effectively clears bacteria from the lungs and prevents dissemination, suggesting a post-antibiotic effect.

The mechanism behind the observed bactericidal activity even after drug concentrations falls below MIC is related to the time required for an organism to recover from exposure [46, 47]. Compounds are readily cleared from the serum but may persist in tissues at submicro-effective levels (PAE-SME) and offer extended killing beyond the visualized T>MIC or AUC/MIC ratios. Over time intracellular concentrations for the drug can build if diffusion is efficient and the compound can linger inside the cell to

continue to be effective, even though demonstrated serum concentrations have fallen below MIC. Phase I metabolites are minimal contributors to the depletion of SBPT04, however, Phase II O-glucuronidation was the primary metabolic modification that affects and limits the levels of SBPT04 in circulation. This information is useful for the design and optimization of next generation compounds. Remodeling SBPT04 to bypass Phase II metabolism would extend serum levels and increase $t_{1/2}$ thereby prolonging antibacterial efficacy at lower doses. Finally, the fact that SBPT04 is a slow onset inhibitor of ftuFabI with an *in vitro* residence time of 140 min may also contribute to the post antibiotic activity of SBPT04 against intracellular pathogens like *F. tularensis* [32] . Notably, triclosan, which has MIC_{90} of ~ 0.000015 μg/ml and is thus significantly more potent *in vitro* than SBPT04, has lower *in vivo* activity, which can be directly related to the much shorter residence time of triclosan on the target [32].

Importantly, in regard to the development of novel chemotherapeutics with broad-spectrum activity, the identification of substituted diphenyl ethers that inhibit FabI affords the opportunity to target other bacterial pathogens. Not only have diphenyl ethers demonstrated efficacy against *F. tularensis*, but with the mechanistic knowledge regarding the enzyme interaction and inhibition, this pharmacophore is suitable for development as a platform for broad-spectrum chemotherapeutics. Notably, animals treated with SBPT04 did not show signs of relapse after withdrawal of treatment. This is particularly significant given the virulence of priority pathogens and the clinical record of currently used drugs for treatment. In summary, these studies provide a platform for the rational design of "next generation" compounds with greater efficacy and less metabolic liability that would allow sufficient efficacy at lower doses.

3.5 ACKNOWLEDGEMENTS

This work was supported by New Opportunities funding from the Rocky Mountain Regional Center of Excellence (AI065357) to RAS and the Northeast Biodefense Center (AI057158) to PJT, as well as NIH grants AI44639 and AI70383 to PJT. We gratefully recognize the post genomics resource and instrumentation, and animal models experitse provided by the Genomics Proteomics Core and the Animal Models Core in the Rocky Mountain Regional Center of Excellence (AI065357), respectively.

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CHAPTER 4

Septum Regulating Protein Induces Dormancy Genes and Alternative Global Regulation Associated with Nonreplicating Persistence in *M. tuberculosis*

This work is dedicated in memory of Dr. Dennis Knudson who performed the initial bioinformatics analyses in a combined effort with Dr. Richard Slayden that identified $MinD_{Mtb}$ encoded by *rv3660c*, previously annotated as a putative septum-site determining protein.

4.1 INTRODUCTION

Mycobacterium tuberculosis has evolved to stall cell cycle progression, alter metabolism, and establish a nonreplicating persistent state, however the regulatory mechanisms involved in this process have yet to be identified. Accumulating evidence indicates that cell division is a key regulatory checkpoint of the cell cycle for entry into a nonreplicating state in association with altered transcriptional and metabolic activity [1-5]. The mechanisms that regulate septum formation and cell division remain undefined in *M. tuberculosis*, however in other bacterial species these events are known to involve protein interactions that are tightly controlled [4, 6-22].

The molecular processes of cell division have been well studied in Gram-positive and Gram-negative bacteria. It is well recognized that the primary committed step in this event is the formation of a septum at the midcell [4, 8, 19, 21, 22]. Septum formation

depends on the assembly of FtsZ into a contractile ring at the midcell with additional proteins recruited to form the components of a new cell wall, dividing the cells into two complete daughter cells (Figure 4.1) [4, 7, 10, 20, 23, 24]. Many proteins involved in cell division, such as FtsZ, are conserved, while others appear limited to specific taxa [8, 17, 27]. Proteins known to interact with FtsZ and direct Z-ring formation include ZipA, ZapA, FtsA, MinD, EzrA, and SulA (Figure 4.2) [10, 11, 17, 18, 24, 28-30]. These proteins along with transcriptional regulators form regulatory networks that are required to coordinate the spatial and temporal control of cell cycle events.

Previous analysis of the *M. tuberculosis* genome did not reveal any homologues of the FtsZ-interacting proteins, and thus it is likely to encode orthologs that fulfill these functions [31]. In 2006, Slayden et al., utilized a two pronged approach to identify cell cycle regulators, using both microarray profiling under demonstrated septum regulating conditions *in vitro* and bioinformatics strategies that identified potential cell division regulatory elements in *M. tuberculosis* [32]. From these studies, two putative orthologs for MinD-like proteins were identified which are encoded by *rv3660* and *rv1708* and are annotated as a putative septum-site determining protein and a putative initiation inhibition protein respectively.

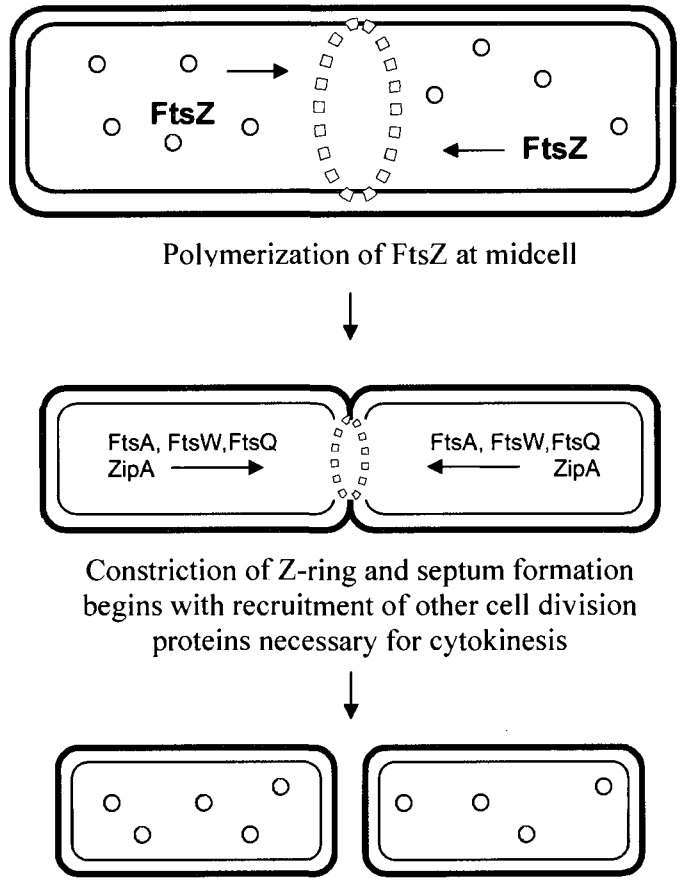

Figure 4.1 Bacterial cell division. FtsZ polymerization into a ring at the midcell is the initial driving force for bacterial cell division. Key players that promote septum formation and cytokinesis include FtsA, FtsW, FtsQ and ZipA. (adapted from Lutkenhaus and Addinall, 1997) [26]

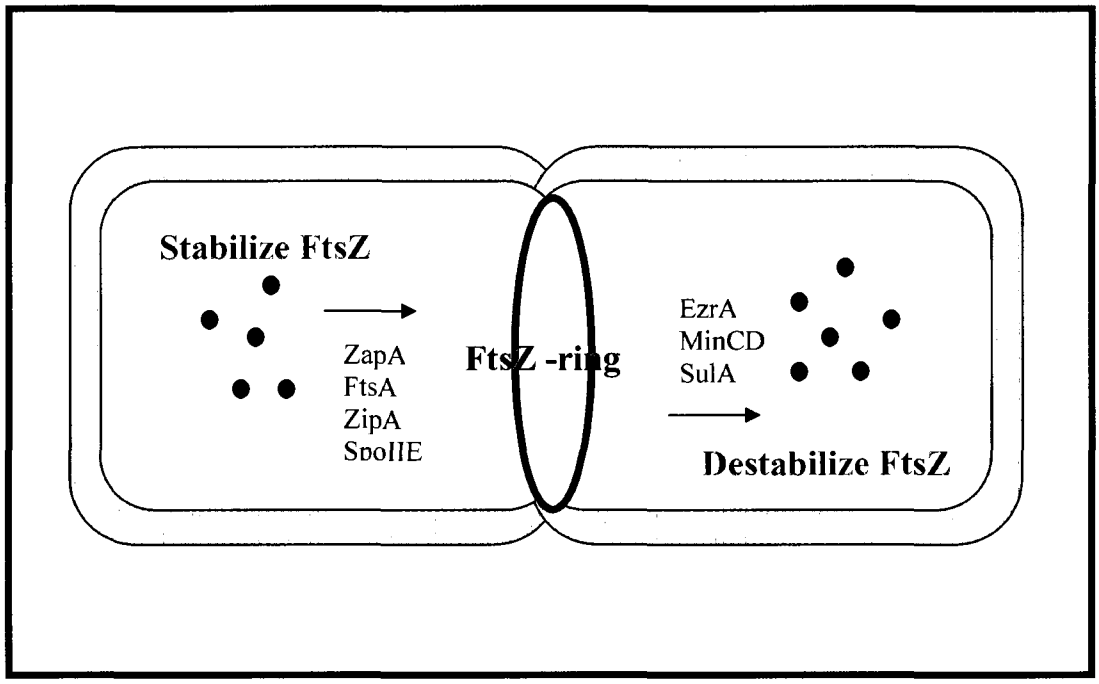

Figure 4.2 Regulation of FtsZ-ring formation. Several proteins have been identified in other bacteria that are involved in stabilizing (FtsA, ZipA, ZapA, SpoIIE) or destabilizing (EzrA, MinCD, SulA, YneA) FtsZ-ring formation. These regulators control cell division events under various cell conditions and coordinate cell cycle events. (modified from Geohring and Beckwith, 2005) [8]

The control of septum formation and placement is regulated in other bacilli by coordinating activities of several proteins, known as Min-system proteins. In Gram-negative organisms, like *Escherichia coli,* the Min-system consists of MinC, MinD and MinE proteins [6, 8-10, 14, 15, 19, 23, 33, 34]. Gram-positive organisms such as *Bacillus subtilis* have a similar system to coordinate septum placement with homologs to MinC, MinD, and ortholog DivIVa [8, 10, 14, 15, 19, 23, 35-37]. These components work together to negatively regulate septum formation and placement by establishing a zone of inhibition until DNA replication is complete and the chromosomes have been properly segregated (Figure 4.3). Studies in other organisms have demonstrated that induced expression of genes that encode Min-system proteins inhibit cell division resulting in filamentous cell morphology [6, 15, 19, 38, 39 , 40]. Mutations in these proteins provide a unique "minicell" morphotype associated with the deregulation of septum placement, resulting in random division events particularly located at the cell poles [12, 15, 19, 37, 40-42].

Regulatory events such as this and elements involved in processes driving or inhibiting cell division are unidentified in *Mycobacterium tuberculosis.* Knowledge of such mechanisms of regulation may lead to an understanding of crucial steps provoking the induction of cells into a nonreplicating state. To identify regulatory elements that influence cell division, bioinformatics, transcriptional profiling and gene dosage strategies were employed. Bioinformatics revealed a protein deemed $MinD_{Mtb}$, encoded by *rv3660,* in *Mycobacterium tuberculosis* with homology to the septum regulating protein MinD. Increased expression of $MinD_{Mtb}$ resulted in filamentous cells, and a $MinD_{Mtb}$ mutant provided minicell morphology with distinct deregulation of septum

placement. Transcriptional analysis showed that $MinD_{Mtb}$ expression induces a unique profile consisting of the dormancy regulon (*dos*R), thought to be a key component of nonreplicative, persistent *Mycobacterium tuberculosis*. In addition, an alternative global transcriptional regulation by alternate sigma factors (σFGHIJLM) was identified. Alternative global regulation of cellular activities induced correlates with the upregulation virulence components and stress responses, which may relate to disease pathogenesis and alternative mechanisms of survival. It is postulated that these responses may be host specific, directing a program required for human infection or one induced during a specific disease state. The identification of a MinD-like protein that stalls cell division and induces gene profiles for alternative metabolism associated with pathogenesis and survival of nonreplicating bacilli, represents a novel regulatory mechanism in *M. tuberculosis*.

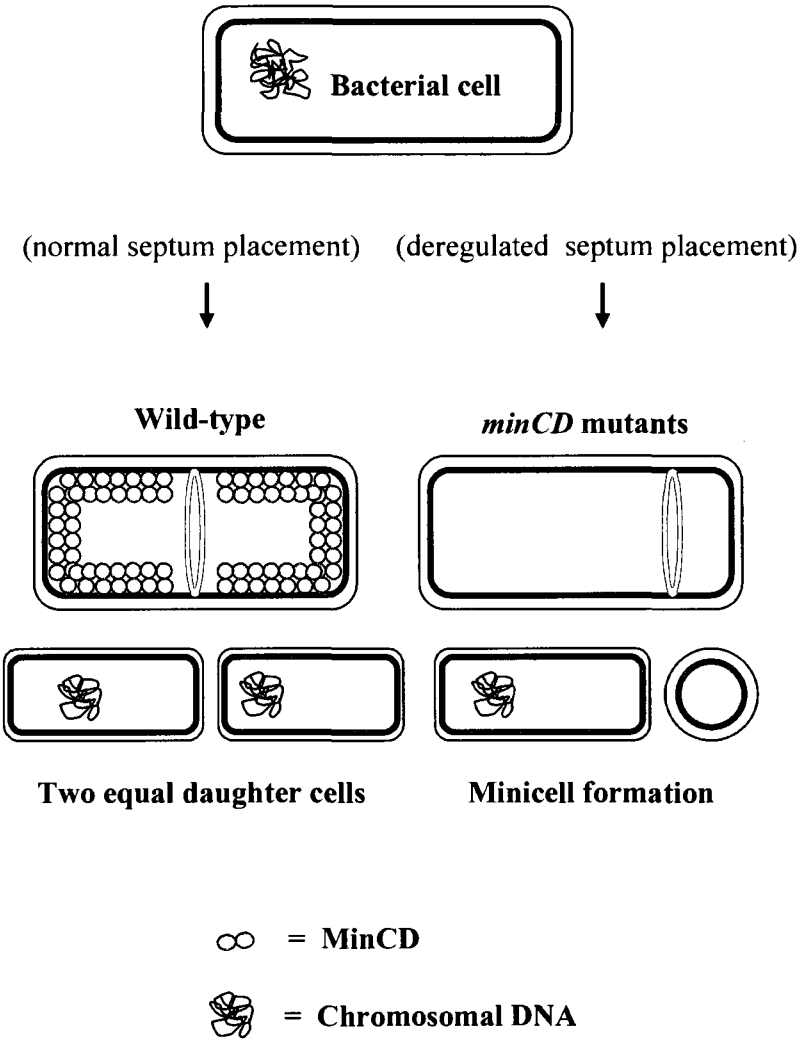

Figure 4.3 Min-system regulation of septum-site placement for *E. coli.* In wild type cells the Z-ring is directed toward the midcell and inhibited from establishing near the poles by the action of MinCD complex. When *minC* or *minD* are mutated, there is no longer control of septum-site placement and thus Z-rings can assemble anywhere within the cell particularly near the poles. Assembly near the poles can form truncated versions of cells or minicells. (adapted from Sullivan and Maddock, 2000) [25]

4.2 METHODS

4.2.1 Bacterial strains, recombinants, Tn mutant, and growth conditions.

The bacterial strains utilized in these experiments were *Mycobacterium smegmatis* mc^2155, *Mycobacterium tuberculosis* H37Rv and a *Mycobacterium tuberculosis* H37Rv Tn mutant E150 (Tn::*rv3660*), which was obtained from the TBVTRM contract funded by NIH, NIAID: HHSN266200400091c. For all experiments mycobacterial recombinants, and the mutant were cultivated at 37°C in Middlebrook 7H9 liquid medium supplemented with 0.2% glycerol, 10% OADC (oleic acid, albumin, dextrose and catalase enrichment), and 0.05% Tween 80 or on supplemented Middlebrook 7H11 agar under kanamycin selection when necessary.

The open reading frame of $minD_{Mtb}$ (*rv3660*) was PCR amplified from *M. tuberculosis* H37Rv genomic DNA using Accuprime *pfx* DNA polymerase (Invitrogen) with primer sequences 5'-*ctgaccgatccgggg* and 3'-*gtgccatcccgccgt* engineered with asymmetric NdeI and HindIII restriction sites respectively, to facilitate cloning into the extrachromosomal mycobacterial vector *p*vv16.

4.2.2 Bioinformatic analysis

To identify *min*-system proteins encoded in *M. tuberculosis,* datasets were created from alignment of 169 MinD protein sequences from a variety of bacterial species from the RefSeq database. MinD alignment was then used to make global and local Hidden Markov Models, which were used to search and identify proteins encoded in the *M. tuberculosis* genome. In all BLAST searches, the percent identity and score were optimized to yield a MinD-like protein.

4.2.3 Ultrastructure analysis by scanning electron microscopy

For visualization by SEM, bacteria were grown to exponential phase, collected by centrifugation and washed three times in PBS, pH 7.4, and fixed with 2.5% gluteraldehyde in Buffer A (0.1 M potassium phosphate (pH 7.4), 1 mM $CaCl_2$ and 1 mM $MgCl_2$) at 4°C for 24hrs. The fixed bacterial cells were again collected by centrifugation, washed three times in Buffer A and treated with 1% OsO_4 in Buffer A for 30 minutes at 4°C. Then cells were washed three times with Buffer A and prepared for SEM with a graded series of ethanol treatments (20-100%). Ultrastructure examination was performed using a JOEL JEM -100CX electron microscope.

4.2.4 Global transcriptional profiling

For transcriptional analysis, bacteria were grown to midlog phase growth. Bacteria were subjected to TRIzol extraction, and total RNA was isolated by physical disruption. Microarray analysis was performed with labeled cDNAs generated using direct labeling from 5 μg of total RNA as described previously [32]. The resulting fluorescence for each channel of the array (Cy3 and Cy5) was normalized to the mean channel intensity and analyzed using Anova single factor analysis. Significance was considered to be a >1.5-fold alteration in expression, with a *p*-value of ≤ 0.05. The transcriptional activity of selected genes identified by DNA microarray analysis was verified using real-time PCR as described previously [32].

4.2.5 Quantitative real-time PCR

Quantitative real-time PCR was performed on selected open reading frames to verify transcriptional expression found by microarray. Quantitative RT-PCR primers were designed according to Manganelli et al., and analyses were performed using SYBR-green (Invitrogen) [43]. PCR amplification was performed with a thermocycling program of 55°C for 5 min then 95°C for 2 minutes, 45 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 45 sec. The relative number of transcripts for each gene was determined based on linear regression analysis of 100 ng, 10 ng, and 1 ng of *M. tuberculosis* genomic DNA. The total number of targets (n) were calculated by the equation n=a + b log (x) where "a" is the intercept and "b" is the slope of the standard curve, and "x" is the threshold cycle obtained by amplifying (n) targets. All reactions were performed in triplicate.

4.3 RESULTS

4.3.1 Bioinformatic identification of $MinD_{Mtb}$ in *M. tuberculosis*

A context-based profile model approach was taken in order to identify MinD orthologs encoded by *M. tuberculosis*. A MinD-protein Hidden Markov Model (HMM) was derived from alignment of proteins annotated as MinD (n=169) in other organisms. Global and local Hidden Markov Models were used to search proteins of *M. tuberculosis*. The search using the global HMM identified 3 MinD putative orthologs (HMM score: 38.8 to 18.2; E-values: 8.5^{e-9} to 0.013), and the search using the local HMM identified 6 MinD putative orthologs (HMM score: 58.5 to 11.7; E-values: 1^{e-144} to 1.2). These represent the two ends of the spectrum from highly related to distantly related proteins.

The ORF, *rv3660*, identified by this bioinformatic analysis, is annotated as a putative septum-site determining protein (http://genolist.pasteur.fr/TubercuList). *rv3660* has BLASTP Bit scores of 123 to MinD orthologs with 30% sequence similarity.

4.3.2 Morphological characterization

Based on the bioinformatic analysis, *rv3660* encodes a MinD-like protein, which we refer to as $MinD_{Mtb}$. Although this gene has been annotated as a putative septum-site determining protein, there has been no current experimentation demonstrating its influence on septum formation or biological role in cell division. Notably, this ORF was recently identified and associated with septum formation by expression profiling [32].

Gene dosage experiments were performed on several identified MinD homologs to experimentally evaluate whether they modulate septum formation. It has been demonstrated that overexpression of MinD [homologous or heterologous] prevents septum formation resulting in filamentous bacteria [9, 19]. Thus, increased expression in mycobacteria should inhibit septum formation, resulting in the production of filamentous cells. Morphological analysis via scanning electron microscopy revealed filamentation, the predominant ultrastructural characteristic, associated with overexpression of *rv3660* ($MinD_{Mtb}$) in *M. smegmatis* and in *M. tuberculosis*, demonstrating a stall in cell division (Figure 4.4, CD and GH, respectively). Analysis of SEM data demonstrated a shift in cell length in *M. smegmatis* and *M. tuberculosis* cell populations for overexpressed systems (Figure 4.5). Control cells in *M. smegmatis* and *M. tuberculosis* ranged from 1.5-2.5 μm, while cells dosed with $MinD_{Mtb}$ exhibited increases in cell length up to 5.5 μm in *M. smegmatis* and 4.5 μm in *M. tuberculosis*. In addition, there was an absence of

concentric rings along the bacterial filament, demonstrating a lack of septum formation. Morphological analysis of Tn mutant E150 (Tn*::* *rv3660*) revealed minicells with lengths ranging from 0.5 - 2.0 μm, (Figure 4.4, I and J). Septum deregulation was noted by the observation of multiple rings at the poles and various locations within the cells. These observations indicate that $MinD_{Mtb}$ influences septum formation and demonstrate a regulatory role in cell division similar to Min-system proteins found in other bacteria.

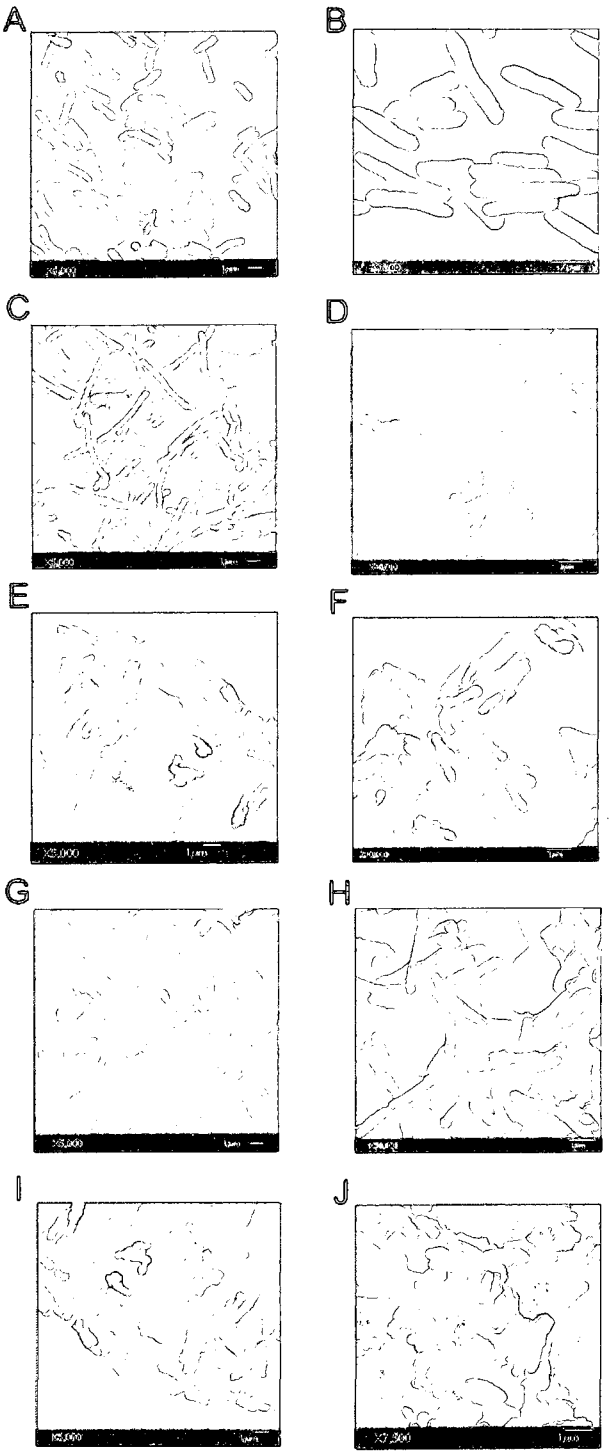

Figure 4.4 Morphological Analysis: Induced $MinD_{Mtb}$ and Tn Mutant. SEM imaging for mycobacterial controls *M smegmatis* and *M. tuberculosis*, Rv3660 recombinants and Tn mutant. SEM images illustrate increases in cell length for recombinant strains and shortened cell lengths for the Mtb:Tn::rv3660c mutant.

(A/B) Msm Control
(C/D) Msm: pVV16-*rv3660*
(E/F) Mtb H37Rv Control
(G/H) Mtb: pvv16-*rv3660*
(I/J) Mtb: Tn:: *rv3660*

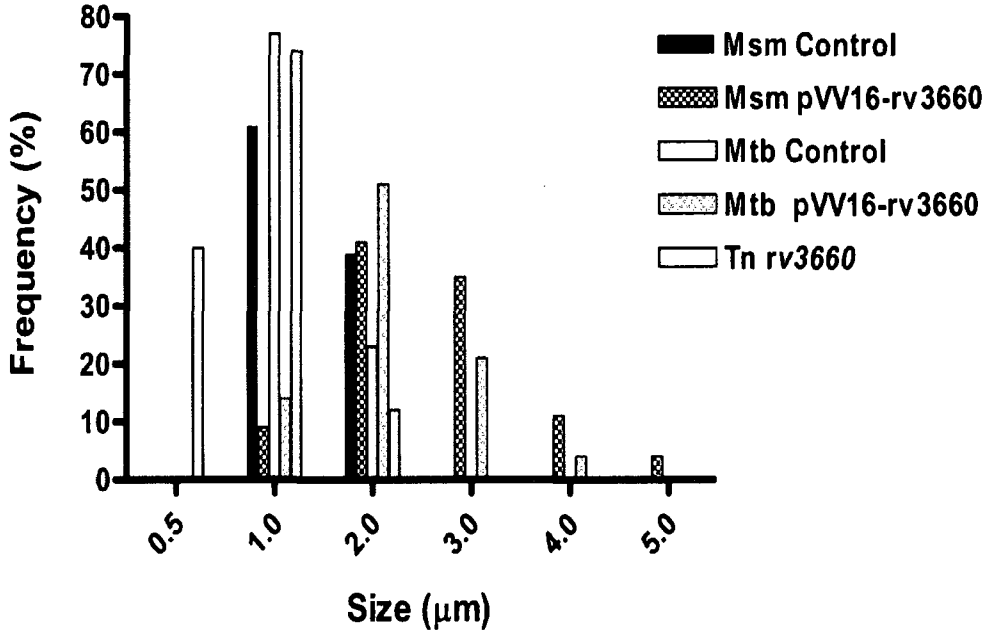

Figure 4.5 Frequency distribution of cell lengths for recombinants and mutant. Length distribution of mycobacterial controls cells, *rv3660* recombinant and Tn mutant cells. Lengths of the bacterial cells were calculated from the co-ordinates of both ends as measured with an electron microscope. Multiple fields were examined and values calculated in 0.5μm increments from a population of over 100 cells.

4.3.3 Gene expression profiling

Gene expression profiles were performed for both mutant and induced $MinD_{Mtb}$ bacterial systems. The transcriptional profile for over produced $MinD_{Mtb}$ provided 3,886 differentially regulated genes of which 2,274 demonstrated p-values ≤ 0.05. A total of 432 significantly active genes displayed a 1.5-fold or greater differential expression (Appendix C). Interestingly, the increase of the septum regulating protein $MinD_{Mtb}$ demonstrated a 2-fold or greater increase in transcriptional activity of genes found in the *dosR* regulon with, *rv3131*, *hspX* and *tgs1* being the highest genes induced (Table, 4.1). A select set of *dosR* genes was use to validated array expression data by QRT-PCR, which verified induced expression of the dormancy regulon profile (Figure 4.6, A).

To validate the role of $MinD_{Mtb}$ in cell cycle regulation, we assessed the transcriptional activity of discriminant genes for cell cycle processes. Selected ORFS representative of cell cycle events involving DNA replication, chromosome segregation, septum formation, cell wall synthesis, and cell division were utilized as previously described [32]. QRT-PCR analysis of cells cycle genes for both the overexpression of $MinD_{Mtb}$ and the mutant were assessed (Figure 4.6, B). In general, the induction of $MinD_{Mtb}$ repressed transcription of genes (*inhA, ftsZ, kasA,*) involved in septum initiation and formation, while the opposite was found for the mutant. Increased expression of these genes in the mutant can be associated with increased septum formation and minicell production. These results correlate with array data demonstrating increased transcriptional activity of genes involved in dormancy, adaptive responses, and conditions associated with nonreplicating cells.

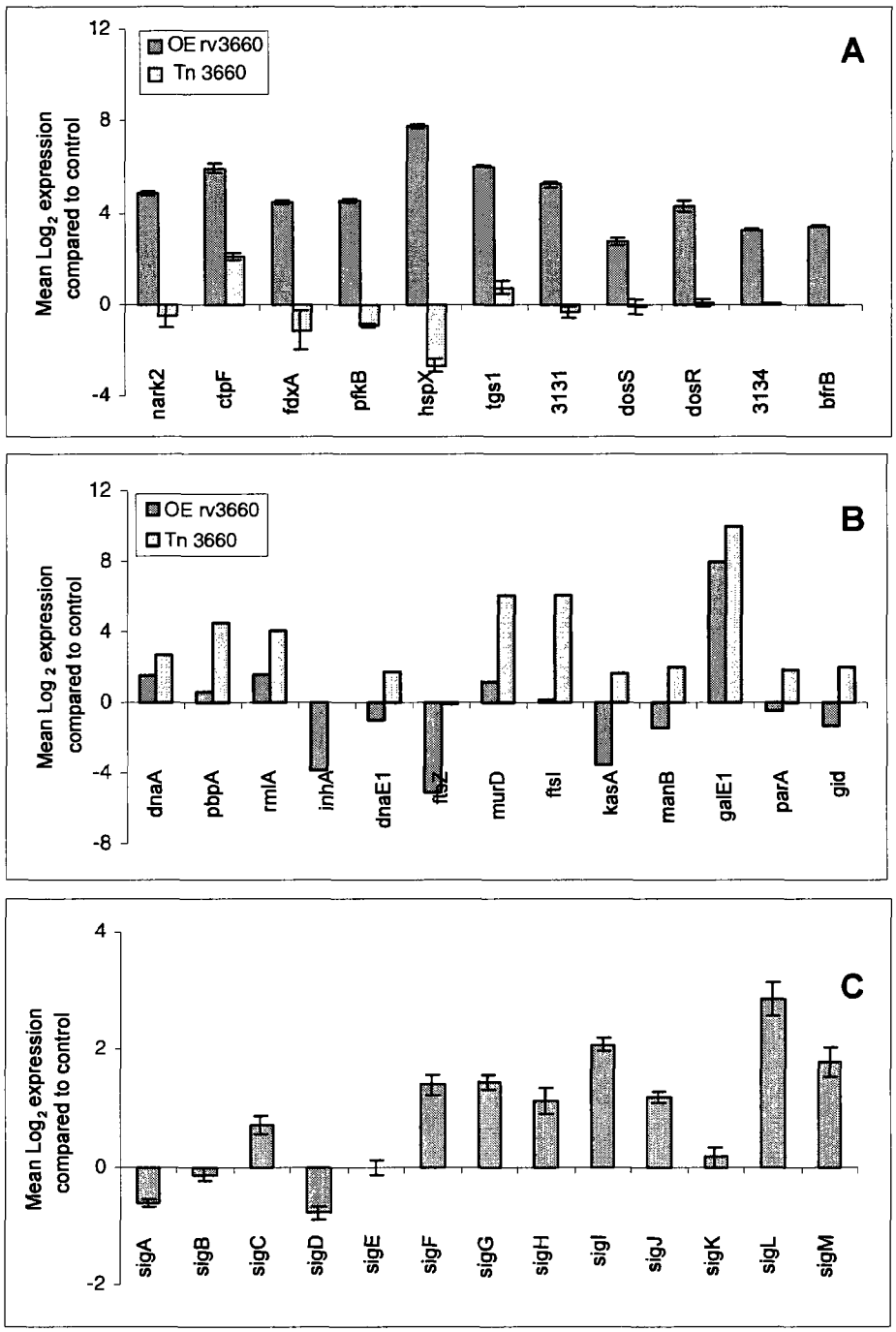

Figure 4.6 Real-time PCR analyses for the induced expression of *minD*$_{Mtb}$. Analyses of (A) cell cycle discriminant genes, (B) a selected set of *dosR* regulon genes, and (C) sigma factor expression from the overexpression of *rv3660*, *minD*$_{Mtb}$ provided support for microarray expression data with respect to inhibition of cell division, induction of the *dosR* regulon and alternative sigma factor expression.

Table 4.1 *dosR* regulon gene expression: $MinD_{Mtb}$ and Tn mutant
Comparative transcriptional profiles for the overexpression of *rv3660* ($MinD_{Mtb}$) vs the Tn mutant of the *dosR* regulon genes.

			OE rv3660c		*Tn mutant*		
Locus	**Gene**	**Product**	**Log_2 exp**	**p-value**	**Log_2 exp**	**p-value**	**Δ**
Rv0079		hypothetical protein	1.31	0.007	0.27	0.000	4.9
Rv0080		hypothetical protein	1.35	0.002	0.20	0.001	6.7
Rv0081		transcriptional regulator (ArsR family)	1.10	0.000	0.20	0.016	5.4
Rv0082		probable oxidoreductase subunit	0.46	0.011	0.28	0.063	1.7
Rv0083		probable oxidoreductase subunit	0.10	0.001	0.88	0.008	0.1
Rv0569		conserved hypothetical protein	1.26	0.000	0.29	0.003	4.3
Rv0570	nrdZ	ribonucleotide reductase, class II	1.19	0.018	-0.08	0.003	-15.0
Rv0571c		conserved hypothetical protein	0.14	0.025	-0.15	0.000	-0.9
Rv0572c		hypothetical protein	0.30	0.002	-0.41	0.013	-0.7
Rv0573c		conserved hypothetical protein	0.83	0.006	0.19	0.000	4.4
Rv0574c		conserved hypothetical protein	0.76	0.009	-0.23	0.006	-3.2
Rv1733c		possible membrane protein	1.99	0.068	0.33	0.002	6.0
Rv1734c		hypothetical protein	0.71	0.013	-0.04	0.009	-18.0
Rv1735c		hypothetical protein	0.50	0.001	0.14	0.012	3.4
Rv1736c	narX	fused nitrate reductase	1.09	0.032	0.07	0.000	15.0
Rv1737c	narK2	nitrite extrusion protein	1.87	0.228	0.20	0.001	9.2
Rv1738		conserved hypothetical protein	2.90	0.230	0.96	0.016	3.0
Rv1812c		probable dehydrogenase	0.03	0.324	-0.15	0.001	-0.2
Rv1813c		conserved hypothetical protein	1.26	0.257	1.83	0.030	0.7
Rv1996		conserved hypothetical protein	2.63	0.046	0.80	0.025	3.3
Rv1997	ctpF	probable cation transport ATPase	1.62	0.001	0.17	0.018	9.4
Rv1998c		conserved hypothetical protein	0.47	0.118	0.10	0.000	4.6
Rv2003c		conserved hypothetical protein	1.26	0.004	0.08	0.010	15.1
Rv2004c		hypothetical protein	1.01	0.008	0.36	0.022	2.8
Rv2005c		conserved hypothetical protein	1.78	0.033	0.33	0.000	5.4
Rv2006	otsB2	trehalose-6-phosphate phosphatase	1.28	0.000	0.02	0.008	78.4
Rv2007c	fdxA	ferredoxin	2.56	0.137	0.64	0.026	4.0
Rv2027c	dosT	sensor histidine kinase	1.35	0.001	0.07	0.044	18.9
Rv2028c		conserved hypothetical protein	0.38	0.009	-0.11	0.004	-3.3
Rv2029c	pfkB	phosphofructokinase II	2.03	0.330	0.26	0.006	7.8
Rv2030c		conserved hypothetical protein	3.37	0.195	0.62	0.004	5.4
Rv2031c	hspX	14kD antigen, heat shock protein Hsp20 family	3.94	0.043	1.50	0.079	2.6
Rv2032	acg	conserved hypothetical protein	2.50	0.277	0.29	0.003	8.6
Rv2617c		hypothetical protein	-0.21	0.012	-0.01	0.000	20.6
Rv2623		conserved hypothetical protein	3.02	0.151	0.15	0.132	19.8
Rv2624c		conserved hypothetical protein	1.34	0.062	0.10	0.024	13.9
Rv2625c		conserved hypothetical protein	-0.03	0.016	-0.94	0.017	0.0
Rv2626c		conserved hypothetical protein	3.35	0.000	0.77	0.184	4.4
Rv2627c		conserved hypothetical protein	2.65	0.285	0.05	0.010	51.0
Rv2628		hypothetical protein	2.22	0.022	0.14	0.038	16.0
Rv2629		hypothetical protein	0.49	0.004	0.28	0.006	1.8
Rv2630		hypothetical protein	1.42	0.003	0.24	0.014	5.9
Rv2631		conserved hypothetical protein	0.70	0.015	-0.17	0.021	-4.1
Rv2830c		similar to phage P1 phd gene	0.29	0.000	-0.07	0.002	-3.9
Rv3126c		hypothetical protein	0.91	0.021	0.07	0.018	12.8
Rv3127		conserved hypothetical protein	2.15	0.044	0.51	0.000	4.2
Rv3128c		conserved hypothetical protein	0.30	0.310	0.13	0.002	2.3
Rv3129		conserved hypothetical protein	1.09	0.002	0.03	0.035	40.6
Rv3130c	tgs1	conserved hypothetical protein	3.92	0.309	0.84	0.013	4.7
Rv3131		conserved hypothetical protein	4.01	0.273	1.66	0.189	2.4
Rv3132c	dosS	sensor histidine kinase	2.00	0.014	0.18	0.001	11.0
Rv3133c	dosR	two-component response regulator	1.00	0.070	0.22	0.009	4.5
Rv3134c		conserved hypothetical protein	2.45	0.024	0.16	0.002	15.0
Rv3841	bfrB	bacterioferritin	1.22	0.106	1.36	0.087	0.9

Previous studies compiling transcriptional profiles for induced hypoxia, starvation, and murine models of infection validated the significance of the *dosR* regulon as a common feature under all conditions [44]. Other common features associated with these dormancy profiles included the upregulation of lipid metabolism, cell wall maintenance and remodeling, alternative respiration and redox balance profiles along with a downregulation of ATP synthesis and ribosomal proteins involved in protein synthesis[44]. In comparison, $MinD_{Mtb}$ overexpression provided some similarities with these findings such that there exhibited and increased expression of genes involved in fatty acid degradation. In fact, of the 217 transcriptionally active genes categorized under lipid metabolism in the profile, 215 encoded proteins involved in fatty acid utilization and displayed increased gene expression. Other correlations include a significant increased expression of genes involved in anaerobic respiration and electron transport or redox-potential, along with the down-regulation of ribosomal proteins and therefore protein synthesis, (Table 4.2). Anomalies included an increase in transcriptional activity of genes involved the synthesis of polyketides and lipoproteins associated with cell surface molecules and an unaltered expression of genes involved in ATP synthesis. Finally, the transcription profile demonstrated a unique sigma factor profile with significant increases in expression of sigma factors σFGHIJLM (Figure 4.6, C).

Table 4.2 Genes differentially regulated for selected cell functions.
(p-value ≤ 0.05).

ORF	Gene	Log $_2$ expression		ORF	Gene	Log $_2$ expression	
		OE Rv3660c	*Tn Mutant*			*OE Rv3660c*	*Tn Mutant*
Fatty acid utilization				***Ribosomal proteins***			
Rv0974c	accD2	1.2	-0.2	Rv0056	rplI	-1.0	-0.6
Rv1935c	echA13	0.9	-0.2	Rv0682	rpsL	-0.9	-0.9
Rv2486	echA14	1.0	-0.1	Rv0700	rpsJ	-1.4	-0.5
Rv0456c	echA2	1.2	-0.1	Rv0701	rplC	-1.5	-0.4
Rv3550	echA20	1.1	0.2	Rv0716	rplE	-1.2	-0.9
Rv0971c	echA7	1.3	-0.1	Rv0722	rpmD	-0.9	-0.3
Rv3546	fadA5	1.1	0.1	Rv0723	rplO	-0.7	-0.2
Rv1715	fadB3	1.0	-0.1	Rv2441c	rpmA	-0.9	-0.5
Rv0099	fadD10	1.2	0.0	Rv3442c	rpsI	-0.9	-0.2
Rv1550	fadD11	1.0	0.2	Rv3443c	rplM	-1.6	-0.5
Rv1058	fadD14	1.2	0.0	Rv3458c	rpsD	-0.8	-0.5
Rv3561	fadD3	0.8	0.5	Rv3460c	rpsM	-1.3	-0.6
Rv0035	fadD34	1.3	0.0	Rv3461c	rpmJ	-1.4	-0.6
Rv0214	fadD4	0.8	-0.2	Rv3924c	rpmH	-1.2	-0.7
Rv0551c	fadD8	0.9	0.0				
Rv2590	fadD9	1.3	-0.5	***Anaerobic respiration***			
Rv0972c	fadE12	1.4	-0.1	Rv0252	nirB	0.8	ndr
Rv0975c	fadE13	1.3	ndr	Rv0253	nirD	1.1	ndr
Rv3061c	fadE22	1.0	-0.1	Rv0267	narU	1.2	ndr
Rv3505	fadE27	1.0	0.0	Rv1161	narG	0.7	ndr
Rv3544c	fadE28	0.8	0.0	Rv1163	narJ	0.7	ndr
Rv3562	fadE31	1.0	0.2	Rv1164	narI	0.6	ndr
Rv3563	fadE32	0.8	0.4	Rv1552	frdA	1.2	ndr
Rv3564	fadE33	1.2	0.3	Rv1553	frdB	0.8	ndr
Rv0752c	fadE9	0.9	-0.1	Rv1554	frdC	1.1	ndr
Rv1492	mutA	1.1	0.2	Rv1736c	narX	1.1	ndr
Rv1493	mutB	1.2	0.5	Rv1737c	narK2	1.9	0.2
Cell surface molecules				***Electron Trpt/Redox***			
Rv0399c	lpqK	0.8	-0.1	Rv0409	ackA	1.0	0.2
Rv0405	pks6	1.2	-0.2	Rv0886	fprB	0.8	0.1
Rv0593	lprL	1.1	0.0	Rv1620c	cydC	1.6	0.0
Rv0604	lpqO	0.8	-0.1	Rv1622c	cydB	2.0	-0.2
Rv0794c	lpdB	0.9	-0.2	Rv1623c	appC	1.0	-0.2
Rv1064c	lpqV	1.1	-0.1	Rv2007c	fdxA	2.6	0.6
Rv1166	lpqW	0.8	0.0	Rv3251c	rubA	0.8	-0.1
Rv1372	pks18	1.1	0.1				
Rv1661	pks7	1.3	-0.2	***ATP synthesis***			
Rv1662	pks8	1.0	0.2	Rv1304	atpB	0.2	-0.6
Rv1663	pks17	1.2	0.2	Rv1305	atpE	0.2	-0.4
Rv1664	pks9	1.1	0.1	Rv1306	atpF	0.0	-0.7
Rv1665	pks11	0.7	0.2	Rv1307	atpH	0.2	-0.6
Rv1921c	lppF	1.4	0.2	Rv1308	atpA	0.3	-0.4
Rv1946c	lppG	1.0	0.1	Rv1309	atpG	-0.1	-0.7
Rv1966	mce3	1.1	0.0	Rv1310	atpD	0.3	-0.4
Rv2270	lppN	0.9	-0.1	Rv1311	atpC	0.2	-0.4
Rv2330c	lppP	0.7	0.1				
Rv2543	lppA	0.9	0.2	*ndr = not differentially regulated*			
Rv2796c	lppV	0.8	0.0				

The transcriptional profile of the Tn mutant provided 65 significantly upregulated genes that displayed a 1.5-fold or greater increase in expression (Appendix D), which included lipid synthesis genes *kasA and kasB,* chromosome partitioning gene *parA*, and the *divIVa* homologue, *wag31* that in known in *B.subtilis* to be involved in regulating septum placement at the cell poles. Again, increased expression of gene products involved in septum formation and partitioning corresponds with minicell production.

4.4 DISCUSSION

Mycobacterium tuberculosis is able to circumvent host responses and establish a latent infection in the lungs where it can silently persist for years. Regulatory mechanisms that reduce growth and promote a nonreplicating persistent state of infection remain unknown. Thus, the identification and study of regulatory elements that control cell cycle progression and that contribute to adaptation under altered growth conditions and environmental stress is paramount to understanding the pathogenesis and survival of the pathogen [45]. Furthermore, understanding the regulation involved in the "off" and "on" persistent phenotypes can provide novel areas to target for drug discovery.

Cell cycle checkpoints are control measures that ensure the fidelity of cell replication and division events. These checkpoints verify whether the each step in the cell cycle has been accurately completed before moving forward in cell cycle progression. Vital processes involved in bacterial cell growth and replication require spatial and temporal coordination of events such as cell wall extension, DNA replication, chromosome partitioning, Z-ring assembly, septum formation, and cytokinesis. It is known that bacterial cells entering different environments adopt adaptive strategies and

regulate cell cycle events in order to maintain viability. Studies in *M. tuberculosis* indicate that DNA replication and cell division events are key "checkpoints" in the cell cycle associated with adaptive responses [46-49]. The importance of entering non-replicating persistence at the correct stage of the cell is so that viability is maintained and resumption of growth can occur. Therefore, regulators involved in controlling septum formation are candidates for participating in the early events of stress responses that reduce growth rate and initiate alternative metabolism involved in survival under stress conditions. However, strong candidates of regulatory proteins known to influence septum formation have not yet been described in *M. tuberculosis*.

To identify putative regulators of septum formation in *M. tuberculosis,* a two-pronged approach of bioinformatics and transcriptional mapping was employed. A protein consensus sequence was generated from alignments of annotated MinD proteins from other organisms. The resulting sequence was then used to probe the open reading frames encoded in the *M. tuberculosis* genome. This approach led to the identification of a Min-like protein encoded by *rv3660* that is annotated as a putative septum site-determining protein. Based on the bioinformatics derived consensus sequence, we deemed the protein encoded by *rv3660* as $MinD_{Mtb}$.

Gene dosage experiments and ultrastructure analysis indicated that the abundance of $MinD_{Mtb}$ directly influenced bacterial morphology. $MinD_{Mtb}$ identified by bioinformatics demonstrated similar cellular morphological traits when induced or mutated as those previously described for Min-system orthologs [6, 12, 38, 39, 41]. Specifically, increased production of $MinD_{Mtb}$ resulted in filamentation devoid of concentric rings. This observation is fully consistent with inhibition of cell division in *M.*

tuberculosis and implicates that filamentation is the result of inhibition of septum formation that is dependent on the presence of $MinD_{Mtb}$ [32, 46, 49].

It has been established that there are shared genes within the transcriptional responses to growth under hypoxia, starvation, and in the murine model of infection [44]. Common genes among these profiles that are induced include genes of the *dosR* regulon and those involved in lipid metabolism, cell wall maintenance and remodeling, and alternative respiration and redox balance. Those that are repressed include genes involved in ATP synthesis and ribosomal proteins for protein synthesis. When gene expression in the $MinD_{Mtb}$ merodiploid strain was evaluated, it was found that in conjunction with induction of the *dosR* regulon there was a *Dos*-like response characterized by an upregulation of genes involved in fatty acid degradation/utilization, anaerobic respiration, electron transport or redox-potential, and a down-regulation of ribosomal proteins and protein synthesis. In addition, despite these signature adaptive responses, ATP synthesis remained unchanged. Importantly, in the $MinD_{Mtb}$ mutant, these genes did not display a significant difference in transcriptional activity, indicating that $MinD_{Mtb}$ plays a role in *Dos*-regulation and cellular adaptation. As with most pathogens, the ability to modulate gene expression under different environments is crucial to survival as the organism experience various stresses under host infection. Adaptation is essential for the organism's pathogenicity and survival.

M. tuberculosis is a slow-growing facultative intracellular parasite which during infection is exposed to various conditions depending on the stage and severity of the disease. These bacilli can replicate inside the macrophage phagosome or extracellularly in open lung cavities. It can disseminate to infect other tissues and organs and lead to

miliary tuberculosis. And finally these bacilli can hide out or evade the immune system inside the hostile environment of the granuloma, or sink into a dormant state and remain viable for years. The necessity to adapt is a major role in the pathogenicity and survival for *M. tuberculosis.*

An important aspect in cell survival and persistence is the utilization of alternative sigma factors for expression of genes required to maintain cell function under various conditions. In addition to the *Dos*-response, increased production of $MinD_{Mtb}$ resulted in an upregulation of the sigma factors σFGHIJLM. Sigma factors provide global regulation of gene expression in response to environmental stimuli, as they are known to combine with the RNA polymerase core enzyme and direct the transcription of new genes [50, 51]. Of the 13 sigma factors in *M. tuberculosis,* several of these have been characterized to respond to environmental stresses while others respond to unknown condition or have no assigned function under *in vitro or in vivo* models. SigF was reported to regulate genes encoding cell wall associated proteins and directly regulate *phoY1* a probable transcriptional regulator involved in phosphate uptake [52]. In addition, SigF was demonstrated to be induced under antibiotic treatment and may regulate genes important for conditions of drug tolerance [53]. SigG was identified as playing a significanct role in the SOS stress response induced by mitomycin C [54]. SigH has been identified as a regulator of heat and oxidative-stress responses likely to be important in pathogenesis and survival [55]. SigI is directly upregulated by SigJ expression, which may control an alternative H_2O_2 resistance pathway for survival with in the macrophage [56]. Studies by Hahn et al. identified the upregulation of genes associated with polyketide synthesis and secreted or membrane proteins required for virulence upon increased expression of sigL.

They postulated that these surface molecules provided an additional modulations to the cell surface required for survival at a particular state of pathogenesis [57]. SigM has been shown to induce a set of esat6-homologs hypothesized as being required for alternative survival and pathogenicity in aspects of the disease cycle such as persistence, survival in necrotic granulomas, or transmission via droplet nuclei [58]. Additionally, it has been postulated that *sigM* is required for survival in other animal models, which more closely mimic human infection, since a Δ*sigM* mutant strain did not demonstrate attenuation of the disease in both the mouse or guinea pig models of tuberculosis. It has been proposed that these rarely expressed sigma factors, σFGIJKLM) are induced under host-specific conditions or at very specific disease conditions. Therefore, the unique alternative sigma profile elicited by the increased production of $MinD_{Mtb}$ may have significance in adaptive responses associated with disease pathogenesis and survival in the host.

Interestingly, previous work by Chauhan et al. (2006), identified a filamentous phenotype of *M. tuberculosis* when grown in macrophages [48]. During infection, *M. tuberculosis* is taken up by macrophages and is introduced to a variety of host responses that are induced to kill the bacilli. RNIs and ROIs, antimicrobials peptides, hydrolytic enzymes, cytokines, and effecter cells are all at work trying to eliminate the infecting bacteria. In addition, the environment during the course of the infection and granuloma formation becomes acidic, hypoxic and nutrient limited. In order to survive, *M. tuberculosis* must adapt to this stressful intracellular environment, which requires an altered program of gene expression that may include those that induce filamentation. This would turn off normal growth processes and conserve energy for processes required

for a dormant survival. This change in balance could promote inhibitory actions affecting Z-ring assembly and septum formation, thus stalling cell division and initiating programmed events directed toward a nonreplicating persistent lifestyle. Notably, $MinD_{Mtb}$ expression induces filamentation and a transcriptional program associated with intracellular adaptive survival strategies related to latency conditions.

While all the components involved in regulation and adaptation strategies for cessation of growth and nonreplicating persistence in *M. tuberculosis* have yet to be defined, these results substantiate that $MinD_{Mtb}$ has a role in regulating cell cycle events leading to a genetic program that promotes a shift into an altered metabolic state and induce a novel program of global regulation. This research provides initial evidence linking a regulatory element of cell division with signature transcriptional events associated with dormant state nonreplicating bacilli in *M. tuberculosis.*

4.5 ACKNOWLEDGEMENTS

This work was support by RO1 AI055298 (RAS). We gratefully acknowledge Dr. Philip Chapman for assistance with the statistical analysis and Ms. Laurel Respicio, Ms. Melissa Boyne, and Ms. Kerry Brookman for technical assistance. We recognize the Rocky Mountain Regional Center of Excellence for providing microarray analysis resources. We acknowledge the post-genomic resources and services provided by the Rocky Mountain Regional Center of Excellence (U54 A1065357).

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CHAPTER 5

rv2216 Encodes an Ortholog of the Cell Division Regulator, YneA and Upregulates Adaptive Responses in *Mycobacterium tuberculosis*

This work is dedicated in memory of Dr. Dennis Knudson who performed the initial bioinformatics analyses in a combined effort with Dr. Richard Slayden that identified $YneA_{Mtb}$ encoded by *rv2216*. In addition, Melissa Boyne performed early work involved in studying the SOS transcriptional response to mitomycin C and piperacillin treatments.

5.1 INTRODUCTION

A hallmark of *Mycobacterium tuberculosis* is its ability to establish and maintain a latent state of infection for long durations despite the host response or chemotherapy. The success of *M. tuberculosis* over the course of an infection lies in its ability to evade the host responses, survive stress conditions such as hypoxia, nutrient starvation, and DNA damage via reactive oxygen and nitrogen species, and tolerate drug treatment [1, 2, 5-16]. While the bacterial responses to stress conditions, as noted above, have been extensively investigated in *M. tuberculosis*; regulatory elements that directly suppress cell division in response to these stresses have not been elucidated.

Mechanisms exist in bacteria to control growth during stress, and mounting experimental evidence indicates that the bacterial response to stress include key regulatory elements to suppress cell division and the cell cycle. Mechanisms governing

cell cycle progression are necessary for the survival of bacteria in response to altered environmental conditions and adaptations to stress. Components of the SOS response are shown to be involved in monitoring completion of cell division and other cell cycle processes in addition to DNA damage repair [17-26]. Furthermore, many components of the SOS system are expressed at potentially significant levels, even in a repressed state, due to self-regulation of RecA and LexA, providing evidence that there may be several proteins involved in governing cell cycle progression under a wide variety of conditions [27-32]. The implication of SOS components in a broad range of stress conditions provides evidence that regulatory elements are checkpoints of cell cycle processes governing adaptation and survival events.

In other bacterial species, stress induced proteins, such as SulA in *Escherichia coli* and YneA in *Bacillus subtilis*, are known to regulate cell division in response to DNA damage resulting from chemical exposure, radiation, or reactive oxidative intermediates (ROIs) [17, 20, 33-40]. These proteins are used in a final survival strategy to halt cells from dividing until chromosomal damage is repaired and then the cell can continue to replicate its chromosome completely. Studies in *E. coli* identified three levels of gene expression during SOS induction [37, 41]. The first genes induced upon DNA damage stress conditions are involved in nucleotide excision repair mechanisms are *uvrA, uvrB uvrC* and *uvrD*. These proteins act to excise damaged nucleotides from double-stranded DNA and gaps are repaired using various single-strand repair mechanisms depending on the extent of the damage. In addition, the LexA and DinI gene products are also induced to repress recombinational repair genes and inhibit translesion DNA synthesis until needed [42]. Expression of these initial

response genes is often less than 10 times constitutive expression. A second defense against DNA lesions is through homologous recombinational repair mechanisms. Genes involved in recombinant repair (*recA* and *recN*) are induced along with other homologous recombination functions. Recombinant repair of lesions on ssDNA regions at replication forks provides a dsDNA substrate for nucleotide excision repair, thus working cooperatively to repair damage and drive through replication events. If DNA damage is not repaired efficiently, a third set of genes (*sulA/YneA, umuD, umuC*) is upregulated to delay cell cycle events and make a final attempt at DNA repair. The SulA (YneA) proteins inhibit cell division while UmuC, and UmuD proteins form an error-prone polymerase (PolV) that inserts random bases opposite noncoding lesions during translesion DNA synthesis often generating mutations. This mutagenic DNA repair is not the best repair system and is only used as a last resort, emphasizing limited use. Typically, either the cell can repair the damage and the cell cycle continues or the damage is beyond repair and the cell is lysed.

In *Mycobacterium tuberculosis* previous work suggested that there are at least two distinct systems associated with DNA repair during induced stress; the classical RecA/LexA-dependent repair genes found in other bacterial species and alternative RecA/LexA-independent mechanism(s) utilizing alternative proteins [30-32, 43-48]. Many of the genes induced in the alternative repair mechanisms included genes involved in nucleotide excision repair, damage reversal and recombination events suggesting that alternative mechanisms were associated with various physiological conditions encountered by the bacilli during host infection. The presence of a group of damage-inducible genes in *M. tuberculosis* that are not directly under the

transcriptional control of LexA supports the notion that there are additional cellular elements responsible for monitoring cell cycle progression.

Although, the regulation and role of SOS genes has been examined in *M. tuberculosis*, these studies have failed to identify the SulA or YneA-like SOS regulatory element that controls cell division during stress. To explore the existence of a SulA or YneA-like homologue in *M. tuberculosis*, we performed protein modeling-based bioinformatic searches to identify putative proteins. This approach led to the identification of a YneA-like protein in *M. tuberculosis* encoded by *rv2216*. Morphological analysis, transcriptional profiling and gene dosage studies allowed us to substantiate that *rv2216* encodes a YneA-like protein ($YneA_{Mtb}$) in *M. tuberculosis*. Gene dosage experiments demonstrated $yneA_{Mtb}$ expression resulted in an inhibition of cell division resulting in a filamentous morphology. Mitomycin and piperacillin were used to induce SOS responses to examine if $YneA_{Mtb}$ was induced under these stress conditions in the cell. Global transcriptional analysis revealed that increased production of $YneA_{Mtb}$ from vector expression induced transcriptional responses relating to conditions of hypoxia, nutrient starvation, and enduring hypoxic conditions associated with a persistent lifestyle. This further substantiates the idea that $YneA_{Mtb}$ functions to suppress cell division under stress related adaptive events. Combined, these results indicate that $YneA_{Mtb}$ encoded by *rv2216* is a regulating element induced to control cell division and adaptive stress responses in *M. tuberculosis*.

5.2 METHODS

5.2.1 Bacteria and growth conditions

M. tuberculosis H37Rv was grown at 37°C in Middlebrook 7H9 liquid medium supplemented with 0.2% glycerol, 10%OADC, 0.05% Tween. Kanamycin sulfate was used at a concentration of 25μg ml^{-1} when necessary. Mitomycin C at a concentration of 0.2μg ml^{-1} was used to induce the SOS response [44]. *rv2216* was cloned into the mycobacterial vector pvv16 using restriction enzymes and transformed into *M. smegmatis* and *M. tuberculosis* as described elsewhere [49].

M. tuberculosis H37Rv was grown to an $O.D._{600nm}$ of 0.1 - 0.2 and subjected to continued growth in the presence of piperacillin and mitomycin C (MMC) over the concentration range of 200 μg ml^{-1} to 0.2 μg ml^{-1} in a two-fold serial dilution of the drug in 100 μl total volumes. IC_{50} was performed in triplicate and was defined as the concentration of drug required to reduce bacterial growth 50% after 7-days incubation. Viability testing was performed at 1, 3, and 5 days post-treatment with 25 μg ml^{-1} piperacillin, 12.5 μg ml^{-1} piperacillin, 0.2 μg ml^{-1} MMC, or 0.1 μg ml^{-1} MMC by determining colony forming units via direct plating and outgrowth.

5.2.2 Bioinformatic analysis

To identify putative YneA-like orthologs several strategies were employed. A reciprocal best-hit (RBH) approach was designed as an initial screen. Datasets of proteins annotated as YneA were created from RefSeq and the UniProt databases. The proteins in each dataset were aligned using MUSCLE. These aligned datasets were used to build YneA profile Hidden Markov Models (HMMs) for YneA using the

HMMER tools. These models were used to search encoded *M. tuberculosis* proteins to identify potential proteins that contained YneA-like motifs using HMM search, a HMMER tool. BLASTX, BLASTP, and TBLASTN analysis against searchable databases prepared from prototype protein datasets of YneA were also used. In all BLAST searches, the percent identity and bit-score were optimized to yield putative *M. tuberculosis* YneA-like proteins. Candidate proteins were further analyzed using InterPro release 11 for motifs or domains and compared with those seen in the prototype YneA assembly protein datasets [50]. HMMpfam BLAST of Rv2216 was performed at the TIGR-CMR web site. Tools from the EMBOSS package were also used to handle, manipulate, or analyze the proteins and datasets [51].

5.3.2 Ultrastructure analysis of *M. tuberculosis* treated with mitomycin C or FtsI inhibitor piperacillin, and morphology from gene dosage experiments.

Bacteria treated with 25 μg ml^{-1} piperacillin, 12.5 μg ml^{-1} piperacillin, 0.2 μg ml^{-1} MMC, or 0.1 μg ml^{-1} MMC at 1, 3, and 5 days and cells that overexpressed *rv2216* taken at mid-log growth were washed three times in PBS and fixed with 2.5% gluteraldehyde in buffer consisting of 0.1 M potassium phosphate, 1.0 mM $MgCl_2$, and 1.0 mM $CaCl_2$ (pH 7.4). Complete fixation was accomplished via incubation for 24 hours at 4°C followed by washing 3-times with 0.1 M potassium phosphate buffer. Cells were post-fixed processed by a 30min treatment with 1% OsO_4 in the same buffer, followed by several washes. The bacteria were centrifuged into pellets and subsequently dehydrated through a graded series of ethanol (20-100%). The cells were examined using a JOEL JEM-100CX electron microscope.

5.2.4 Quantitative real-time PCR

M. tuberculosis H37Rv was treated with 0.2 μg ml^{-1} MMC or 25 μg ml^{-1} piperacillin at 37°C for time intervals up to 72 hrs. Following incubation, the cells were harvested, resuspended in TRIzol reagent (Invitrogen™), and total RNA was liberated by physical disruption as previously described [52]. cDNA was generated from total RNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen™). Primer sequences were designed using Primer3 [53]. QPCR was performed with an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories) using Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen™). PCR was performed at 55°C for 5 minutes then 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. The final steps involved an increase of 1°C every 10 seconds beginning at 30°C for 65 cycles to establish melt curves. All reactions were performed in triplicate on two independent total RNA preparations. Performance of each primer-set was evaluated using genomic DNA to establish specific amplification characteristics. The number of targets in each sample for each gene corresponding to the threshold cycle for each PCR was determined based on the specific standard curves generated. The relative number of transcripts for each gene was determined from standard curves established for each primer set by linear regression of data obtained using 100 ng, 10 ng, and 1 ng of H37Rv genomic DNA. Amplification results were visualized and analyzed using BioRad iCycler software and quantification of each gene was determined in each treated sample relative to a time zero base-line sample. In addition, a gene dosage experiment profile

of a set of cell cycle discriminant genes was also analyzed at mid-log growth by QRT-PCR as described above.

5.2.5 Global transcriptional profiling

For transcriptional analysis recombinant bacteria were grown to mid-log phase growth. Whole bacteria were subjected to TRIzol extraction, and total RNA was isolated by physical disruption. Microarray analysis was performed with labeled cDNAs generated using direct labeling from 5 μg of total RNA as described previously [54]. The resulting fluorescence for each channel of the array (Cy3 and Cy5) was normalized to the mean channel intensity and analyzed using Anova single factor analysis. Significance was considered to be a >1.5-fold alteration in expression, with *p*-value of ≤ 0.05. The transcriptional activity of selected genes identified by DNA microarray analysis was verified using real-time PCR as described previously [54].

5.3 RESULTS

5.3.1 Identification of the $YneA_{Mtb}$ ortholog encoded by *rv2216*

A putative YneA ortholog in *M. tuberculosis* was identified using a reciprocal best-hit (RBH) strategy constructed from YneA-specific local and global Hidden Markov Models (HMMs) from 85 bacterial genomes built from RefSeq datasets to search for the H37Rv proteins. Both models led to the identification of a 301 amino acid protein, encoded by *rv2216,* annotated as a conserved hypothetical protein of unknown function with an E value of ~ 10^{-55}. Rv2216 alignments with the models revealed motifs throughout the sequence, which were conserved HMMs and no simple motif or block appeared to be more significant than another. These analyses revealed that *rv2216* encodes the YneA ortholog in *M. tuberculosis*, $YneA_{Mtb}$. Further, RBH analysis using $YneA_{Mtb}$ versus 85 bacterial genomes showed that $YneA_{Mtb}$ is conserved in actinomycetales mycobacteria, corynebacteria, and norcardia, substantiating the identification of $YneA_{Mtb}$ as the conserved YneA ortholog.

5.3.2 Mitomycin C and piperacillin treatments produce filamentous cells

In order to assess the relationship of $YneA_{Mtb}$ to the SOS response and cell cycle progression in *M. tuberculosis*, a clearer picture of each of these processes was needed. To accomplish this, we induced an SOS response in the bacteria using mitomycin C, or the FtsI inhibitor, piperacillin. Morphological analysis was performed to determine the effects of each of these conditions on the overall macrostructure of *M. tuberculosis*, in regards to which step in the cell cycle is affected. It was previously determined that inhibition of septum formation *via* prevention of FtsZ polymerization

produces filamentous cells which are generally devoid of cell septa while inhibition of FtsI produces cells with concentric rings marking the location of septum formation [22, 52, 54, 55]. Since a correlation between treatment with the β-lactam antibiotic piperacillin and induction of the SOS response has been reported, then a similar bacterial morphology should be observed [21]. Bacterial ultrastructural characteristics were visualized via microscopy following 1 and 3 days of treatment with 25 μg ml^{-1} piperacillin (Figures 5.1, A and B) and 0.2 μg ml^{-1} mitomycin C (Figures 5.1, C and D). In comparison to untreated bacteria (Figures 5.1, E and F), both piperacillin and mitomycin C treated cells were elongated. As illustrated in figure 5.1 A and B, treatment with piperacillin after 3 days of drug exposure provided elongated cells lacking initially observed concentric septal rings seen on day 1. Mitomycin C treatment led to cells with and early and more exaggerated smooth filamentous phenotype (Figures 5.1, C and D). Together, these results indicate that stress induced responses in *M. tuberculosis* stalls cell division and prevents septum formation.

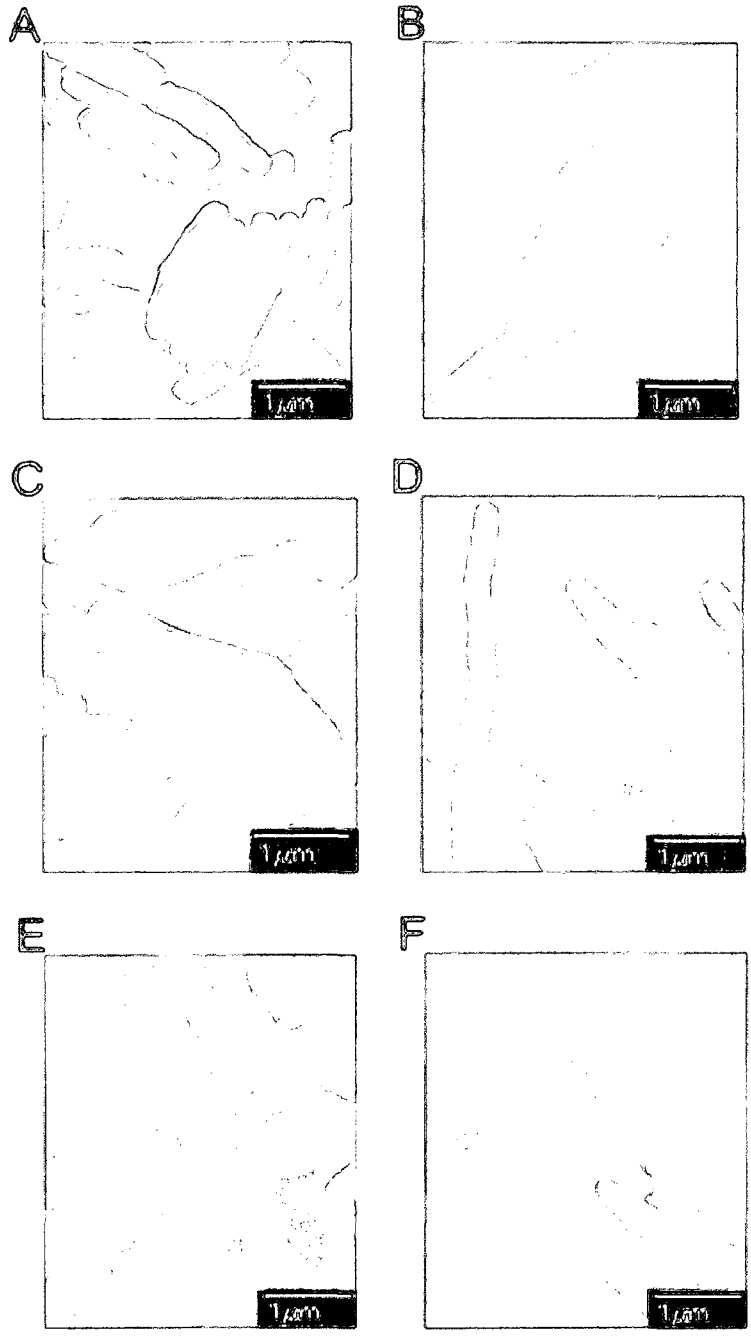

Figure 5.1 SOS induced morphology. Morphological analysis was performed on H37Rv cells treated with piperacillin (AB), or mitomycin C (CD), with representative untreated control *M. tuberculosis* (EF). Images represent cultures treated for 1 day (ACE) or 3 days (BDF). Images provided are at 10,000x magnification.

5.3.3 Mitomycin C and piperacillin treatment increases *yneA*$_{Mtb}$ expression

In order to further investigate YneA$_{Mtb}$ as a member of the SOS-system, and involved in regulation of septum formation we assessed the transcriptional activity of SOS genes and discriminant genes for cell cycle processes in response to mitomycin C and piperacillin treatment. The selected ORFs that were considered representative of the cell cycle are involved in DNA replication, chromosome segregation, cell division, and cell wall synthesis as previously reported [22, 55]. Both mitomycin C treatment and piperacillin treatment repressed expression of several cell cycle discriminant genes involved in cell division. Mitomycin C treatment reduced most all genes in the profile, while piperacillin treatment primarily suppressed *ftsZ* and *ftsI,* which initiate and control septum formation (Figures 5.2, A and B). This suggests that overall cell cycle progression is significantly reduced following induction of the SOS response in *M. tuberculosis* from these treatments. In contrast, the SOS genes were upregulated (Figure 5.3, A and B), with the exception of *rv3164 (moxR3)*, *rv3260 (whiB2)*, and *rv3261 (fbiA)*, (data not shown) which fall into the category of genes that may not be SOS responsive [43]. Importantly, upregulation of *yneA*$_{Mtb}$ *(rv2216)* following induction of the SOS response, provides experimental evidence that supports the information obtained by bioinformatic analysis; specifically, analogous to the ~300 amino acid protein members of the YneA-family in other organisms, *yneA*$_{Mtb}$ encodes an SOS responsive gene in *M. tuberculosis.* Similar to a recent report, inhibition of FtsI and cell division with piperacillin treatment led to an induction of the SOS response, albeit transient, despite the continued presence of drug (Figure 5.3, B) [22, 55]. Specifically, the SOS genes *ruvA, ruvC, rv2719c, lexA,*

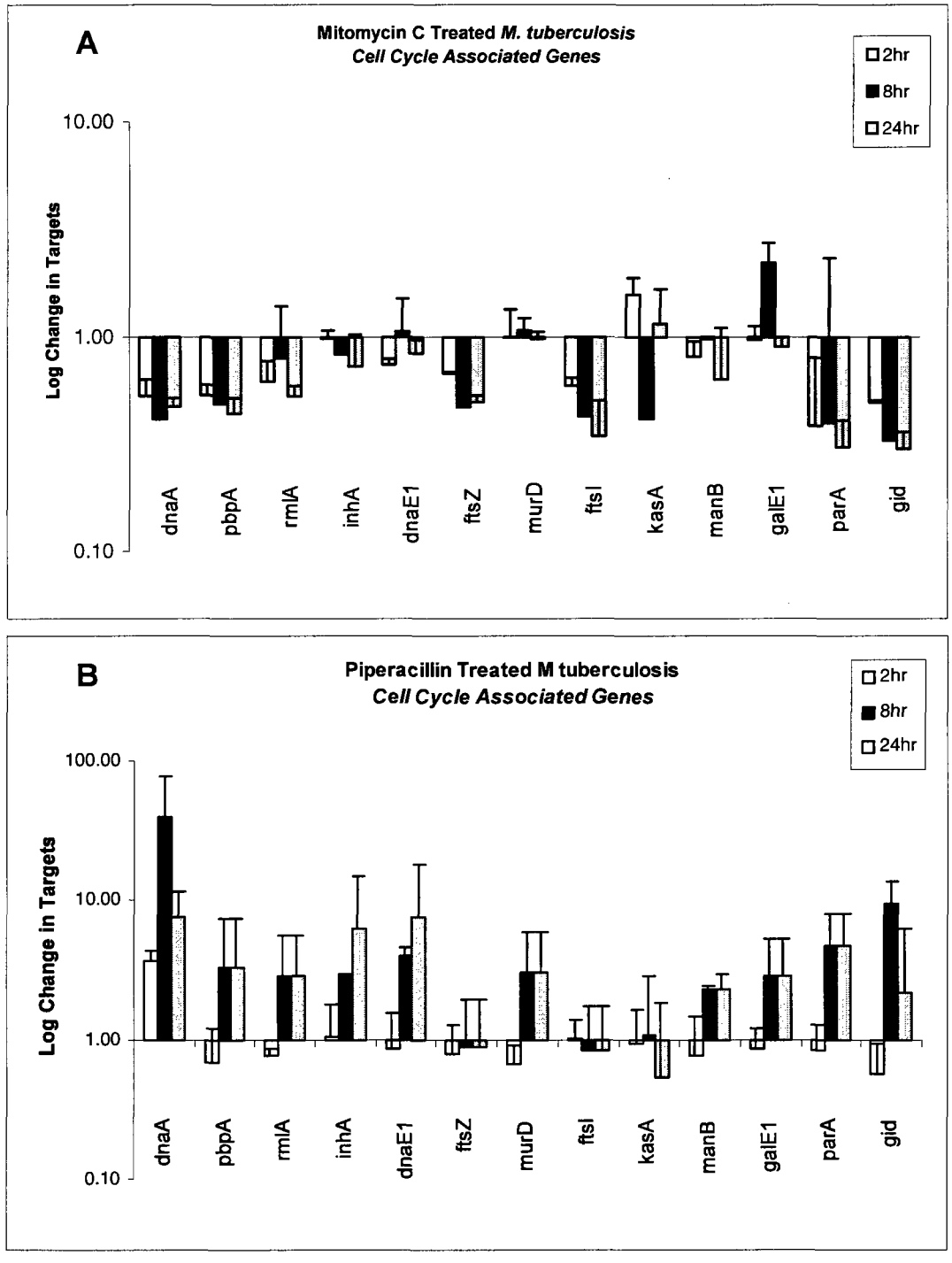

Figure 5.2 Real-time PCR analysis of cell cycle genes for drug treated bacilli. The transcriptional expression of cell cycle associated genes from cultures of *M. tuberculosis* treated with (A) mitomycin C and (B) piperacillin were evaluated at 2, 8, and 24hrs of drug exposure.

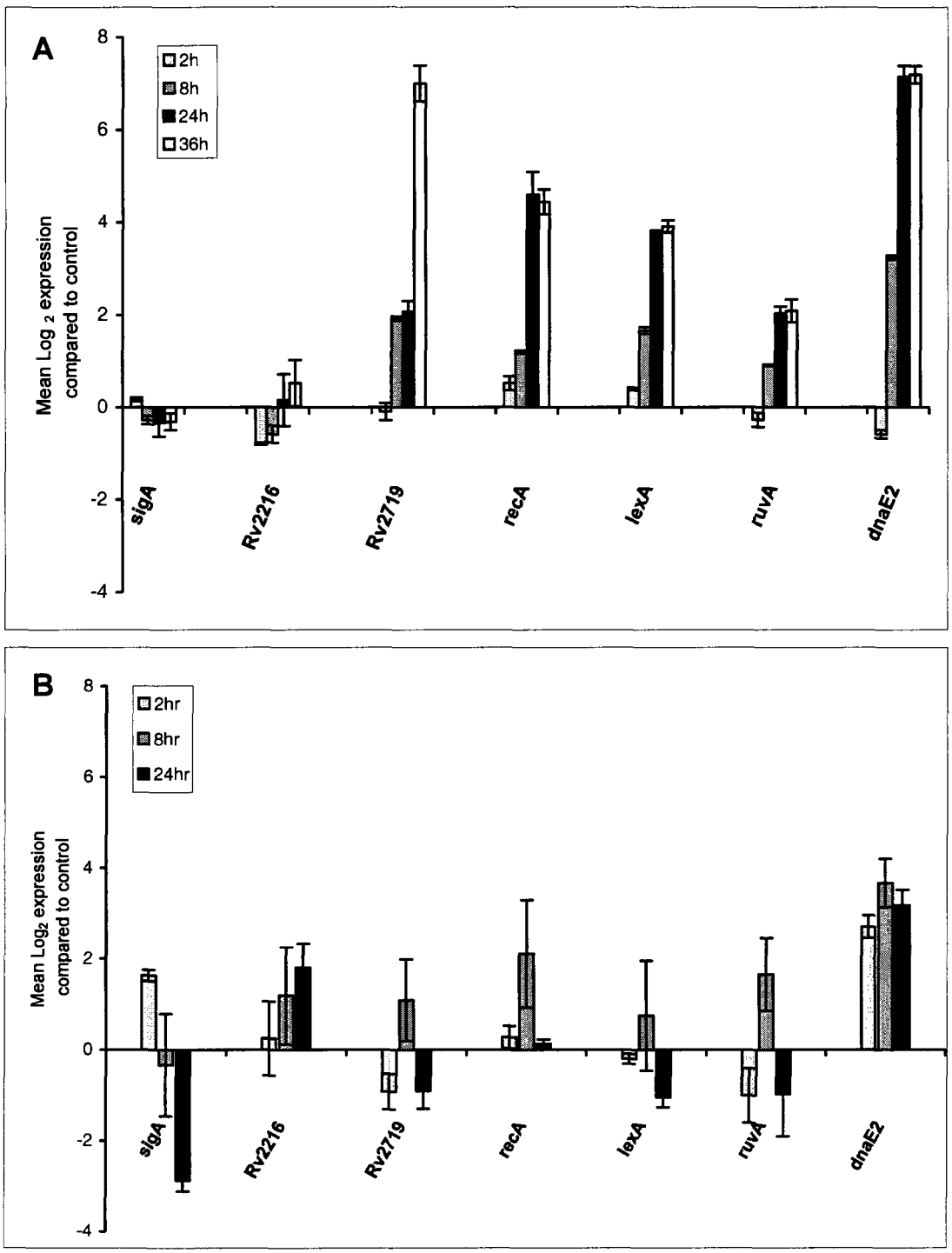

Figure 5.3. Real-time PCR analysis of SOS response genes for drug treated bacilli. The transcriptional expression of SOS response genes from cultures of *M. tuberculosis* treated with (A) mitomycin C and (B) piperacillin were evaluated at 2,8,24, and 36hrs of drug exposure.

and *recA,* show an initial decrease in expression as compared to the control, followed by an increase in expression at 8 hours and then a downregulation after 24 hours of FtsI inhibition. Importantly, the transcriptional response of $yneA_{Mtb}$ to mitomycin C treatment along with demonstrated inhibition of cell division supports the notion that $YneA_{Mtb}$ plays a role in the SOS response.

5.3.4 $yneA_{Mtb}$ expression inhibits cell division leading to filamentation

To assess the role of $YneA_{Mtb}$ in regulation of septum formation, the morphological effects of overexpression of $yneA_{Mtb}$ in *M. smegmatis* and *M. tuberculosis* were examined. Scanning electron microscopy revealed that $yneA_{Mtb}$ expression leads to the generation of filamentous cells for both recombinants strains *M. smegmatis* (Figure 5.4, C and D) and *M. tuberculosis* (Figure 5.4, G and H) as compared to vector controls (Figure 5.4, A and B or E and F). Interestingly, $yneA_{Mtb}$ overexpression resulted in a bimodal distribution of cell lengths between 1.5-2.5 μm and 3.5-5.5 μm (Figure 5.5). In addition to cell elongation, ultrastructural features indicative of septum formation were not seen and are consistent with the inhibition of FtsZ-ring formation. This finding has also been observed previously with septum inhibition in mycobacteria [22, 54, 55].

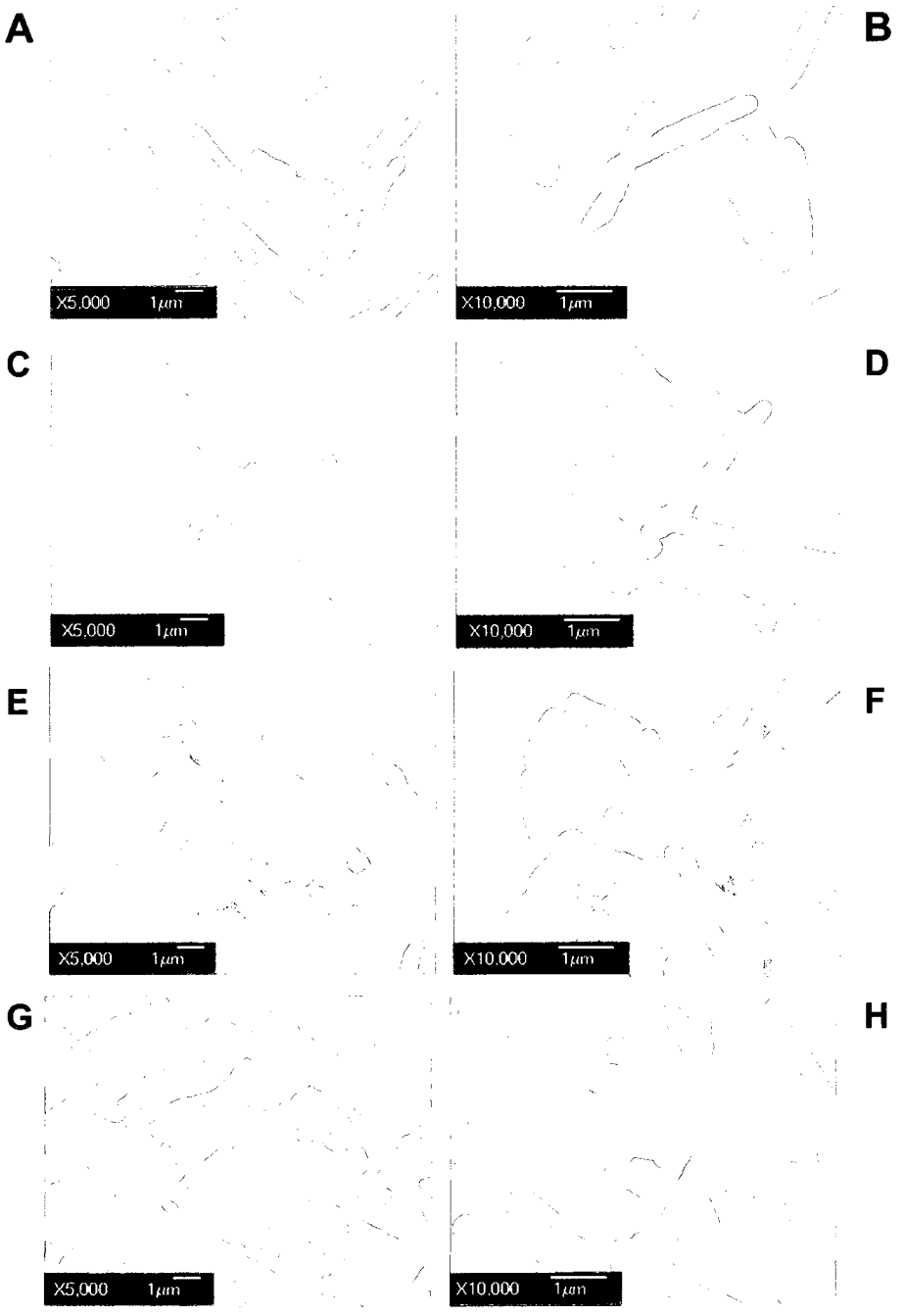

Figure 5.4 Morphological analyses for induced expression of *yneA*$_{Mtb}$. Gene dosage experiments were done to assess morphology changes representative for cell division inhibition in mycobacteria. SEM images illustrate cell morphologies for (AB) *M. smegmatis* vector control, (CD) *M. smegmatis*: pvv16-*rv2216* (EF) *M. tuberculosis* vector control, (GH) *M. tuberculosis*: pvv16-*rv2216* at 5000x and 10,000x magnification.

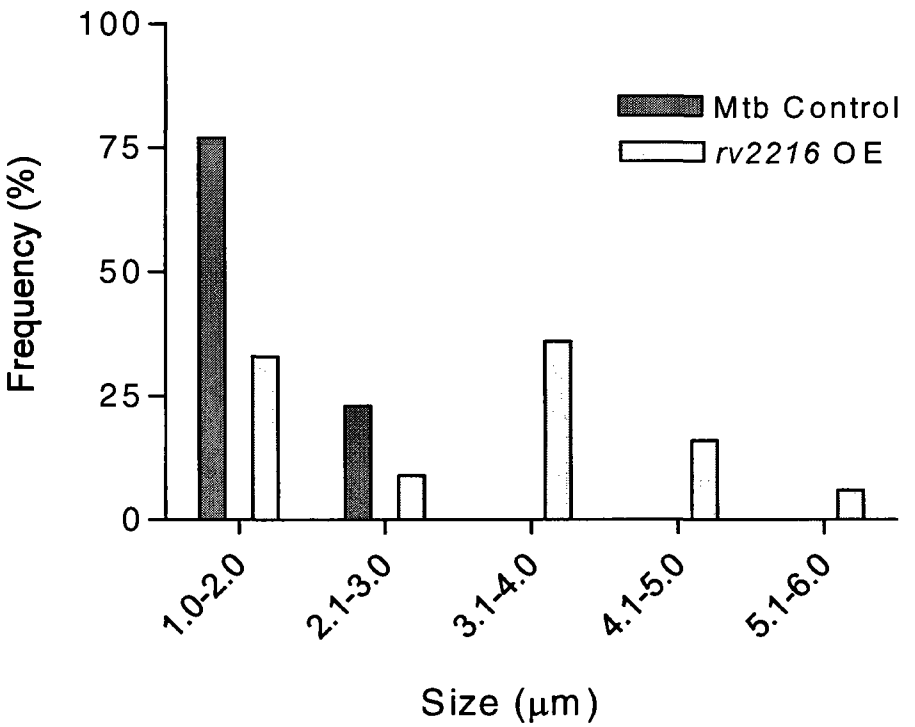

Figure 5.5 Frequency distribution of cell length for *yneA*$_{mtb}$ merodiploid. Cultures were taken at mid-log growth and visualized by SEM. Lengths were calculated from coordinates of both ends as measured by SEM. Multiple fields were examined and values are reported in 1μm increments to achieve evaluation of greater than 100 bacterial cells.

5.3.5 Real-time PCR of cell cycle genes from induced *yneA*$_{Mtb}$ expression

Overexpression of *yneA*$_{Mtb}$ repressed transcription of genes encoding proteins involved chromosome replication (*dnaA, dnaE1*) and partitioning (*parA*), along with *ftsZ* the initiator of septum formation. Induced gene expression was noted for genes involved in septum resolution and cell wall synthesis (*ftsI, pbpA, murD, galE1, and kasA*) (Figure 5.6). This indicates a mechanisms for inhibition through the regulation of FtsZ-ring assembly as described for YneA orthologs [56]. Increased *ftsI* expression and other cell wall synthesis genes represent increased activities in cell wall production for cell elongation as demonstrated in the scanning electron micrographs (Figures 5.6, G and H).

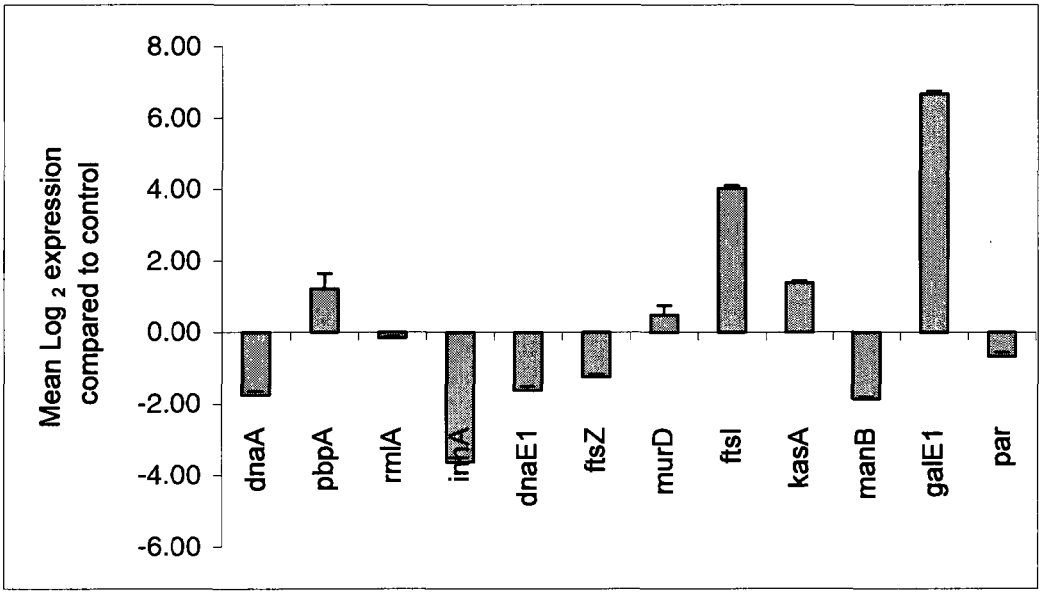

Figure 5.6 Real-time PCR of cell cycle discriminant genes from overexpression of *yneA*$_{Mtb}$. Transcriptional data obtained from induced production of YneA$_{Mtb}$ illustrates a shutdown of genes involved in replication and division events.

5.3.4 Expression of *yneA*$_{Mtb}$ results in the induction of adaptive response genes.

The global transcriptional response of *M. tuberculosis* upon overexpression of *YneA*$_{Mtb}$ revealed broad metabolic changes associate with cessation of cell division and adaptation to altered growth. A total of 1,048 ORFs (SNR>2, p value $\leq$0.05) were identified as transcriptionally active, and 277 ORFs displayed a $\geq$ 2-fold change in expression (Appendix E). This represents an altered expression of ~7% of the encoded ORFs indicating global effects on cell cycle processes, which is consistent with values reported previously for inhibition of septum formation [54]. Genes found in functional classes associated with hypothetical and conserved hypothetical proteins along with intermediary metabolism and respiration were the most differentially regulated (Figure 5.7). Interestingly, several of the upregulated genes were found in association with dormancy conditions such as hypoxia (33%) and nutrient starvation (33%) as well as induction of genes found in the *dosR* regulon (33%), (Table 5.1 and 5.2). In further analysis, 41% of these genes were also associated with the EHR response previously reported by Rustad et al. (2008) [2]. It is important to note that since *yneA*$_{Mtb}$ was overexpressed and not innately induced by DNA damage or stress conditions there are no SOS related gene elements differentially regulated.

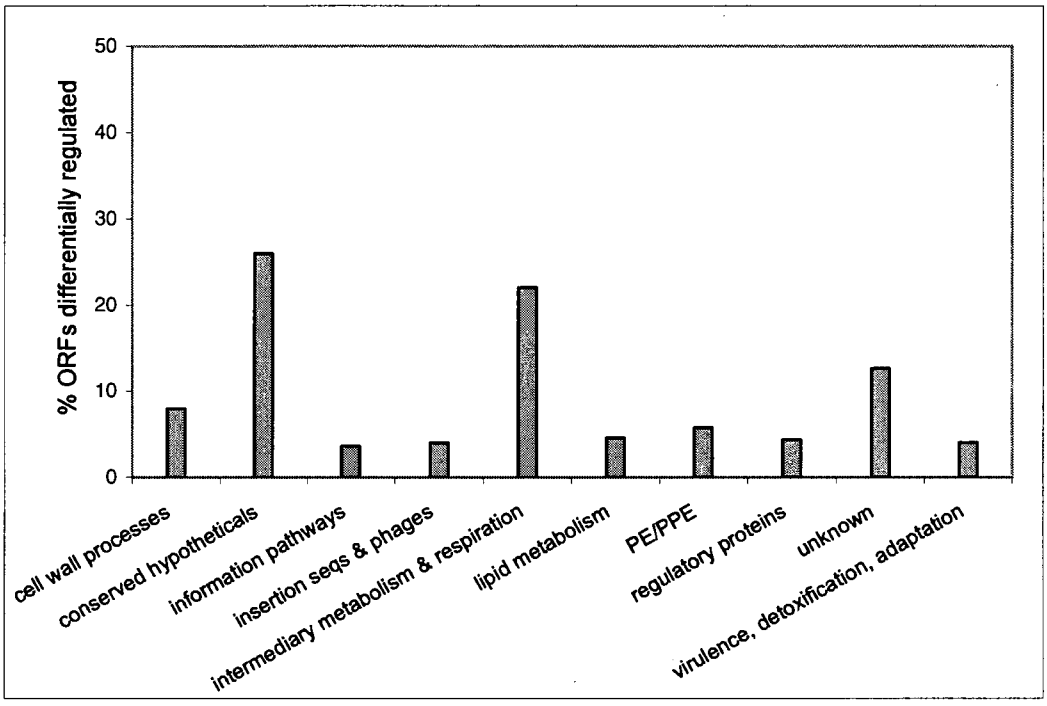

Figure 5.7 Functional classification of genes differentially regulated from the overexpression of *yneA*$_{Mtb}$. Induced production of $YneA_{Mtb}$ in *M. tuberculosis* significantly regulated gene expression for genes found in the functional categories of conserved hypotheticals, intermediary metabolism and respiration, and unknowns. Many of these genes are noted as part of adaptive responses to low oxygen, nutrient starvation, and dormant state conditions (*dosR*-regulon).

Table 5.1 Transcriptionally active genes from induced expression of *yneA*$_{Mtb}$. Significantly differentially regulated ORFs demonstrating a 2-fold change in expression involved in adaptive responses associated with intracellular life and latency conditions.

ORF	Gene	Gene product	Mean Log_2 expression	p value
Rv3189		conserved hypothetical protein	3.66	0.0001
Rv0791c		possible monooxygenasemonoxygenase	3.63	0.0151
Rv1997	ctpF	probable cation transport ATPase	3.03	0.0004
Rv2032		conserved hypothetical protein	3.01	0.0004
Rv3131		conserved hypothetical protein	2.88	0.0111
Rv2662		hypothetical protein	2.86	0.0002
Rv2624c		conserved hypothetical protein	2.85	0.0002
Rv3841	bfrB	bacterioferritin	2.83	0.0013
Rv2016		hypothetical protein	2.76	0.0334
Rv2659c		phiRV2 integrase	2.75	0.0252
Rv3127		conserved hypothetical protein	2.70	0.0009
Rv0140		conserved hypothetical protein	2.65	0.0439
Rv3287c	rsbW	anti-sigma B factor	2.62	0.0025
Rv2626c		conserved hypothetical protein	2.55	0.0327
Rv0563	htpX	probable (transmembrane) heat shock protein	2.41	0.0451
Rv0570	nrdZ	ribonucleotide reductase, class II	2.40	0.0050
Rv2630		hypothetical protein	2.30	0.0522
Rv1585c		phiRV1 phage related protein	2.26	0.0009
Rv2629		hypothetical protein	2.20	0.0079
Rv0064		possible membrane protein	2.12	0.0068
Rv1471	trxB	thioredoxin reductase	2.07	0.0439
Rv3288c		conserved hypothetical protein	2.07	0.0001
Rv0678		hypothetical protein	1.95	0.0185
Rv2466c		conserved hypothetical protein	1.91	0.0108
Rv3130c		conserved hypothetical protein	1.83	0.0031
Rv2913c		probable D-amino acid aminohydrolase	1.81	0.0000
Rv3327		hypothetical protein	1.78	0.0411
Rv3133c		two-component response regulator	1.78	0.0040
Rv3334		transcriptional regulator (MerR family)	1.57	0.0125
Rv2031c	hspX	14kD antigen, heat shock protein Hsp20 family	1.56	0.0131
Rv3543c	fadE29	acyl-CoA dehydrogenase	1.48	0.0123
Rv2030c		conserved hypothetical protein	1.46	0.0271
Rv3290c	lat	lysine-[epsilon] aminotransferase	1.42	0.0159
Rv1809	PPE	PPE-family protein	1.40	0.0070
Rv3270	ctpC	cation transport ATPase	1.37	0.0059
Rv2497c		pyruvate dehydrogenase E1 component	1.32	0.0008
Rv1813c		conserved hypothetical protein	1.25	0.0002
Rv1874		hypothetical protein	1.24	0.0085
Rv1957		hypothetical protein	1.23	0.0022
Rv2465c	rpi	phosphopentose isomerase	1.23	0.0345
Rv1128c		REP-family protein	1.20	0.0073
Rv2028c		conserved hypothetical protein	1.19	0.0022
Rv1875		conserved hypothetical protein	1.14	0.0470
Rv3088		conserved hypothetical protein	1.12	0.0451

Rv2495c	pdhC	dihydrolipoamide acetyltransferase	1.11	0.0068
Rv1082		similar to S. lincolnensis lmbE	1.07	0.0265
Rv3545c		cytochrome p450	1.03	0.0477
Rv1733c		possible membrane protein	1.03	0.0004
Rv1152		transcriptional regulator (GntR family)	1.02	0.0001
Rv2499c		putative aldehyde dehydrogenase	1.01	0.0363
Rv2399c	cysT	sulphate transport system permease protein	1.0	0.0000
Rv2412	rpsT	30S ribosomal protein S20	-1.0	0.0297
Rv3864		conserved hypothetical protein	-1.16	0.0388
Rv0745		conserved hypothetical protein	-1.18	0.0071
Rv3597c	lsr2	conserved hypothetical protein	-1.25	0.0007
Rv2450c		conserved hypothetical protein	-2.07	0.0476
Rv2386c	trpE2	anthranilate synthase component I	-2.74	0.0130

Table 5.2 Summary (%) of genes induced in adaptive response categories. Induced production of $YneA_{Mtb}$ in the cell upregulated genes in adaptive response categories associated with low oxygen conditions, nutrient starvation, and latency as previously reported [1-4].

Latency associated	*Low oxygen (1%)*	*dosR regulon*	*Nutrient starvation*	*EHR Response (7 day)*	*Total genes induced and associated with NRP and Latency*
6	19	17	17	23	41
12%	33%	33%	33%	39%	71%

5.4 DISCUSSION

One of the most important yet least understood aspects of *M. tuberculosis* physiology is how cell cycle processes are coordinated. It is known that cell cycle regulators exist to regulate cell cycle processes in times of stress for other bacteria; however, few checkpoints have been described during normal or adaptive growth for *M. tuberculosis* [20, 36, 38, 57-59]. Investigational searches of putative regulators can provide critical information toward developing an understanding of complex regulatory networks employed by *M. tuberculosis*. Therefore, we designed a series of experiments to delineate the function of the *rv2216* gene product, which bioinformatic analysis identified as a putative YneA ortholog. YneA is a well-established modulator of cell division in response to bacterial stress in other Gram-positive bacilli [20, 60, 61]. Thus, the existence of YneA-like proteins in *M. tuberculosis* that regulate septum formation and cell cycle processes under stress conditions are likely. Specifically, the YneA-family of proteins consists of the 300 amino acid YneA-family members involved in the inhibition of cell division and the 160 amino acid YneA-family members that inhibit FtsZ polymerization during the SOS response. The size of *rv2216* (~300 amino acids) provided the first indication that this particular gene product may play a role in governing cell cycle progression in addition to stress responses.

Treatment with antibiotics like piperacillin, a known inhibitor of the cell cycle protein FtsI, causes an induction of the SOS response, leading to the stall in cell division observed following drug exposure [21, 24, 62, 63]. Visualization of *M. tuberculosis* following inhibition of FtsI or induction of the SOS response revealed important ultrastructural features. First, as predicted, both treatments led to the

generation of filamentous cells. FtsI inhibited cells displayed a single concentric ring at midcell after 1 day of exposure, indicative of a present but unresolved septum. However, further exposure to piperacillin did not increase the number of concentric rings observed along the bacterial filament. The discontinued formation of concentric rings as a result of extended incubation with piperacillin provides evidence that continued filamentation does not result from inhibition of FtsI. Rather, a regulatory element that is responsive to completion of cell division prevents formation of additional septa, leading to elongated bacteria with generally smooth morphologies. In contrast to FtsI inhibition, treatment with mitomycin C led to the early generation of extremely filamentous cells with smooth morphologies. In accordance with this observation, previous work has shown that induction of the SOS response inhibits the polymerization of FtsZ, which results in smooth filaments lacking septal rings [35, 36, 64]. The initial macrostructure of these two treatments, in which septal rings are present immediately following FtsI inhibition and absent during induction of the SOS response, substantiates the notion that inhibition of FtsI does not produce filamentous cells by inducing the SOS response. Alternately, these results indicate that the observed cell elongation of *M. tuberculosis* that occurs following treatment with both agents results from different regulatory mechanisms. This argument was also validated based on the differing transcriptional profiles of sets of discriminant genes for both the SOS response and the cell cycle. These finding showed that although the SOS response is transiently expressed following piperacillin treatment, the response of *M. tuberculosis* to each of these agents is clearly different. Furthermore, the transcriptional activity of $yneA_{Mtb}$ to mitomycin C treatment suggest a role for this

protein in the SOS response, and is most likely responsible for regulating cell division similar to other YneA-family proteins.

Historically, cell cycle control under normal growth conditions was considered to be unique and separate from mechanisms associated with adaptation to stress such as the SOS response. Accordingly, there are significant efforts toward defining the processes associated with the establishment of non-replicating persistent states of growth for *M. tuberculosis*. The *M. tuberculosis* genome encodes a large number of regulatory elements, which hinders efforts to define adaptive strategies. The transcriptional response and the observed ultrastructural characteristics in mitomycin C and piperacillin treated bacteria establish that $YneA_{Mtb}$ is involved in stress-associated responses. $YneA_{Mtb}$ also demonstrated involvement in regulating cytokinesis via prevention of septum formation prior to completion of the previous round of cell division. In this study, the filamentous phenotype typically identified with the inhibition of cell division associated with DNA damage, demonstrates an adaptive response profile to an alternative program designed to promote bacterial survival. This infers that $YneA_{Mtb}$ has either of two roles: 1) inhibition of septum formation and cell division, or 2) induction of gene regulons involved in adaptation to dormant state conditions (or that it is a part of a domino effect in which the development of filamentous cell morphology triggers the induction of genes associated with adaptive responses).

There is an accumulation of evidence supporting the role of filamentation in pathogen survival against environmental stress, phagocytosis, predation, and enhanced virulence and pathogenesis [12, 18, 19, 21, 22, 24, 56, 62, 63, 65-70]. However,

initiators involved in transformed morphology are still unknown. Suggested causes for altered morphology involve responses to intracellular stress, nutrient limitation, altered metabolism, or an attribute of virulence. An organism like *Candida albicans* is known for the ability to alter its morphology, which is thought to be required for different stages of pathogenesis [69]. Antibiotic-induced *B. pseudomallei* filaments have demonstrated alterations in adherence and phagocytosis, which prevent internalization of the bacterium and thus inhibits intracellular killing by activated host cells [62]. In addition, reversion of these treated filaments demonstrated enhanced antibiotic resistance and altered virulence, which could enhance disease severity with discontinued therapy. Studies on UPEC filamentation during infection provide evidence that altered morphology serves as a mechanism to evade host innate immune responses [18, 19]. Inactivation of *sulA* in UPEC distinctly attenuated virulence in mice, suggesting that filamentation is a critical evasive tool against the innate immune response and required for bacterial survival. Other hypotheses suggest that components of the SOS response may serve as signals in lethal environments that direct transcriptional and morphological changes to ensure pathogen survival [18].

Previous work by Chauhan et al. (2006) reported the transformation of *M. tuberculosis* bacilli into filamentous bacteria lacking septum after phagocytosis by macrophages [56]. This change in morphology is suggested as advantageous for intracellular survival. Because inhibition of cell division can also lead to resistance to antimicrobials, it is possible that the clinical need for prolonged antituberculosis therapy is due to the existence of intracellular drug-resistant filaments.

It is evident from previous work that there are many components involved in regulating cell division that induce filamentation. However, the morphological transition seems to be a multifaceted survival strategy. Thus, inhibiting regulatory elements involved in filamentation are presumable good drug targets. Antifilamentation agents directed against *M. tuberculosis* persistent bacilli may provide supportive therapy for current drug regimens.

Based on bioinformatics, it appears that Rv2216 originated from a YneA-like protein with a cell division regulatory function. Accordingly we have deemed the *rv2216* gene product as $YneA_{Mtb}$. This study identifies a YneA-like protein member in *M. tuberculosis* as having structural and transcriptional responses consistent with septum inhibition. More important, this study alludes to a unique association with septum inhibition and the induction of genes responsible for the survival and adaptation of bacilli under dormancy conditions. Significantly, this report is in agreement with a newly evolving theme in which cellular components adapt to serve as novel regulatory "check points" during the progression of the bacterial cell cycle and in the case of *M. tuberculosis*, can lead to innovative adaptive survival programs.

5.5 ACKNOWLEDGEMENTS

This work was support by RO1 AI055298 (RAS). We gratefully acknowledge Dr. Philip Chapman for assistance with the statistical analysis and Ms. Laurel Respicio, Ms. Melissa Boyne, and Ms. Kerry Brookman for technical assistance. We recognize the Rocky Mountain Regional Center of Excellence for providing microarray analysis resources. We acknowledge the post-genomic resources and services provided by the Rocky Mountain Regional Center of Excellence (U54 A1065357)

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CHAPTER 6

Final Discussion and Concluding Remarks

6.1 FINAL DISCUSSION

A few decades after the antibiotic era and early antimicrobial drug development, there was a common belief that diseases caused by microorganisms were soon to be a part of history. Nearly seven decades later, infectious disease remains an ever-present, evolving, and challenging threat to human health. Currently, infectious diseases are the second-leading cause of death in the world, directly linked to nearly fifteen million deaths annually [1].

Several factors contribute to the demand for new antimicrobials (or anti-infectives) which include 1) the continued development of drug resistance, 2) the abuse and misuse of current antimicrobials, 3) the re-emergence of pathogens considered controlled or diminished, 4) the emergence of newly identified pathogens, 5) the global climate changes that provide new habitats and promote the evolutionary success of organisms through adaptation, 6) the changes in populations and social factors that promote transmission and spread, and 7) the increased threat of bioterrorism [2]. A rise in incidence of established communicable diseases like tuberculosis is due to the lack of treatment and poor managed care in the developing world, increases in drug resistance,

and socioeconomic factors. In the years 1999-2003, we witnessed the rapid migration of a nonendemic disease (West Nile) into the western hemisphere [3, 4]. Emergent and re-emergent diseases (Ebola fever, SARS, *Leptospirosis,* Plague, Nipah virus, H5N1 influenza, Cholera) have encroached into human populations, due to human expansion into new geographic regions, consumption of exotic animal products, the domestication of exotic pets, global environmental changes, the aftermath of war, and a rise in population placing demands on infrastructures of public health, sanitation, and overall living conditions within communities primarily in the developing world [1, 2, 5, 6]. Additionally, the threat of biowarfare or bioterrorism with resistant or engineered bioagents has created a new risk leading to the demand for alternative chemotherapeutics for currently treatable diseases or those of rare incident infections. Thus, the development of innovative chemotherapies with alternative mechanisms of action and novel drug targets is required to keep up with the continuous and enduring battle between microbe and man.

The research outlined in this dissertation utilized rational drug design strategies to direct drug discovery research for two intracellular pathogens, *F. tularensis* and *M. tuberculosis.* Structure-based design methods were employed to find a lead compound active against select agent *F. tularensis.* Tularemia has recently been brought into drug discovery research efforts because its etiologic agent, *F. tularensis,* was given Category A select agent status. Current recommended treatments for *F. tularensis* have several drawbacks 1) the most effective drugs are delivered parenterally and have ototoxic and nephrotoxic effects, 2) many of the recommended drugs used for treatment have issues concerning relapse of disease upon withdrawal of treatment, and 3) there are concerns as

to the existence and potential use of drug-resistant strains engineered under past or present bioweapons programs. Secondly, the use of genomic strategies aided in identifying unique targets involved in regulating cell cycle events in *M. tuberculosis*. Tuberculosis is a longstanding public health burden and the organism has complex adaptive strategies that have pushed the limits of current treatment programs and created difficult challenges for new drug design. Current treatment regimens for tuberculosis have become less effective due to the extensive use of these drugs for other infections, poor patient compliance during treatment, the length of the treatment, drug toxicity issues, increased development of resistance, and the fact that there are few compounds in these treatment profiles that can eliminate latent TB bacilli. The focus of my research has been the identification of alternative drug targets and new effective compounds for these two pathogens.

6.1.1 Diphenyl ethers: a broad-spectrum alternative for priority pathogens

The FASII system has been recognized as an attractive target for novel antibacterials [7-18]. Fatty acids are essential for bacterial growth, and they are not scavenged from the host, but are synthesized *de novo*. The enzymes of the FASII system are highly conserved across many bacteria and are very distinct from the multienzyme FASI-complex found in mammals. Compounds targeting enzymes in this pathway offer a conserved mechanism of action leading to prospective broad-spectrum therapy. My research has expanded on previous work targeting the enoyl reductase enzymes, FabI, in the FASII lipid biosynthesis pathway.

Utilizing a library of substituted-diphenyl ether compounds designed from crystallographic and enzyme inhibition studies for the enoyl reductase enzyme, ftuFabI, of *Francisella tularensis,* we identified lead compound SBPT04. This work demonstrated SBPT04 to be a selective, potent, and effective compound *in vitro* and *in vivo* with suitable bioavailability and limited cellular toxicity. Properties associated with *in vivo* activity include tight-binding enzyme inhibition with enhanced residence time, bactericidal activity effecting cell metabolic functions and overall cell viability, low minimal inhibitory concentrations that are effective substantially below toxic cell concentrations, and single dose concentration-dependent killing. Attributes regarding stability were defined from *in vitro* metabolism studies using liver microsomes. Limited Phase I metabolism by human liver microsomes suggests that SBPT04 will have a higher bioavailability with an increased half-life prolonging antibacterial efficacy at lower doses for human treatment of *F. tularensis.* In addition, Phase II metabolism was defined as the major eliminator of active compound from circulation. This information is critical in order to optimize and remodel SBPT04 to bypass Phase II metabolism. The most important finding was that the treatment of pulmonary tularemia in mice for a limited duration of 5 days by i.p. injection (200mg/kg) cleared infection with no signs of relapse for over a 30 day period. In addition, survival rates by oral delivery at the same concentration suggest that SBPT04 is an oral drug candidate that can be improved with alterations, either in structure to enhance bioavailability and limit metabolism, or in formulation. Importantly, this research substantiates the use of substituted diphenyl ethers as broad-spectrum compounds active against FabI enzymes, and affords the opportunity to target other bacterial pathogens. With our current understanding of the

mechanistic action of inhibition, active site molecular specificities, the significance of residence time, relative bioavailability, and the general metabolism of these compounds, substituted diphenyl ethers have proven suitable for development as a platform for broad-spectrum chemotherapeutic alternatives.

6.1.2 Cell division regulators: novel drug targets for tuberculosis

TB drug discovery efforts today are primarily focused on the identification of new drug targets involved in alternative cellular activities, particularly those associated with latent TB bacilli. Most of the current TB drugs target cell wall biogenesis with a few exceptions. My research focuses on regulatory elements of cell division that may have control over bacilli at critical phases in cell cycle events, which may lead to nonreplicating persistence or reactivation.

Many research groups designing inhibitors for cell division have focused on targeting the primary initiator of cell division, FtsZ, a protein that sets the stage for septum formation and promotes a cascade of events leading to cytokinesis [19-25]. Inhibitors of FtsZ polymerization and ring formation at the midcell prevent cell cycle progression, inducing cell death. Regulators of septum formation and FtsZ polymerization are therefore considered unique target candidates for drug discovery.

The research presented here focused on two cell cycle regulatory systems that are well defined in other bacteria, the Min-system for regulating septum placement and the SOS response which regulates cell progression under DNA damage conditions. Specific regulatory elements in these two systems have not been defined in *M. tuberculosis*. Our research identified and characterized of two putative regulators of cell division, $MinD_{Mtb}$

and $YneA_{Mtb}$, through the use of bioinformatics and gene dosage experiments. These experiments provided signature cell morphologies associated with an inhibition of septum formation and cell division events. In addition, gene expression profiles suggesting that these regulators play a role in the induction of adaptive responses related to intracellular survival.

Bacterial mechanisms of damage control under induced stress during infection are not yet understood for *M. tuberculosis*. This research identified a YneA-like protein noted for involvement in stress responses for other Gram-positive bacteria [26-28]. YneA is a component of an SOS induced response in other bacterial species, and is a known cell division inhibitor. Although various works on SOS response genes in tuberculosis have identified several components, there are limited reports on specific cell division inhibitors induced under stress that control cell cycle events. This work identifies a protein encoded by *rv2216* ($YneA_{Mtb}$) with roles in both regulating cell division and inducing a program for adapting to stress.

Induction of the SOS response by mitomycin C and piperacillin treatment resulted in elongated cells demonstrating a delay in cell division. Transcriptional analysis by QRT provided evidence for the increase in expression of $yneA_{Mtb}$ under both treatment conditions. The results of these experiments supported the hypothesis that Yne_{Mtb} has a definite role under stress-induced conditions. In fact, it appears to have a role not only in the DNA damage SOS stress response, but regulates division events under other elements of stress, (i.e. damage due to drug treatment). Global array analysis of gene expression from gene dosage experiments also indicates decreased expression of elements involved in septum formation and cell division, along with increases in gene expression for

adaptive responses associated with hypoxia and dormant state conditions. However, it remains undetermined whether the protein is involved in regulating these responses or the act of inhibiting cell division promoting cell elongation induces these adaptive responses.

This research also provides experimental evidence of a MinD-like protein that controls septum formation and cell division while inducing transcriptional responses relating to dormancy state conditions with an alternative global regulation program. Induced and repressed expression of $MinD_{Mtb}$ demonstrates typical morphologies associated with septum regulating proteins. Transcriptional analyses report a unique profile involving the upregulation of *dosR* genes and alternative sigma factors σFGHIJLM, suggesting a role for this protein in the induction of nonreplicating adaptive responses associated with stress, and conditions required for intracellular survival and pathogenesis.

Notably, both regulatory elements $MinD_{Mtb}$ and $YneA_{Mtb}$ demonstrated a role in preventing cell division, and induced transcriptional responses programmed for adaptation to stress conditions associated with intracellular life, disease pathogenesis, and survival for *M. tuberculosis*. The primary questions that arise are, "Are these responses a result of the inhibition of cell division and filamentation?" "Is this protein a regulator involved in programming the induction of a nonreplicating persistent state?", and "Does the induced expression of this protein cause stress within the cell that activates adaptive transcriptional responses?"

There is increasing evidence supporting the role of filamentation in bacterial survival during environmental stress, phagocytosis, predation, virulence, and pathogenesis [29-40]. Studies have demonstrated that UPEC filamentation is a

mechanism used to evade the host innate immune response [35, 36]. Filamentation enhanced the survival of Salmonella species under altered environmental conditions [29]. Research involving protist grazing models demonstrated that filamentous bacteria are resistant to predation and phagocytosis [32]. These models have been used to study intracellular pathogens that infect humans, as many of them can undergo filamentation under *in vitro* stress conditions similar to host conditions or upon phagocytosis [33]. Adaptation via filamentation has also been noted after antibiotic treatment [31, 34, 39]. Even studies by Wayne and Hayes identified increased cell lengths for *M. tuberculosis* under low oxygen conditions during the shiftdown into nonreplicating cells [38]. It is uncertain in these experiments as to whether filamentation is induced by intracellular stress, nutrient limitation, altered metabolism, or an action of virulence. Most likely, filamentation was a primitive adaptive mechanism required for survival. The program for induced filamentation may have evolved various elements of regulation over time to be triggered under different conditions. Selective regulation would offer a tight control as the organism may have acquired other ways to handle various elements of change. These selective regulators may have alternative actions that promote the transcription of unique sets of genes required for specialized responses.

Our experiments found regulatory elements that induce filamentation and provide different genetic programs in order to adapt and cope with different elements of stress in *M. tuberculosis*. Questions that now arise are "What induces the transcription of these regulatory elements?" "Is this morphology change advantageous for intracellular survival and persistence?" and "What role does filamentation play in TB drug resistance?"

It is postulated that the need for extended TB therapy is due to the formation of intracellular drug-resistant filaments, i.e. nonreplicating persistent bacilli. Inhibiting regulatory elements that induce filamentation could provide a unique set of drug targets for *M. tuberculosis*. Agents controlling events of cell division in *M. tuberculosis* may provide supportive therapy for current drug regimens by preventing cells from entering a dormant state or controlling reactivation events.

6.2 FUTURE DIRECTIONS

6.2.1 Diphenyl ether actives: future development

Efforts for the optimization and development of compounds like SBPT04 or other diphenyl ether analogs into broad-spectrum alternatives for many bacterial pathogens are promising. Typically, the primary goal is to have an orally bioavailable drug that has limited toxicity with single daily dosing over a short period of time. Although issues of solubility, bioavailability, and long term toxic effects may prove problematic for some compounds, there are several ways to approach these challenges. One option would be to develop a "prodrug" that is activated at a crucial phase of metabolism to establish higher levels of active compound at the site of infection. Another option would include the use of alternative formulations to increase bioavailability and absorption for a more effective oral therapeutic.

Today, there are various approaches to the formulation and to the delivery of drug compounds with poor ADME properties. Many are designed to directly deposit active drug at the primary site of infection, or offer slow continuous release of treatment over time. Implants, nanoparticles, and alternative carrier-based delivery systems are

being investigated to target the site of infection, reduce dosing frequency or treatment duration, and eliminate problems associated with bioavailability [41, 42]. Current trends in drug delivery include the microencapsulation of compounds in biodegradable polymers, and carrier delivery systems such as liposomes, microsomes, and lipid based carriers in order to sustain delivery and improve absorption [41 , 43-45]. Utilizing these alternatives could enhance the development of active diphenyl ether analogs into highly effective alternative treatments. Therefore, future goals in the development of lead compound SBPT04 would be to investigate the possibility of designing a prodrug that will bypass Phase II metabolism, or to try alternative delivery methods that include inhaled aerosol-drug delivery, or to design an improved formulation in order to enhance oral bioavailability.

Additionally, studies are required to define the levels of overall animal toxicity, to identify dose-susceptibility relationships in order to establish dosing regimens, and to determine rates of elimination or excretion of the compound from the body. Furthermore, these compounds need to be assessed for efficacy in a more human-like infection model for *F. tularensis.* It is well noted that efficacy in mice is not always representative of the efficacy found under human infection conditions. And since pulmonary tularemia is rare, Phase III clinical trials on human patients are unethical. However, the FDA has established a "two-animal rule" instead of human Phase III trials in which animal testing can be considered sufficient to prove efficacy for new drugs against select agents. Even though there is much more work needed for the development of SBPT04 as a treatment for human infections, our initial work is extremely promising.

6.2.2 Targeting cell division: defining the mechanism and decoding the response

The identification of $YneA_{Mtb}$ and $MinD_{Mtb}$ as having regulatory functions in cell division with programmed adaptive strategies provides new insight into tuberculobacilli survival. It has yet to be demonstrated whether the actual events of septum inhibition and cell elongation stimulate adaptive responses or the actual presence of these proteins within the cell promotes adaptation. The most substantial outcome from this research is the enticing evidence that the establishment of nonreplicating bacilli (or slow replicating bacilli) may control or regulate events important for cell survival. Clearly, further experimentation is needed to verify this hypothesis and elucidate the precise roles these proteins play in *M. tuberculosis* survival and pathogenesis.

Future research should involve defining the interactions of these proteins that induce cell filamentation and stall cell division, in addition to verifying the activity associated with the established transcriptional responses. Protein-protein interaction studies and localization experiments can identify important circuitry that will define regulatory networks and define mechanisms involved in inhibiting cell division. Utilizing mutants in animal infections would provide evidence to the actual importance of these elements in pathogenesis and survival for the bacilli *in vivo*. If these regulatory elements are needed for the bacilli to establish a latent persistent infection, or adapt to harsh environments during infection, then an absence of these elements may promote a more rapid clearance by host immune responses due to the inability to turn on programmed stress adapters, thus resulting in attenuated virulence. Nonetheless, the identification of $YneA_{Mtb}$ and $MinD_{Mtb}$ and their connections with inhibitory activity of cell cycle progression may help to unravel regulatory circuitry involved in controlling

cell growth and allude to mechanisms associated with the establishment of latent persistent bacilli with adaptive strategies for a dormant survival.

6.3 CONCLUDING REMARKS

6.3.1 Drug design: moving out-of-the-box

Designing and optimizing inhibitors for FabI and identifying key components involved in cell division regulation will provide direction for future development of novel drug candidates with broad-spectrum use. However, there are several more stages involved in the development pipeline that could eliminate prospective leads. But that is the risk we take as drug discovery researchers. The question we must ask is how we can streamline this process to make if more efficient and cost-effective.

Utilizing genomic and structure based strategies for drug discovery can provide fruitful results along the path to drug development; however, these approaches have their shortcomings. First, target-based strategies depend on the protein-target as being essential for viability. The question is, "What is essential?" With pathogens like *M. tuberculosis* that can have various phenotypes and strategies for adaptive survival, genes that are deemed essential for growth *in vitro* are not necessarily what is required *in vivo*. Looking for growth essential gene targets is limiting. Pathogen genomes are stocked with genes required for life under a variety of conditions. Bacteria have evolved and continue to evolve in order to adapt to various lifestyles. These organisms insert and delete genes or induce selective mutations as they go through various modes of gene acquisition or genetic shuffling required for adaptive survival. All genes in the genome have a likely role in the disease processes at various stages, or are required at some point

for the organism's survival. Therefore, all genes are genuinely essential for life. Secondly, although target-based strategies may produce a lead compound with high affinity for the target and sufficient pharmacokinetic properties, it may still not be able to get to the site of infection or remain within the targeted microbe. Again, bacteria have been around for billions of years, evolving ways to eliminate toxic molecules and survive harsh environments. Efflux systems and enzymes that degrade or modify compounds are mechanisms bacteria utilize to eliminate toxins that can penetrate the selectively permeable cell wall. Also, interesting aspects of pathology like granuloma formation can add another dimension to effective delivery of the drug to the targeted site. Not only does the drug have to get into circulation, avoid metabolism, and move through tissues, but also granulomas establish new barriers that prevent the drug from getting to internalized bacilli. In addition, the center of the granuloma is known to have various environments often filled with a reactive intermediates, lipophilic acids, and mineralized tissue. These conditions add to the complexity of drug design for targeting latent bacilli.

Historically, most active antimicrobials have been identified through cell-based screening, thus the problem of choosing a druggable target and the issue of cell permeability is avoided. However, improving affinity, bioavailability and other ADME properties is difficult since the target and the mechanism of action are undefined. Evolving trends move beyond the methods of target-based and structure-based drug design in search for more efficient alternatives. Newer alternatives involve a more systems-type approach using large-scale databases and robust statistical methods to accelerate these processes. Identifying affected pathways or specific gene mutations through microarray gene expression profiling, rapid-whole genome sequencing, or

transcriptome analysis can define the mode of action for active drug compounds or illustrate specific pathways induced during infection and disease processes. Systems biology uses mathematical and computational approaches to understand specific aspects of disease, such as granuloma formation in *M. tuberculosis* infections [46]. Data obtained by these methods can aid in identifying key information about pathophysiology and immunoregulation related to a particular stage of disease. Understanding not only how the pathogen functions during actively growing conditions but defining disease specific host-pathogen interactions will provide a greater perspective of pathogen specific infections and define disease-related processes to target. Thus, drug discovery must move beyond conventional strategies that define targets as essential, selective, druggable, or that focus on specific structure-function relationships, and move "outside-the-box" to find alternative approaches to subverting microbes.

6.3.2 Combination therapy: the best solution

Challenges that accompany rapid infections involving highly virulent pathogens like *Francisella,* latent chronic infections such as with tuberculosis, or super drug-resistant infections of VRE, MRSA, and XDR-TB require researchers take a "backdoor" approach to drug design and treatment strategies. Combinational therapy is one way of utilizing existing drug arsenals in a "blitzkrieg" strategy, targeting multiple aspects of infection. Attacking pathogens from all sides – metabolic processes, virulence elements, cell replication and division events, altering resistance mechanisms (i.e. efflux inhibitors) with added directed immune modulators – may work like guerilla warfare, catching the bacterium "off guard", preventing it from eliciting a tactical defense. With the increase in

drug resistance and a developing understanding of phenotypic diversity within a bacterial population, attacking from various directions may be the best strategy to eradicate drug-resistant organisms and challenging infections posed by longstanding problematic diseases like *M. tuberculosis*.

The use of drug susceptibility testing to define treatment programs for each individual patient has shown promising results in the treatment of resistant organisms like *M. tuberculosis* [47]. Therefore with proper diagnosis and treatment, increases in resistance can be reduced and thus the threat of super bugs like MRSA, VRE, or MDR/XDR-TB prevented. Better management of therapy and disease control measures can reduce the need for high-powered drugs. For example, the rate of development and spread of drug resistant organisms (i.e. *M. tuberculosis*) depends on several factors, 1) the rate of mutation coding for resistance, 2) the portion of patients carrying resistant phenotypes, 3) the different survival rates of resistant and susceptible genotypes, which determine amplification and magnitude of infection, 4) the transmissibility of various genotypes, and 5) the portion of resistant strains that convert to active disease [47]. These factors determine case reproduction numbers and determine the fitness of various strains across the transmission cycle. Successful management of initial primary cases (either susceptible or resistant) limits reproduction numbers and opportunities for the bacteria to acquire additional resistance. Simply treating patients without profiling resistance increases the risk of the amplification of resistant strains in the bacterial populations. Clearly, treatment of resistant bacilli will be more successful with drugs to which these strains are more susceptible.

Drug combinations have proven to be most effective in preventing the development and spread of resistant bacteria [48-53]. Using two drugs with two different modes of action that work in synergy, decreases the probability of developing resistance mutations from 10^{-8} to 10^{-16} per generation [47, 54]. In regard to resistance development using combination therapy to treat TB, problems arise primarily due to poor treatment compliance due to the duration of treatment or development of toxic side effects, and poor managed care in developing countries. Both factors can increase the development, survival and spread of resistant genotypes, and increase the frequency and prevalence of resistant strains in a population. Historically, the sporadic appearance of resistance genes by mutation is inevitable and under the control of selective pressures. However, the spread of these resistant strains is preventable through proper treatment and well-managed care and control measures (DOTS).

6.3.3 Global drug regulations: preventing misuse and abuse

One of the most significant problems associated with infectious disease evolution is the mismanagement of health care and the abuse of currently available drugs. We have many drugs that work against bacterial infections, however, there is limited oversight globally in the management and availability of commonly used antimicrobials. Prescriptions for respiratory illnesses are handed out irresponsibly without proper determination if the cause of infection is viral or bacterial [55-57]. In developing countries, many broad-spectrum antibiotics are available over-the-counter with no restrictions [58-60]. The cyber age now provides easy access to a variety of drugs *via* the Internet through websites like www.epharmacies.com and www.247-pharmacy.com.

Internet trafficking of prescription drugs increases the possibility of unnecessary or in appropriate drug exposure to human infections, and thus an additional opportunity for the development of resistance [73]. In addition, poor quality drugs or less effective compounds are being sold in bulk at reduced prices to developing countries for the treatment of challenging diseases like *M. tuberculosis*, therefore, leading to increases in drug resistance [61].

The increased use of antibiotics on the farm has also lead to an increase in antibiotic resistant food borne illnesses. In developed countries antibiotics are routinely used to promote enhanced growth of livestock, and thus increasing the potential for making disease-causing bacteria more resistant to drugs, many of which are used for human infection [62-66]. In other words, people infected with bacteria from various food sources are at risk of developing a resistant infection. Currently, several developed countries including the United States are rethinking the use of powerful antibiotics in the food industry. The controversy lies in the health risks associated with untreated products verses the correlation with increased development of super resistant bacteria. However, the real battle is associated with the loss of capitol in the food industry, as restriction on antibiotic use would ultimately decrease overall food quality and production.

Hospitals in developed countries are becoming problematic as they have become cesspools for superbugs with resistance to top hitters like vancomycin or linezolid [67-70]. Studies have demonstrated that resistance rates are higher in bacterial isolates from in-patients than out-patients and multiple antibiotic resistance is linked to hospital cross-infection [54]. Additionally, doctors often use broad-spectrum antibiotics (sometimes more than one) to treat high-risk patients that enter the emergency rooms in order to keep

them alive for a lab to culture or get PCR results identifying the organism in order to make a proper diagnosis [71, 72]. Doctors are also advocates for their patients and not the global population. Therefore, if their patient is dying they have an ethical obligation to do all they can do, even if that means pull out the "big guns" for treatment. Multiply these scenarios by hundreds of thousands of patients and you have an overuse of powerful antibiotics. Without global government restrictions on the use and distribution of antimicrobials, we are faced with daunting challenges for infectious disease management and control, along with an urgent demand for novel chemotherapeutics.

6.3.4 Final statement

The discovery of antibiotics was a leap in modern medicine and the continued development of chemotherapy has been able to stop the growth or kill a variety of microorganisms. However, microbes have proven to be much more innovative and adaptive than ever imagined. Therefore, as modern life grows increasingly complex, humans and pathogens will continue to cross paths and evolve together. Continued efforts in drug discovery research, such as presented in this body of work, contribute to resolving current treatment challenges and promote additional research in the development of much needed alternatives for combating infectious disease.

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APPENDIX A

APPENDIX A: **Latency Drug Targets**: Murphy et al. (2007): Pathways and associated genes, which are potential TB dormancy phase targets.

Rv number*		Up-regula-tion Score	Down-regula-tion Score	Growth-attenua-tion Score	Gene ID	Protein ID	†Brief Annotation
devS/devT devR (dosR) Two component response regulator (dosR regulon)							
Rv3132c		13.815	-4.935	7.495	devS	NP_217648.1	sensor histidine kinase
Rv3133c		16.175	-3.085	0	devR	NP_217649.1	two-component response regulator [=1ZLK, =1ZLJ]
Rv2027c		3.47	-3.99	0	devT	NP_216543.1	sensor histidine kinase
mprAB 2 component response regulator							
Rv0981		13.21	-3.56	0	mprA	NP_215496.1	two-component response regulator [=1YS6]
Rv0982		8.14	-0.485	3.48	mprB	NP_215497.1	sensor histidine kinase [1YS3, 2C2A]
triacylglycerol biosynthesis							
Rv3130c		20.135	0	0	tgs1	NP_217646.1	triacylglycerol synthase
Rv3734c		3.635	-6.835	0	tgs2	NP_218251.1	triacylglycerol synthase
Rv3234c		1.8	-5.8	0	tgs3	NP_217751.1	triacylglycerol synthase
Rv3088		7.325	0	0	tgs4	NP_217604.1	triacylglycerol synthase
Rv1760		4.51	0	0		NP_216276.1	triacylglycerol synthase
Rv2285		0	-0.77	0		NP_216801.1	triacylglycerol synthase
Rv0221		1.995	-5.015	0		NP_214735.1	triacylglycerol synthase
Rv3740c		2.69	-1.675	0		NP_218257.1	triacylglycerol synthase
Rv3087		3.315	-4.785	4.64		NP_217603.1	triacylglycerol synthase
Rv3371		9.255	-2.515	4.945		NP_217888.1	triacylglycerol synthase
Rv3480c		0	-7.355	4.97		NP_217997.1	triacylglycerol synthase
Rv3233c		0	-8.12	0		NP_217750.1	triacylglycerol synthase
Rv1425		0	-2.66	0		NP_215941.1	triacylglycerol synthase
Rv0895		4.015	-2.575	0		NP_215410.1	triacylglycerol synthase
Rv2484c		2.04	-3.325	0		NP_217000.1	triacylglycerol synthase
pantothenate biosynthesis							
Rv1820		0.765	-0.35	0	ilvG	NP_216336.1	acetolactate synthase II [NP_006835, 5e-72, 2C31]
Rv3003c		3.185	-3.495	4.75	ilvB1	YP_177917.1	acetolactate synthase I large subunit [NP_036392, 2e-44,

							1JSC]
Rv3470c		8.27	-2.7	0	ilvB2	NP_217987.1	acetolactate synthase large subunit NP_006835, 4e-26, 1JSC]
Rv3002c		7.19	-6.545	4.275	ilvN	NP_217518.1	acetolactate synthase I small subunit [2FGD]
Rv3509c		2.065	-7.875	0	ilvX	NP_218026.1	probable acetohydroxyacid synthase I large subunit [1YNO]
Rv3001c		3.045	-6.77	3.7	ilvC	NP_217517.1	ketol-acid reductoisomerase [1NP3]
Rv0189c		4.415	0	3.37	ilvD	NP_214703.1	dihydroxy-acid dehydratase [2GP4]
Rv2225		9.315	-1.985	2.845	panB	NP_216741.1	3-methyl-2-oxobutanoate hydroxymethyltransferase [1OY0]
Rv2210c		0	-4.715	3.63	ilvE	NP_216726.1	branched-chain-amino-acid transaminase [NP_005495, 5e-61, 2COG]
Rv2573		2.22	-6.76	0		NP_217089.1	putative 2-dehydropantoate 2-reductase [2EW2]
Rv3602c		1.555	0	3.815	panC	NP_218119.1	pantoate-[beta]-alanine ligase [=1MOP]
Rv3601c		0	-2.135	0	panD	NP_218118.1	aspartate 1-decarboxylase [2C45]
Rv1092c		0	-6.31	8.025	coaA	NP_215608.1	pantothenate kinase [2GES]
Rv1391		2.205	-5.995	3.42	dfp	NP_215907.1	flavoprotein [NP_068595, 3e-15, 1U7U]
Rv2965c		2.22	-2.805	0	kdtB	NP_217481.1	lipopolysaccharide core biosynthesis protein [1TFU]
Rv1631		0	-5.93	3.61	coaE	NP_216147.1	conserved hypothetical protein [NP_079095, 3e-22, 1VHL]
Rv2523c		0	-4.875	0	acpS	NP_217039.1	CoA:apo-[ACP] pantethienephosphotransferase [1F7T]
Isoprene biosynthesis							
Rv2682c		0	-0.23	3.335	dxs1	YP_177898.1	1-deoxy-D-xylulose 5-phosphate synthase [NP_115512, 3e-17, 2O1S]
Rv1086		9.25	-0.6	0		NP_215602.1	conserved hypothetical protein [NP_079163, 6e-29, 1F75]

Rv3379c		9.465	-4.31	0	dxs2	NP_217896.1	unknown transketolase, interrupted by IS6110 [NP_115512, 7e-16, 2O1X]
Rv2870c		4.275	0	0	dxr	NP_217386.2	conserved hypothetical protein [=2C82]
Rv3582c		11.555	-1.47	4.565	ispD	NP_218099.1	conserved hypothetical protein [1VPA]
Rv1011		3.59	-5.15	3.165	ispE	NP_215527.1	conserved hypothetical protein [1UEK]
Rv3581c		10.78	0	3.795	ispF	NP_218098.1	conserved hypothetical protein [1H47]
Rv2868c		5.075	-4.515	0	gcpE	NP_217384.1	essential gene of unknown function
Rv1110		4.145	-2.335	0	lytB2	YP_177788.1	very similar to LytB
Rv3382c		0.13	0	0	lytB1	YP_177967.1	LytB protein homologue
Universal stress proteins							
Rv2005c		15.5	-1.28	0		NP_216521.1	conserved hypothetical protein
Rv2623		20.825	-2.29	2.9		NP_217139.1	conserved hypothetical protein
Rv3134c		19.29	-2.46	0		NP_217650.1	conserved hypothetical protein
Rv2624c		17.475	-0.185	0		NP_217140.1	conserved hypothetical protein
Rv1996		13.63	-2.71	0		NP_216512.1	conserved hypothetical protein
Rv2028c		9.605	-3.085	0		NP_216544.1	conserved hypothetical protein [1WJG]
Rv2026c		7.19	0	2.485		NP_216542.1	conserved hypothetical protein [|NP_079368, 4e-27, 1H54]
Rv1636		0.505	-7.255	0		NP_216152.1	conserved hypothetical protein [1TQ8]
Sulfate fixation							
Rv1285		17.68	0	4.255	cysD	NP_215801.1	ATP:sulphurylase subunit 2 [NP_003866, 6e-21]
Rv1286		12.78	0	3.755	cysN	NP_215802.1	ATP:sulphurylase subunit 1 [NP_005434, 3e-45, 1ZUN]
Rv3340		9.995	0	0	metC	NP_217857.1	cystathionine [beta]-lyase [NP_001893, 3e-56, 2CTZ]
Rv0848		12.8	0	0	cysK2	YP_177762.1	putative cysteine synthase [NP_000062, 6e-26, 1VE1]
Rv1373		4.07	-2.935	0		NP_215889.1	slight similarity to sulfotransferases [NP_814444, 2e-12]
Rv2392		1.215	-9.135	3.17	cysH	NP_216908.1	3'-phosphoadenylylsulfate (PAPS) reductase

							[2GOY]
Sulfate transporter							
Rv1707		11.61	0	0		NP_216223.1	probable sulphate permease [NP_075062, 5e-25]
Rv1739c		10.5	-5.47	0		NP_216255.1	possible sufate transporter [NP_075062, 8e-57]
Rv2398c		7.73	-2.995	3.615	cysW	NP_216914.1	sulphate transport system permease protein [2ONK]
Rv2399c		9.59	-0.735	3.27	cysT	NP_216915.1	sulphate transport system permease protein [2ONK]
NAD biosynthesis							
Rv0212c		11.785	0	0	nadR	NP_214726.1	similar to E.coli NadR [NP_079509, 6e-07, 1LW7]
Nitroreductase							
Rv2032		19.4	-2.755	0	acg	NP_216548.1	conserved hypothetical protein
Rv3131		16.055	-3.31	3.345		NP_217647.1	conserved hypothetical protein
Rv3127		14.055	-6.615	0		NP_217643.1	conserved hypothetical protein
Rv1736c		6.795	-3.145	0	narX	NP_216252.1	fused nitrate reductase [1Q16]
Respiratory chain							
Rv3054c		15.8	-0.85	0		NP_217570.1	conserved hypothetical protein [NP_000895, 2e-20, 1RTT]
Rv0082		11.51	-2.55	0		NP_214596.1	probable oxidoreductase subunit [NP_077718, 1e-19, 2FUG]
Rv1812c		7.565	-1.88	0		NP_216328.1	probable dehydrogenase
Rv1854c		7.39	-3.525	0	ndh	NP_216370.1	probable NADH dehydrogenase [NP_116186, 5e-10, 1XHC]
Rv1552		12.925	0	0	frdA	NP_216068.1	fumarate reductase flavoprotein subunit [NP_004159, 4e-95, 1KF6]
Rv1553		5.96	-2.81	0	frdB	NP_216069.1	fumarate reductase iron sulphur protein [1KF6]
Rv1554		6.32	-2.675	0	frdC	NP_216070.1	fumarate reductase 15kD anchor protein
Rv1555		4.735	-2.72	0	frdD	NP_216071.1	fumarate reductase 13kD anchor protein
nitrite extrusion protein							

Rv1737c	13.04	-2.915	0	narK2	NP_216253.1	nitrite extrusion protein
protease regulating envelope composition						
Rv2869c	2.22	-1.76	2.585		NP_217385.1	probable integral membrane protein [1KY9]
cation transport ATPase						
Rv1997	16.165	-3.32	0	ctpF	NP_216513.1	probable cation transport ATPase [NP_001001486, 1e-150, 2EAR]
Rv1992c	14.45	0	0	ctpG	NP_216508.1	probable cation transport ATPase [NP_000044, 1e-70, 2B8E]
Chaperonins/hsp						
Rv0251c	21.935	-1.81	0	hsp	NP_214765.1	possible heat shock protein [1GME]
Rv2031c	15.78	0	0	hspX	NP_216547.1	14kD antigen, heat shock protein Hsp20 family [1GME]
Rv0353	6.265	0	0	hspR	NP_214867.1	heat shock regulator
Rv0563	15.86	-2.125	0	htpX	NP_215077.1	probable (transmembrane) heat shock protein [NP_005848, 6e-26, 1P7B]
Rv0384c	15.625	-0.37	4.025	clpB	NP_214898.1	heat shock protein [NP_004784, 1e-39, 1JBK]
Rv0440	8.565	-9.995	3.655	groEL 2	NP_214954.1	60 kD chaperonin 2 [NP_955472, 1e-138, =1SJP]
Rv3417c	8.74	-3.985	2.31	groEL 1	NP_217934.1	60 kD chaperonin 1 [NP_955472, 1e-117, =1SJP]
Rv3418c	6.92	-6.04	3.895	groE S	NP_217935.1	10 kD chaperone [NP_002148, 3e-12, =1HX5]
Rv3610c	7.115	-2.995	3.23	ftsH	NP_218127.1	inner membrane protein, chaperone [NP_006787, e-120, 2CE7]
Ribonucleotide reductase						
Rv0233	9.05	0	0	nrdB	NP_214747.1	ribonucleoside-diphosphate reductase B2 [1XSM]
Rv3051c	7.085	-5.02	4.415	nrdE	NP_217567.1	ribonucleoside diphosphate reductase [alpha] chain [NP_001024, 8e-46, 1PEM, 4R1R]
Rv1981c	8.98	-1.695	0	nrdF1	YP_177853.1	ribonucleotide reductase small subunit [1KGN]

Rv3048c	15.845	-0.49	2.085	nrdF2	YP_177921.1	ribonucleoside-diphosphate small subunit [=1UZR]
Rv3053c	9.72	-3.08	0	nrdH	NP_217569.1	glutaredoxin electron transport component of NrdEF [1R7H]
Rv3052c	10.97	-3.145	0	nrdI	NP_217568.1	NrdI/YgaO/YmaA family [1RLJ]
lysine-e-aminotransferase						
Rv3290c	19.385	0	0	lat	NP_217807.1	lysine-[epsilon] aminotransferase NP_000654, 1e-39, =2CIN]
	Redox balance					
Rv0467	12.25	-1.385	0	icl	YP_177728.1	isocitrate lyase [=1F61]
Rv1915	5.545	-6.31	0	aceAa	NP_216431.1	isocitrate lyase, [alpha] module [1F61, 1F8IA]
Rv1916	2.605	-4.555	0	aceAb	NP_216432.1	isocitrate lyase, [beta] module
Rv2780	15.115	0	0	ald	NP_217296.1	L-alanine dehydrogenase [NP_036475, 3e-32, 1PJB, 1F8G]
Rv0211	8.635	-1.875	0	pckA	NP_214725.1	phosphoenolpyruvate carboxykinase [NP_002582, e-176, 1KHB]
Rv1131	11.92	-1.675	0	gltA1	NP_215647.1	citrate synthase 3 [NP_004068, 5e-14, 1A59]
Rv2332	8.69	0	0	mez	NP_216848.2	probable malate oxidoreductase [NP_002386, 3e-96, 1LLQ]
sigma factors						
Rv0182c	4.1	-4.59	0	sigG	NP_214696.1	sigma-70 factors ECF subfamily [1H3L, 1OR7]
Rv0445c	2.335	-12.13	0	sigK	NP_214959.1	ECF-type sigma factor
Rv0735	0.805	-4.64	0	sigL	NP_215249.1	sigma-70 factors ECF subfamily
Rv1189	2.2	-2.085	0	sigI	NP_215705.1	ECF family sigma factor
Rv1221	13.005	-3.67	0	sigE	NP_215737.1	ECF subfamily sigma subunit [1OR7]
Rv2069	6.48	-5.88	0	sigC	NP_216585.1	ECF subfamily sigma subunit [2O7G]
Rv2703	0.47	-3.82	3.33	sigA	NP_217219.1	RNA polymerase sigma factor (aka MysA, RpoV) [NP_005372|NP_005372, 3e-13, 1L9Z]
Rv2710	18.915	0	2.005	sigB	NP_217226.1	RNA polymerase sigma factor (aka MysB) [1IW7]

Rv3223c		9.98	-1.76	4.885	sigH	NP_217739.1	ECF subfamily sigma subunit [1H3L]
Rv3286c		6.365	-2.07	0	sigF	NP_217803.1	ECF subfamily sigma subunit [1L0O]
Rv3328c		0	-1.86	0	sigJ	NP_217845.1	similar to SigI, ECF family [1OHS]
Rv3414c		1.66	-4.91	0	sigD	NP_217931.1	ECF subfamily sigma subunit [1OR7]
Rv3911		4.265	-4.19	0	sigM	NP_218428.1	probable sigma factor, similar to SigE [1OR7]
relA							
Rv2583c		3.775	-3.995	0	relA	NP_217099.1	(p)ppGpp synthase I [NP_940929, 5e-13, 1VJ7]

*Multiple genes in the same pathway are included for score comparisons. †In brackets are the closest human ortholog, BLASTP [123] expect (E) value, and relevant 3-D structure (pdb entry), if any. An E-value cutoff of 1e-10 was used for inclusion of a human ortholog. An equal sign before the pdb entry indicates the protein is from *M. tuberculosis.*

APPENDIX B

APPENDIX B: Complete data set of significantly ($p \leq 0.05$) differentially regulated ORFs (>1-fold) for SBPT04 drug treated LVS at 2XMIC for 2hrs

Locus	Gene	Avg Log_2 Expresssion	p-value	Gene product
FTT1695	groS	2.10	0.009	Chaperonin protein, groES
FTT1349		2.01	0.000	hypothetical protein
FTT0890c		1.75	0.001	Type IV pili fiber building block protein
FTT1704		1.67	0.034	hypothetical protein
FTT1699	pdpA1	1.66	0.006	hypothetical protein
FTT1293c		1.61	0.000	Sua5_yciO_yrdC family protein
FTT1709		1.56	0.023	hypothetical protein
FTT1178c		1.51	0.005	hypothetical membrane protein
FTT1705		1.48	0.045	hypothetical protein
FTT1350		1.44	0.004	hypothetical protein
FTT1435c	yagD	1.36	0.003	ABC transporter, ATP-binding protein
FTT1762c		1.36	0.004	Acetyltransferase protein
FTT1708		1.32	0.016	hypothetical protein
FTT1334c		1.30	0.015	hypothetical protein
FTT1534c		1.30	0.011	hypothetical protein
FTT0988		1.28	0.039	hypothetical protein
FTT0594c		1.27	0.025	hypothetical protein
FTT0459	sohB	1.27	0.037	peptidase family S49 protein
FTT1601c	recG	1.25	0.022	ATP-dependent DNA helicase RecG
FTT1217c	visC	1.25	0.000	monooxygenase family protein
FTT1524c	hrpA	1.24	0.021	ATp-dependent helicase
FTT1624c		1.22	0.030	hypothetical protein
FTT0863c		1.22	0.028	LemA-like protein
FTT1715c	pdpD1	1.22	0.011	hypothetical protein
FTT1636	lolA	1.19	0.035	lipoprotein releasing system, subunit A, outer membrane lipoproteins carrier
FTT0942c	folK	1.14	0.004	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase/dihydropteroate synthase
FTT1443c		1.13	0.014	ATPase, AAA family
FTT0267		1.13	0.000	hypothetical protein
FTT0918		1.10	0.025	hypothetical protein
FTT0433		1.08	0.031	hypothetical protein
FTT1426c		1.07	0.009	conservered hypothetical membrane protein
FTT1149c		1.07	0.001	amino acid transporter family protein
FTT1197c	murI	1.06	0.014	glutamate racemase
FTT1007c		1.04	0.019	hypothetical protein
FTT0785		1.04	0.023	hypothetical protein
FTT0623	tig	1.00	0.008	trigger factor
FTT1295c	glk	0.99	0.047	glucose kinase
FTT0424		0.97	0.017	hypothetical protein

FTT1684		0.96	0.010	transcriptional regulator
FTT1140		0.96	0.039	hypothetical protein
FTT0682c		0.95	0.003	hypothetical protein
FTT1188		0.95	0.047	hypothetical membrane protein
FTT0164c		0.94	0.024	Eflux protein
FTT1010		0.92	0.000	Helix-turn-helix family protein
FTT0496		0.92	0.002	hypothetical protein
FTT1390	panC	0.92	0.027	Pantoate-beta-alanine ligase
FTT0501c		0.91	0.009	hypothetical protein
FTT1517c		0.90	0.001	hypothetical protein
FTT1547		0.90	0.044	Transposase
FTT1403c	feoA	0.89	0.000	ferrous iron transport protein A
FTT1220		0.87	0.002	5-formyltetrahydroformate cycloligase family protein
FTT0797		0.86	0.004	glycosyl transferase family protein
FTT1574c	dxr	0.86	0.029	1-deoxy-D-xylulose 5-phosphate reductoisomerase
FTT0075	sdhB	0.86	0.000	succinate dehydrogenase catalytic subunit
FTT1027c	yrbI	0.84	0.030	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase
FTT0552		0.84	0.047	aldehyde dehydrogenase
FTT0566		0.84	0.047	hypothetical protein
FTT1023c		0.83	0.000	fumarylacetoacetate hydrolase family protein
FTT1300c		0.82	0.001	hypothetical protein
FTT1206		0.82	0.044	hypothetical lipoprotein
FTT1075		0.81	0.000	Nif3 family protein
FTT1119		0.81	0.007	transcriptional regulator
FTT1356c	iglD	0.81	0.012	intracellular growth locus, subunit D
FTT1787c		0.81	0.002	Transporter, LysE family
FTT1432c	ppnK	0.80	0.042	inorganic phosphate/ATP-NAD kinase
FTT1244c	yfiO	0.78	0.006	conserved hypothetical lipoprotein
FTT1401		0.77	0.044	prophage repressor protein
FTT0356	htpG	0.77	0.010	heat shock protein 90
FTT0879	sodC	0.77	0.010	superoxide dismuate (Cu-Zn) precusor
FTT0713c	tdh	0.77	0.021	L-threonine 3-dehydrogenase
FTT1219c		0.77	0.006	hypothetical protein
FTT0395		0.75	0.029	hypothetical protein
FTT0519		0.74	0.005	LysR transcriptional regulator family protein
FTT1374		0.73	0.032	malonyl coA-acyl carrier protein transacylase
FTT1242		0.73	0.043	hypothetical protein
FTT1330	serS	0.72	0.019	seryl-tRNA synthetase
FTT1444c	ppx	0.71	0.030	Exopolyphosphatase
FTT1722c	gad	0.70	0.039	glutamate decarboxylase
FTT0555		0.70	0.041	hypothetical protein
FTT1555c	rnc	0.69	0.034	Ribonuclease III
FTT1156c	pilQ	0.68	0.002	Type IV pilin multimeric outer membrane protein
FTT0848		0.68	0.025	hypothetical protein
FTT0627	hupB	0.68	0.000	Histone-like protein HU form B
FTT1385c		0.67	0.047	hypothetical protein
FTT0845		0.67	0.024	hypothetical protein

FTT1375	fabG	0.66	0.042	3-oxoacyl-(acyl-carrier-protein) reductase
FTT0499		0.66	0.002	hypothetical protein
FTT1245	rluD	0.65	0.038	ribosomal large subunit pseudouridine synthase D
FTT0105c		0.63	0.000	Transporter AcrB/AcrD/AcrF family
FTT1249		0.62	0.008	mycobacterial cell entry (mce) related family protein
FTT1590c	ubiG	0.62	0.029	3-demethylubiquinone-9 3-methyltransferase
FTT1707		0.61	0.010	hypothetical protein
FTT1532		0.61	0.018	hypothetical protein
FTT1093c	talA	0.61	0.012	transaldolase
FTT0085c		0.61	0.041	hypothetical protein
FTT1775c	clcA	0.61	0.001	chloride channel protein
FTT0874c		0.60	0.013	hypothetical protein
FTT1408c	hemD	0.60	0.008	Uroporphyrinogen III synthase
FTT1645		0.59	0.003	hypothetical protein
FTT1711c	iglD1	0.59	0.030	intracellular growth locus, subunit D
FTT1531	fadA	0.59	0.001	acetyl-CoA acetyltransferase
FTT1399		0.58	0.000	conservered hypothetical membrane protein
FTT0376c		0.57	0.047	hypothetical membrane protein
FTT1124	metN	0.57	0.042	D-methionine transport protein, ABC transporter,ATP-binding subunit
FTT0611c		0.56	0.011	beta-lactamase
FTT1113c		0.56	0.009	hypothetical protein
FTT0027c	lysA	0.56	0.007	diaminopimelate decarboxylase
FTT0425c	asd	0.56	0.006	aspartate-semialdehyde dehydrogenase
FTT0688c	hslV	0.56	0.001	ATP-dependent protease peptidase subunit
FTT0097		0.56	0.000	hypothetical protein
FTT0624	clpP	0.56	0.027	ATP-dependent Clp protease subunit P
FTT0621	tdk	0.55	0.028	thymidine kinase
FTT0536		0.55	0.043	hypothetical membrane protein
FTT0558		0.55	0.000	short chain dehydrogenase
FTT0256c		0.55	0.000	Lipopolysaccharide protein
FTT1168c	nagA	0.54	0.013	N-acetylglucosamine-6-phosphate deacetylase
FTT0652c	ftnA	0.54	0.008	Ferritin-like protein
FTT1761		0.54	0.002	hypothetical protein
FTT0851		0.54	0.001	hypothetical protein
FTT0995		0.53	0.005	major facilitator superfamily (MFS) transport protein
FTT0304c	nfnB	0.53	0.001	Oxygen-insensitive NAD(P)H nitroreductase
FTT1499	muts	0.53	0.020	DNA mismatch repair protein
FTT1043		0.53	0.039	FKBP-type peptidyl-prolyl cis-trans isomerase family protein
FTT1484c	aceF	0.52	0.010	pyruvate dehydrogenase, E2 component
FTT0968c		0.52	0.017	amino acid antiporter
FTT0541c	yqaB	0.51	0.000	haloacid dehalogenase
FTT0451	murD	-0.50	0.001	UDP-N-acetylmuramoylalanine--D-glutamate ligase
FTT0035	nuoE	-0.50	0.026	NADH dehydrogenase I, E subunit
FTT0168	prfA	-0.51	0.047	peptide chain release factor 1
FTT0116	nupC	-0.51	0.009	nucleoside permease NUP family protein
FTT0133	glpF	-0.51	0.010	glycerol uptake facilitator protein

FTT0130	glpK	-0.52	0.006	glycerol kinase
FTT1608		-0.54	0.025	ABC transporter, ATP-binding protein
FTT0333	rpmC	-0.54	0.001	50S ribosomal protein L29
FTT0115	nupC1	-0.55	0.031	nucleoside permease NUP family protein
FTT0835		-0.55	0.000	CDP-alcohol phosphatidyltransferase
FTT1674	ribH	-0.55	0.050	riboflavin synthase beta subunit (6,7-dimethl-8-ribityllumazine synthase)
FTT1800c		-0.56	0.001	hypothetical protein
FTT1273	rplM	-0.57	0.009	50S ribosomal protein L13
FTT0120	ftsY	-0.57	0.014	signal recognition particle receptor FtsY
FTT0174		-0.58	0.000	YggT family protein
FTT1470c	gmk	-0.59	0.008	guanylate kinase
FTT0018		-0.59	0.010	Secretion protein
FTT1037c		-0.60	0.012	hypothetical protein
FTT0112		-0.60	0.001	Sigma-54 modulation protein
FTT0007	aspS	-0.60	0.000	Aspartyl-tRNA synthetase
FTT0014c		-0.61	0.030	hypothetical protein
FTT0346	rpmJ	-0.62	0.000	50S ribosomal protein L36
FTT0017		-0.62	0.000	hypothetical protein
FTT0487		-0.63	0.019	hypothetical membrane protein
FTT1611		-0.64	0.024	conserved hypothetcial protein
FTT1372	plsX	-0.64	0.012	fatty acid/phospholipid synthesis protein
FTT1343c		-0.65	0.029	hypothetical protein
FTT0332	rplP	-0.65	0.006	50S ribosomal protein L16
FTT0443		-0.66	0.002	hypothetical protein
FTT0896	purE	-0.67	0.023	phosphoribosylaminoimidazole carboxylase,catalyic subunit
FTT0282	cyoB	-0.67	0.024	Cytochrome O ubiquinol oxidase subunit I
FTT0291		-0.67	0.016	hypothetical protein
FTT0892	folD	-0.68	0.017	methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase putative bifunctional protein
FTT1571c	lpxD	-0.68	0.000	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase
FTT0331	rpsC	-0.68	0.011	30S ribosomal protein S3
FTT0705	rumA	-0.68	0.042	23S rRNA (Uracil-5-)-methyltransferase
FTT0834	aroQ	-0.68	0.025	chorismate mutase
FTT1553c	rnr	-0.69	0.005	Ribonuclease R
FTT0525		-0.69	0.020	hypothetical protein
FTT1382	suhB	-0.70	0.009	Inositol-1-monophosphatase
FTT0510	gyrB	-0.70	0.000	DNA gyrase subunit B
FTT1332		-0.70	0.018	polysaccharide biosynthesis protein (export protein)
FTT1659		-0.71	0.032	hypothetical protein
FTT0114	deoC	-0.72	0.038	Deoxyribose-phosphate aldolase
FTT0209c		-0.73	0.040	periplasmic solute binding family protein
FTT0814c		-0.74	0.011	hypothetical protein
FTT1724c	tolC	-0.74	0.018	outer membrane protein tolC precursor
FTT1496c		-0.75	0.047	hypothetical membrane protein
FTT0061	atpH	-0.75	0.050	ATP synthase delta chain
FTT0773	rpmA	-0.76	0.006	50S ribosomal protein L27

FTT0321	rpsL	-0.77	0.050	30S ribosomal protein S12
FTT1778c		-0.79	0.005	hypothetical membrane protein
FTT0039	nuoI	-0.79	0.001	NADH dehydrogenase subunit I
FTT0759		-0.80	0.007	hypothetical protein
FTT1042		-0.80	0.001	hypothetical protein
FTT0062	atpA	-0.81	0.009	ATP synthase subunit A
FTT1753	tdcD	-0.82	0.003	propionate kinase
FTT1274	rpsI	-0.82	0.002	30S ribosomal protein S9
FTT1557c		-0.82	0.027	trp operon repressor
FTT0820	rplT	-0.82	0.003	50S ribosomal protein L20
FTT0183c	rpsA	-0.82	0.005	30S ribosomal protein S1
FTT0312c	folA	-0.83	0.038	dihydrofolate reductase type I
FTT0562	potG	-0.84	0.001	polyamine transporter, ABC transporter,ATP-binding protein
FTT0450	mraY	-0.84	0.007	Phospho-N-acetylmuramoyl-pentapeptide transferase
FTT0320	pgsA	-0.86	0.020	phosphatidylglycerophosphate synthetase
FTT0800		-0.88	0.019	haloacid dehalogenase-like hydrolase family protein
FTT0058	atpB	-0.88	0.000	ATP synthase subunit A
FTT0804		-0.89	0.041	major facilitator superfamily (MFS) transport protein
FTT0798		-0.89	0.043	glycosyl transferase family protein
FTT0329	rpsS	-0.89	0.000	30S ribosomal protein S19
FTT0481	potF	-0.91	0.000	Putrescine-binding periplasmic protein
FTT0318	cdsA	-0.91	0.045	phosphatidate cytidylyltransferase
FTT0349	rpsD	-0.91	0.002	30S ribosomal protein S4
FTT0351	rplQ	-0.92	0.050	50S ribosomal protein L17
FTT0897	purK	-0.94	0.001	phosphoribosylaminoimidazole carboxylase, ATPase subunit
FTT1296	ubiE	-0.95	0.042	menaquinone biosynthesis methyltransferase
FTT0109	valA	-0.95	0.008	Lipid A transport protein, ABC transporter, ATP-binding and membrane protein
FTT0051	rbfA	-0.96	0.037	Ribosome-binding factor A
FTT1259	nadE	-0.96	0.002	NH(3)-dependent NAD(+) synthetase
FTT1291		-0.97	0.004	major facilitator superfamily (MFS) transport protein
FTT0338	rpsN	-1.01	0.005	30S ribosomal protein S14
FTT0632c		-1.02	0.001	hypothetical protein
FTT1041		-1.03	0.029	hypothetical protein
FTT0235c	mpA	-1.03	0.035	Ribonuclease P protein component
FTT1369c	tktA	-1.05	0.002	transketolase
FTT0063	atpG	-1.05	0.003	ATP synthase gamma chain
FTT1373	fabH	-1.06	0.015	3-oxoacyl-[acyl carrier protein] synthase III
FTT0881c		-1.06	0.020	amino acid permease
FTT1688		-1.08	0.000	aromatic amino acid transporter of the HAAAP family
FTT0325	rplC	-1.09	0.010	50S ribosomal protein L3
FTT0347	rpsM	-1.11	0.024	30S ribosomal protein S13
FTT0031	nuoA	-1.12	0.001	NADH dehydrogenase I, A subunit
FTT0919		-1.16	0.003	hypothetical protein
FTT0064	atpD	-1.17	0.049	ATP synthase subunit B
FTT0348	rpsK	-1.18	0.006	30S ribosomal protein S11
FTT0053		-1.20	0.003	major facilitator superfamily (MFS) transport protein

FTT0319	dut	-1.20	0.005	deoxyuridine 5'-triphosphate nucleotidohydrolase
FTT0041	nuoK	-1.20	0.001	NADH dehydrogenase I, K subunit
FTT0564	potI	-1.22	0.002	polyamine transporter, subunit I, ABC transporter, membrane protein
FTT0038	nuoH	-1.24	0.010	NADH dehydrogenase I, H subunit
FTT0789	rpe	-1.25	0.042	D-ribulose-phosphate 3-epimerase
FTT0139	nusG	-1.28	0.000	transcription antitermination protein nusG
FTT0315	pyrH	-1.29	0.035	uridylate kinase
FTT0336	rplX	-1.30	0.000	50S ribosomal protein L24
FTT0138	secE	-1.30	0.034	preprotein translocase, subunit E, membrane protein
FTT0340	rplF	-1.31	0.000	50S ribosomal protein L6
FTT0342	rpsE	-1.32	0.001	30S ribosomal protein S5
FTT0249	feoB	-1.33	0.000	ferrous iron transport protein
FTT0311c		-1.36	0.005	hypothetical protein
FTT0137	tufA	-1.37	0.002	elongation factor Tu (EF-Tu)
FTT1442c	rpoA2	-1.39	0.015	DNA-directed RNA polymerase alpha subunit
FTT0044	nuoN	-1.43	0.005	NADH dehydrogenase I, N subunit
FTT1750	recA	-1.45	0.004	Recombinase A protein
FTT0142	rplJ	-1.46	0.017	50S ribosomal protein L10
FTT0151	rimM	-1.48	0.016	16S rRNA processing protein rimM
FTT1668	sdaC2	-1.52	0.030	serine transporter
FTT0626	lon	-1.52	0.001	DNA-binding, ATP-dependent protease La
FTT0339	rpsH	-1.53	0.000	30S ribosomal protein S8
FTT0818	infC	-1.53	0.025	translation initiation factor IF-3
FTT1006		-1.58	0.016	hypothetical membrane protein
FTT0141	rplA	-1.58	0.044	50S ribosomal protein L1
FTT0250	ppdK	-1.60	0.013	pyruvate phosphate dikinase
FTT0048		-1.66	0.007	hypothetical protein
FTT1638	trkH	-1.73	0.031	potassium uptake protein
FTT0350	rpoA1	-1.75	0.002	DNA-directed RNA polymerase alpha subunit
FTT0583	fopA	-1.84	0.051	outer membrane associated protein
FTT0043	nuoM	-1.85	0.006	NADH dehydrogenase I, M subunit
FTT0343	rpmD	-1.87	0.027	50S ribosomal protein L30
FTT0145	rpoC	-2.01	0.007	DNA-directed RNA polymerase, beta subunit
*NC_006570-PR1149		1.59	0.021	Unknown
*NC_006570-PR1763		1.54	0.001	Unknown
*NC_006570-PR385		1.38	0.003	Transposase
*NC_006570-PR1475		1.34	0.013	Unknown
*NC_006570-PR838		1.33	0.002	Transposase
*NC_006570-PR100		1.31	0.041	Transposase
*NC_006570-PR1585		1.27	0.003	Unknown
*NC_006570-PR790		1.24	0.004	Transposase
*NC_006570-PR570		1.22	0.005	Transposase
*NC_006570-PR1623		1.20	0.023	Transposase
*NC_006570-PR1200		1.18	0.003	Unknown
*NC_006570-PR1659		1.18	0.036	Transposase
*NC_006570-PR526		1.17	0.015	Unknown

*NC_006570-PR102	1.16	0.020	Unknown
*NC_006570-PR1709	1.15	0.006	Unknown
*NC_006570-PR1283	1.14	0.011	Transposase
*NC_006570-PR937	1.13	0.006	Unknown
*NC_006570-PR1788	1.13	0.045	Unknown
*NC_006570-PR360	1.12	0.006	Unknown
*NC_006570-PR1738	1.09	0.036	Transposase
*NC_006570-PR548	1.06	0.003	Unknown
*NC_006570-PR1160	1.05	0.006	Transposase
*NC_006570-PR1764	1.05	0.025	Unknown
*NC_006570-PR1395	1.04	0.006	Unknown
*NC_006570-PR1228	1.04	0.002	Transposase
*NC_006570-PR1083	1.04	0.000	Transposase
*NC_006570-PR232	1.03	0.023	Transposase
*NC_006570-PR1762	1.02	0.011	Unknown
*NC_006570-PR1280	1.01	0.005	Transposase
*NC_006570-PR371	1.00	0.036	Transposase
*NC_006570-PR436	1.00	0.001	Unknown
*NC_006570-PR776	0.99	0.003	Transposase
*NC_006570-PR506	0.99	0.024	Unknown
*NC_006570-PR1250	0.96	0.009	Transposase
*NC_006570-PR1683	0.96	0.017	Unknown
*NC_006570-PR253	0.96	0.001	Transposase
*NC_006570-PR280	0.96	0.000	Transposase
*NC_006570-PR1654	0.96	0.008	Transposase
*NC_006570-PR1802	0.95	0.031	Unknown
*NC_006570-PR1638	0.92	0.000	Transposase
*NC_006570-PR259	0.90	0.009	Transposase
*NC_006570-PR701	0.87	0.002	Transposase
*NC_006570-PR1416	0.87	0.023	Transposase
*NC_006570-PR1732	0.83	0.044	Transposase
*NC_006570-PR1162	0.82	0.000	Unknown
*NC_006570-PR1122	0.78	0.006	Unknown
*NC_006570-PR1826	0.74	0.011	Unknown
*NC_006570-PR1464	0.74	0.000	Unknown
*NC_006570-PR734	0.73	0.012	Unknown
*NC_006570-PR133	0.72	0.014	Transposase
*NC_006570-PR1787	0.69	0.009	Unknown
*NC_006570-PR1198	0.69	0.040	Unknown
*NC_006570-PR538	0.69	0.028	Unknown
*NC_006570-PR1518	0.64	0.014	Unknown
*NC_006570-PR1573	0.62	0.003	Unknown
*NC_006570-PR9	0.60	0.017	Unknown
*NC_006570-PR1791	0.60	0.028	Unknown
*NC_006570-PR816	0.54	0.035	Unknown
*NC_006570-PR125	0.54	0.016	Unknown
*NC_006570-PR91	0.54	0.001	Unknown
*NC_006570-PR868	0.52	0.017	Unknown

*NC_006570-PR1371	-0.51	0.017	Unknown
*NC_006570-PR817	-0.54	0.029	Unknown
*NC_006570-PR207	-0.58	0.026	Unknown
*NC_006570-PR212	-0.59	0.006	Unknown
*NC_006570-PR1603	-0.60	0.000	Unknown
*NC_006570-PR141	-0.60	0.032	Unknown
*NC_006570-PR1372	-0.65	0.042	Unknown
*NC_006570-PR1743	-0.72	0.009	Unknown
*NC_006570-PR1679	-0.76	0.001	Unknown
*NC_006570-PR744	-0.80	0.006	Unknown
*NC_006570-PR1373	-0.82	0.000	Unknown
*NC_006570-PR140	-0.87	0.008	Unknown
*NC_006570-PR1444	-1.34	0.005	Unknown
*NC_006570-PR1539	-1.35	0.029	Unknown

APPENDIX C

APPENDIX C: Complete data set of differentially regulated ORFs (>1.5 fold)p-values of ≤ 0.5 for overexpressed *rv3660*

Overexpressed *rv3660c*

Locus	Gene	*Avg Log_2 Expression*	*p value*	Gene product
Rv2031c	hspX	3.94	0.0427	14kD antigen, heat shock protein Hsp20 family
Rv2626c		3.35	0.0000	conserved hypothetical protein
Rv1996		2.63	0.0465	conserved hypothetical protein
Rv2160c		2.53	0.0088	hypothetical protein
Rv3134c		2.45	0.0237	conserved hypothetical protein
Rv2628		2.22	0.0217	hypothetical protein
Rv3127		2.15	0.0443	conserved hypothetical protein
Rv3132c	dosS	2.00	0.0143	sensor histidine kinase
Rv1622c	cydB	1.99	0.0445	cytochrome d ubiquinol oxidase subunit II
Rv3230c		1.93	0.0003	similar to various oxygenases
Rv2005c		1.78	0.0335	conserved hypothetical protein
Rv1620c	cydC	1.63	0.0019	ABC transporter
Rv1997	ctpF	1.62	0.0010	probable cation transport ATPase
Rv3290c	lat	1.61	0.0089	lysine-[epsilon] aminotransferase
Rv0395		1.58	0.0041	hypothetical protein
Rv3448		1.56	0.0015	probable membrane protein
Rv0915c	PPE	1.55	0.0068	PPE-family protein
Rv2492		1.55	0.0322	hypothetical protein
Rv0987		1.51	0.0025	potential integral membrane protein
Rv0988		1.50	0.0007	conserved hypothetical protein
Rv1726		1.49	0.0060	6-hydroxy-d-nicotine oxidase
Rv3171c	hpx	1.49	0.0150	probable non-heme haloperoxidase
Rv2481c		1.48	0.0064	hypothetical protein
Rv0560c		1.46	0.0042	methyl transferase
Rv0990c		1.45	0.0046	hypothetical protein
Rv1921c	lppF	1.45	0.0004	lipoprotein
Rv0393		1.44	0.0064	conserved hypothetical protein
Rv0982		1.44	0.0026	sensor histidine kinase
Rv0989c	grcC2	1.43	0.0151	heptaprenyl diphosphate synthase II
Rv3177		1.43	0.0001	probable non-heme haloperoxidase
Rv3470c	ilvB2	1.43	0.0007	acetolactate synthase large subunit
Rv1909c	furA	1.43	0.0252	ferric uptake regulatory protein
Rv0459		1.42	0.0026	conserved hypothetical protein
Rv3652	PE_PGRS	1.42	0.0002	PE_PGRS-family protein
Rv2385	lipK	1.42	0.0012	probable acetyl-hydrolase
Rv2652c		1.42	0.0000	phiRV2 phage related protein
Rv0983		1.42	0.0010	probable serine protease
Rv2812		1.42	0.0229	low similarity to transposases
Rv2630		1.42	0.0027	hypothetical protein
Rv2488c		1.41	0.0066	transcriptional regulator (LuxR/UhpA family)

Rv0033		1.41	0.0000	possible acyl carrier protein
Rv0986		1.40	0.0034	Probable ABC transporter
Rv0969	ctpV	1.40	0.0038	cation transport ATPase
Rv0978c	PE_PGRS	1.40	0.0123	PE_PGRS-family protein
Rv0972c	fadE12	1.40	0.0242	acyl-CoA dehydrogenase
Rv1931c		1.39	0.0124	transcriptional regulator (AraC/XylS family)
Rv0991c		1.39	0.0137	hypothetical protein
Rv1153c	omt	1.39	0.0021	PKS o-methyltransferase
Rv3854c		1.39	0.0178	probable monooxygenase
Rv1927		1.38	0.0016	hypothetical protein
Rv2232		1.38	0.0010	hypothetical protein
Rv3192		1.37	0.0001	hypothetical protein
Rv2250c		1.37	0.0358	putative transcriptional regulator
Rv0492c		1.37	0.0007	gmc-type oxidoreductase
Rv2269c		1.37	0.0031	hypothetical protein
Rv0912		1.37	0.0005	conserved hypothetical protein
Rv3468c	rmlB3	1.37	0.0225	dTDP-glucose 4,6-dehydratase
Rv2690c		1.36	0.0413	possible transport protein
Rv0368c		1.36	0.0342	conserved hypothetical protein
Rv2044c		1.36	0.0011	hypothetical protein
Rv0965c		1.36	0.0077	conserved hypothetical protein
Rv3428c		1.35	0.0250	hypothetical protein
Rv3616c		1.35	0.0195	conserved hypothetical protein
Rv0967		1.35	0.0003	conserved hypothetical protein
Rv0080		1.35	0.0021	hypothetical protein
Rv0918		1.35	0.0015	conserved hypothetical protein
Rv1902c	nanT	1.35	0.0192	probable sialic acid transporter
Rv0963c		1.35	0.0000	conserved hypothetical protein
Rv2027c		1.35	0.0007	sensor histidine kinase
Rv0975c	fadE13	1.35	0.0001	acyl-CoA dehydrogenase
Rv3351c		1.35	0.0364	conserved hypothetical protein
Rv3746c	PE	1.35	0.0052	PE-family protein
Rv0979c		1.35	0.0037	hypothetical protein
Rv0971c	echA7	1.34	0.0297	enoyl-CoA hydratase/isomerase superfamily
Rv1116		1.34	0.0043	hypothetical protein
Rv3830c		1.33	0.0024	transcriptional regulator (TetR/AcrR family)
Rv0258c		1.33	0.0008	conserved hypothetical protein
Rv0961		1.33	0.0292	hypothetical protein
Rv0051		1.32	0.0041	probable membrane protein
Rv1203c		1.32	0.0022	hypothetical protein
Rv0035	fadD34	1.32	0.0012	acyl-CoA synthase
Rv1949c		1.32	0.0107	conserved hypothetical protein
Rv2590	fadD9	1.32	0.0000	acyl-CoA synthase
Rv0650		1.32	0.0056	transcriptional regulator (ROK family)
Rv0079		1.31	0.0066	hypothetical protein
Rv3352c		1.31	0.0277	defective/truncated oxidoreductase
Rv3080c	pknK	1.31	0.0263	serine-threonine protein kinase
Rv1979c		1.31	0.0076	unknown permease
Rv2689c		1.31	0.0025	weak similarity to RNA methyltransferases
Rv3611		1.31	0.0041	hypothetical protein
Rv3444c		1.31	0.0055	conserved hypothetical protein
Rv0396		1.31	0.0002	hypothetical protein

Rv3343c	PPE	1.31	0.0002	PPE-family protein
Rv0246		1.30	0.0001	probable membrane transport protein
Rv3114		1.30	0.0000	hypothetical protein
Rv1148c		1.30	0.0030	REP-family protein
Rv0968		1.29	0.0212	conserved hypothetical protein
Rv0209		1.29	0.0350	hypothetical protein
Rv0465c		1.29	0.0031	transcriptional regulator (PbsX/Xre family)
Rv0962c		1.29	0.0178	hypothetical protein
Rv2340c	PE	1.29	0.0312	PE-family protein
Rv3350c	PPE	1.29	0.0044	PPE-family protein
Rv0851c		1.29	0.0005	Short-chain dehydrogenases/reductase
Rv1974		1.28	0.0141	conserved hypothetical protein
Rv3195		1.28	0.0029	conserved hypothetical protein
Rv2316	uspA	1.28	0.0260	sugar transport protein
Rv2824c		1.28	0.0000	hypothetical protein
Rv1530	adh	1.28	0.0100	alcohol dehydrogenase (Zn)
Rv2570		1.28	0.0019	hypothetical protein
Rv2311		1.28	0.0124	hypothetical protein
Rv0136		1.28	0.0009	cytochrome p450
Rv2686c		1.28	0.0114	possible membrane protein
Rv1930c		1.28	0.0060	conserved hypothetical protein
Rv1594	nadA	1.28	0.0003	quinolinate synthase
Rv2006	otsB	1.28	0.0002	trehalose-6-phosphate phosphatase
Rv0843		1.27	0.0024	similar to various dehydrogenases
Rv2040c		1.27	0.0131	probable sugar transporter
Rv0097		1.27	0.0007	conserved hypothetical protein
Rv3174		1.27	0.0013	putative oxidoreductase
Rv0031		1.27	0.0010	conserved hypothetical protein
Rv0193c		1.27	0.0441	hypothetical protein
Rv1661	pks7	1.27	0.0074	polyketide synthase
Rv0762c		1.27	0.0138	hypothetical protein
Rv0943c		1.27	0.0003	probable monooxygenase
Rv2003c		1.26	0.0039	conserved hypothetical protein
Rv3344c	PE_PGRS	1.26	0.0230	PE_PGRS-family protein
Rv0569		1.26	0.0000	conserved hypothetical protein
Rv0160c	PE	1.26	0.0171	PE-family protein
Rv0484c		1.26	0.0010	oxidoreductase
Rv0106		1.26	0.0025	conserved hypothetical protein
Rv0976c		1.26	0.0061	hypothetical protein
Rv0330c		1.25	0.0038	hypothetical protein
Rv3125c	PPE	1.25	0.0000	PPE-family protein
Rv3000		1.25	0.0381	conserved hypothetical protein
Rv0985c	mscL	1.25	0.0123	highly similar to large-conductance mechanosensitive
Rv0371c		1.25	0.0141	Possible membrane protein
Rv0304c	PPE	1.25	0.0054	PPE-family protein
Rv3449		1.25	0.0250	probable precursor of serine protease
Rv0318c		1.25	0.0000	
Rv1058	fadD14	1.24	0.0009	acyl-CoA synthase
Rv3073c		1.24	0.0232	conserved hypothetical protein
Rv3612c		1.24	0.0110	hypothetical protein
Rv1358		1.24	0.0057	transcriptional regulator (LuxR/UhpA family)
Rv0771		1.24	0.0040	probable 4-carboxymuconolactone decarboxylase

Rv3558	PPE	1.24	0.0007	PPE-family protein
Rv3338		1.24	0.0016	conserved hypothetical protein
Rv3553		1.24	0.0133	similar to dioxygenasesdiooxygenases
Rv0101	nrp	1.24	0.0018	unknown non-ribosomal peptide synthase
Rv1917c	PPE	1.24	0.0000	PPE-family protein
Rv0098		1.23	0.0008	hypothetical protein
Rv3469c	mhpE	1.23	0.0005	probable 4-hydroxy-2-oxovalerate aldolase
Rv0329c		1.23	0.0017	conserved hypothetical protein
Rv1969		1.23	0.0000	part of mce3 operon
Rv0874c		1.23	0.0000	conserved hypothetical protein
Rv3555c		1.23	0.0007	conserved hypothetical protein
Rv3643		1.23	0.0040	hypothetical protein
Rv1490		1.23	0.0149	unknown putative membrane protein
Rv0797		1.23	0.0003	hypothetical protein
Rv2355		1.22	0.0087	possible IS6110 transposase
Rv0458		1.22	0.0464	aldehyde dehydrogenase
Rv3664c	dppC	1.22	0.0281	probable peptide transport system permease
Rv0876c		1.22	0.0023	possible membrane protein
Rv0456c	echA2	1.22	0.0055	enoyl-CoA hydratase/isomerase superfamily
Rv2874		1.22	0.0019	integral membrane protein
Rv0103c	ctpB	1.22	0.0034	cation transport ATPase
Rv0099	fadD10	1.21	0.0007	acyl-CoA synthase
Rv3112	moaD	1.21	0.0012	molybdopterin converting factor subunit 1
Rv1947		1.21	0.0002	hypothetical protein
Rv2835c	ugpA	1.21	0.0004	sn-glycerol-3-phosphate permease
Rv3531c		1.21	0.0147	hypothetical protein
Rv0768	aldA	1.21	0.0005	aldehyde dehydrogenases
Rv2748c	ftsK	1.21	0.0422	chromosome partitioning
Rv2670c		1.21	0.0360	conserved hypothetical protein
Rv2668		1.21	0.0202	hypothetical protein
Rv0095c		1.21	0.0049	REP-family protein
Rv3359		1.21	0.0271	probable oxidoreductase
Rv3124		1.21	0.0172	transcriptional regulator (AfsR/Dndl/RedD family)
Rv0977	PE_PGRS	1.21	0.0002	PE_PGRS-family protein
Rv0105c	rpmB	1.21	0.0013	50S ribosomal protein L28
Rv3166c		1.21	0.0162	conserved hypothetical protein
Rv1714		1.20	0.0001	Probable oxidoreductase/gluconate 3-dehydrogenase
Rv3163c		1.20	0.0077	possible membrane protein
Rv1504c		1.20	0.0050	conserved hypothetical protein
Rv2780	ald	1.20	0.0000	L-alanine dehydrogenase
Rv1563c	glgY	1.20	0.0015	putative [alpha]-amylase
Rv1619		1.19	0.0322	conserved hypothetical protein
Rv2811		1.19	0.0031	conserved hypothetical protein
Rv3064c		1.19	0.0006	conserved hypothetical protein
Rv1922		1.19	0.0080	probable penicillin binding protein
Rv1506c		1.19	0.0007	hypothetical protein
Rv1663	pks17	1.19	0.0148	polyketide synthase
Rv1134		1.19	0.0060	hypothetical protein
Rv0974c	accD2	1.19	0.0020	acetyl/propionyl-CoA carboxylase, [beta] subunit
Rv0570	nrdZ	1.19	0.0179	ribonucleotide reductase, class II
Rv2123	PPE	1.19	0.0012	phosphoribosyl-AMP cyclohydrolase
Rv0793		1.19	0.0047	conserved hypothetical protein

Rv3537		1.19	0.0398	3-oxosteroid 1-dehydrogenase
Rv0152c	PE	1.19	0.0015	PE-family protein
Rv3384c		1.18	0.0034	conserved hypothetical protein
Rv0942		1.18	0.0010	hypothetical protein
Rv0405	pks6	1.18	0.0288	polyketide synthase
Rv3415c		1.18	0.0136	conserved hypothetical protein
Rv3768		1.18	0.0510	hypothetical protein
Rv1973		1.18	0.0028	conserved hypothetical protein
Rv0773c	ggtA	1.18	0.0000	putative [gamma]-glutamyl transpeptidase
Rv1802	PPE	1.18	0.0038	PPE-family protein
Rv3021c	PPE	1.18	0.0028	PPE-family protein
Rv3324c	moaC3	1.18	0.0176	molybdenum cofactor biosynthesis, protein C
Rv0110		1.18	0.0005	transmembrane protein
Rv1146		1.18	0.0022	probable drug transporter
Rv0507	mmpL2	1.18	0.0005	conserved large membrane protein
Rv1510		1.17	0.0353	probable membrane proteinvery similar to
Rv0339c		1.17	0.0002	conserved hypothetical protein
Rv1552	frdA	1.17	0.0347	fumarate reductase flavoprotein subunit
Rv2834c	ugpE	1.17	0.0036	sn-glycerol-3-phosphate transport system protein
Rv3564	fadE33	1.17	0.0006	acyl-CoA dehydrogenase
Rv3902c		1.17	0.0008	hypothetical protein
Rv2122c	hisI	1.16	0.0379	conserved hypothetical protein
Rv0941c		1.16	0.0027	hypothetical protein
Rv2281	pitB	1.16	0.0019	phosphate permease
Rv2313c		1.16	0.0000	hypothetical protein
Rv2363	amiA2	1.16	0.0023	putative amidase
Rv2369c		1.16	0.0106	hypothetical protein
Rv2318	uspC	1.16	0.0066	sugar transport protein
Rv3288c		1.16	0.0055	conserved hypothetical protein
Rv3366	spoU	1.16	0.0123	probable rRNA methylase
Rv0819		1.16	0.0101	conserved hypothetical protein
Rv2280		1.16	0.0001	similar to D-lactate dehydrogenase
Rv0746	PE_PGRS	1.16	0.0015	PE_PGRS-family protein
Rv0417	thiG	1.16	0.0028	thiamine synthesis, thiazole moiety
Rv0228		1.15	0.0126	similar to acyltransferases
Rv1493	mutB	1.15	0.0218	methylmalonyl-CoA mutase, [alpha] subunit
Rv1755c	plcD	1.15	0.0120	partial CDS for phospholipase C
Rv0267	narU	1.15	0.0060	similar to nitrite extrusion protein 2
Rv0817c		1.15	0.0042	probable exported protein
Rv0481c		1.15	0.0009	hypothetical protein
Rv3534c		1.15	0.0018	4-hydroxy-2-oxovalerate aldolase
Rv3070		1.15	0.0107	unknown membrane protein (3 TM segments)
Rv3639c		1.15	0.0183	conserved hypothetical protein
Rv0354c	PPE	1.15	0.0024	PPE-family protein
Rv3370c	dnaE2	1.15	0.0309	DNA polymerase III [alpha] chain
Rv1720c		1.15	0.0142	conserved hypothetical protein
Rv1834		1.15	0.0049	conserved hypothetical protein
Rv0593	lprL	1.15	0.0433	part of mce2 operon
Rv0769		1.15	0.0155	similar to 7-alpha-hydroxysteroid dehydrogenase
Rv3453		1.15	0.0253	hypothetical protein
Rv3895c		1.14	0.0336	conserved hypothetical protein
Rv1027c	kdpE	1.14	0.0242	two-component response regulator

Rv0109	PE_PGRS	1.14	0.0004	PE_PGRS-family protein
Rv1777		1.14	0.0007	probable cytochrome p450
Rv2041c		1.14	0.0030	probable sugar transporter
Rv2026c		1.14	0.0002	conserved hypothetical protein
Rv0323c		1.14	0.0008	conserved hypothetical protein
Rv1350	fabG2	1.14	0.0325	3-oxoacyl-[ACP] reductase
Rv2025c		1.14	0.0076	possible membrane protein
Rv0488		1.14	0.0117	probable membrane protein
Rv0597c		1.14	0.0218	conserved hypothetical protein
Rv0415		1.14	0.0039	conserved hypothetical protein
Rv0388c	PPE	1.14	0.0139	PPE-family protein
Rv0777	purB	1.14	0.0000	adenylosuccinate lyase
Rv3786c		1.14	0.0019	hypothetical protein
Rv0782	ptrBa	1.14	0.0211	protease II, [alpha] subunit
Rv0509	hemA	1.13	0.0020	glutamyl-tRNA reductase
Rv0347		1.13	0.0020	conserved hypothetical protein
Rv2408	PE	1.13	0.0029	PE-family protein
Rv3430c		1.13	0.0173	transposase
Rv1526c		1.13	0.0189	possible rhamnosyl/glycosyl transferase
Rv3079c		1.13	0.0068	probable monooxygenase
Rv3559c		1.13	0.0159	short-chain alcohol dehydrogenase
Rv2566		1.13	0.0300	hypothetical protein
Rv1554	frdC	1.13	0.0010	fumarate reductase 15kD anchor protein
Rv2305		1.13	0.0046	hypothetical protein
Rv0385		1.13	0.0107	similar to oxidoreductases
Rv0540		1.12	0.0034	conserved hypothetical protein
Rv3894c		1.12	0.0183	transmembrane ATP/GTP binding protein
Rv3107c		1.12	0.0159	Some similarity to D-lactate dehydrogenase
Rv0372c		1.12	0.0007	conserved hypothetical protein
Rv0690c		1.12	0.0021	hypothetical protein
Rv0775		1.12	0.0135	conserved hypothetical protein
Rv2848c	cobB	1.12	0.0033	cobyrinic acid a,c-diamide synthase
Rv2723		1.12	0.0088	probable membrane protein, tellurium resistance
Rv0320		1.12	0.0138	conserved hypothetical protein
Rv2337c		1.12	0.0118	hypothetical protein
Rv2638		1.12	0.0084	conserved hypothetical protein
Rv1291c		1.12	0.0146	conserved hypothetical protein
Rv1972		1.12	0.0305	conserved hypothetical protein
Rv1063c		1.12	0.0108	conserved hypothetical protein
Rv0359		1.11	0.0147	conserved hypothetical protein
Rv0235c		1.11	0.0494	conserved hypothetical protein
Rv1372	pks18	1.11	0.0079	polyketide synthase
Rv0311		1.11	0.0392	hypothetical protein
Rv0294		1.11	0.0156	conserved hypothetical protein
Rv1995		1.11	0.0075	hypothetical protein
Rv1664	pks9	1.11	0.0004	polyketide synthase
Rv1705c	PPE	1.11	0.0091	PPE-family protein
Rv0218		1.11	0.0385	some similarity with sulphitesulfite oxidases
Rv0600c		1.11	0.0390	sensor histidine kinase
Rv0245		1.10	0.0350	probable monooxygenase
Rv0306		1.10	0.0118	similar to BluB cobalamin synthesis protein R.
Rv0084	hycD	1.10	0.0455	formate hydrogenlyase subunit 4

Rv3175		1.10	0.0082	Probable amidase
Rv0081		1.10	0.0001	transcriptional regulator (ArsR family)
Rv0585c		1.10	0.0452	possible membrane protein
Rv1496		1.10	0.0158	conserved hypothetical protein
Rv2765		1.10	0.0037	hypothetical protein
Rv2546		1.10	0.0299	conserved hypothetical protein
Rv1852	ureG	1.10	0.0018	urease accessory protein
Rv3550	echA20	1.10	0.0245	enoyl-CoA hydratase/isomerase superfamily
Rv3546	fadA5	1.10	0.0018	acetyl-CoA C-acetyltransferase
Rv1668c		1.09	0.0017	probable ABC transporter
Rv0728c		1.09	0.0137	similar to D-3-phosphoglycerate dehydrogenases
Rv1736c	narX	1.09	0.0317	fused nitrate reductase
Rv0539		1.09	0.0217	probable glycosyltransferase
Rv2265		1.09	0.0043	putative integral membrane protein
Rv2248		1.09	0.0010	conserved hypothetical protein
Rv0387c		1.09	0.0000	conserved hypothetical protein
Rv0630c	recB	1.09	0.0173	exodeoxyribonuclease V
Rv0377		1.09	0.0071	transcriptional regulator (LysR family)
Rv0964c		1.09	0.0053	hypothetical protein
Rv3181c		1.09	0.0053	conserved hypothetical protein
Rv2319c		1.09	0.0013	hypothetical protein
Rv1843c	guaB1	1.09	0.0355	inosine-5'-monophosphate dehydrogenase
Rv1966	mce3	1.09	0.0405	cell invasion protein
Rv2292c		1.09	0.0001	hypothetical protein
Rv0493c		1.09	0.0024	conserved hypothetical protein
Rv2387		1.09	0.0001	conserved hypothetical protein
Rv2896c		1.09	0.0196	conserved hypothetical protein
Rv3129		1.09	0.0018	conserved hypothetical protein
Rv3060c		1.09	0.0011	transcriptional regulator (GntR family)
Rv2819c		1.08	0.0006	hypothetical protein
Rv2159c		1.08	0.0115	hypothetical protein
Rv3764c		1.08	0.0003	sensor histidine kinase
Rv2317	uspE	1.08	0.0184	sugar transport protein
Rv1124	ephC	1.08	0.0003	probable epoxide hydrolase
Rv0564c	gpdA1	1.07	0.0232	glycerol-3-phosphate dehydrogenase
Rv3398c	idsA	1.07	0.0473	geranylgeranyl pyrophosphate synthase
Rv0194		1.07	0.0002	Probable ABC transporter
Rv1764		1.07	0.0175	possible IS6110 transposase
Rv0766c		1.07	0.0109	cytochrome p-450
Rv0195		1.07	0.0216	transcriptional regulator (LuxR/UhpA family)
Rv0966c		1.07	0.0098	conserved hypothetical protein
Rv1064c	lpqV	1.07	0.0019	lipoprotein
Rv2608	PPE	1.06	0.0000	PPE-family protein
Rv2649		1.06	0.0035	possible IS6110 transposase
Rv1104		1.06	0.0124	probable esterase pseudogene
Rv0253	nirD	1.06	0.0063	probable nitrite reductase small subunit
Rv1896c		1.06	0.0065	conserved hypothetical protein
Rv1492	mutA	1.06	0.0162	methylmalonyl-CoA mutase, [beta] subunit
Rv0032	bioF2	1.06	0.0037	C-terminal similar to B. subtilis BioF
Rv2774c		1.06	0.0025	hypothetical protein
Rv0779c		1.06	0.0397	conserved hypothetical protein
Rv1403c		1.06	0.0025	similar to phosphatidylethanolamine N-methyltransferase

Rv3447c		1.06	0.0009	probable membrane protein
Rv2644c		1.05	0.0013	hypothetical protein
Rv2646		1.05	0.0003	phiRV2 integrase
Rv1514c		1.05	0.0268	involved in polysaccharide synthesis
Rv0376c		1.05	0.0281	conserved hypothetical protein
Rv1866		1.05	0.0045	conserved hypothetical protein
Rv2401		1.05	0.0148	hypothetical protein
Rv1953		1.05	0.0132	conserved hypothetical protein
Rv3360		1.05	0.0429	possible ABC transporter
Rv0102		1.05	0.0002	membrane protein
Rv0591		1.05	0.0328	part of mce2 operon
Rv0899	ompA	1.05	0.0396	member of OmpA family
Rv0355c	PPE	1.05	0.0161	PPE-family protein
Rv3076		1.05	0.0453	conserved hypothetical protein
Rv3097c	PE	1.04	0.0191	PE-family protein
Rv3098c		1.04	0.0068	hypothetical protein
Rv2479c		1.04	0.0018	possible IS6110 transposase
Rv2767c		1.04	0.0045	hypothetical protein
Rv0087	hycE	1.04	0.0090	probable formate hydrogenlyase subunit 5
Rv1623c	appC	1.04	0.0106	cytochrome bd-II oxidase subunit I
Rv2650c		1.04	0.0014	phiRV2 phage related protein
Rv2088	pknJ	1.04	0.0185	serine-threonine protein kinase
Rv0305c	PPE	1.04	0.0143	PPE-family protein
Rv2383c	mbtB	1.03	0.0137	mycobactin/exochelin synthesis (serine/threonine
Rv1767		1.03	0.0339	conserved hypothetical protein
Rv3278c		1.03	0.0360	conserved hypothetical protein
Rv1138c		1.03	0.0126	conserved hypothetical protein
Rv3170		1.03	0.0013	Probable flavin-containing monoamine oxidase
Rv1892		1.03	0.0005	hypothetical protein
Rv1940	ribA	1.03	0.0098	GTP cyclohydrolase II
Rv1482c		1.03	0.0258	conserved hypothetical protein
Rv0216		1.03	0.0142	conserved hypothetical protein
Rv2684	arsA	1.03	0.0253	probable arsenical pump
Rv0508		1.03	0.0282	hypothetical protein
Rv0394c		1.03	0.0001	hypothetical protein
Rv0649	fabD2	1.02	0.0384	malonyl CoA-[ACP] transacylase
Rv1908c	katG	1.02	0.0230	catalase-peroxidase
Rv1255c		1.02	0.0296	transcriptional regulator (TetR/AcrR family)
Rv0326		1.02	0.0021	hypothetical protein
Rv2483c		1.02	0.0060	possible transferase
Rv1128c		1.02	0.0006	REP-family protein
Rv1688		1.02	0.0183	probable 3-methylpurine DNA glycosylase
Rv3312c		1.02	0.0155	conserved hypothetical protein
Rv0800	pepC	1.02	0.0025	aminopeptidase I
Rv0866	moaE2	1.02	0.0020	molybdopterin-converting factor subunit 2
Rv0674		1.02	0.0115	hypothetical protein
Rv0398c		1.02	0.0001	hypothetical protein
Rv3179		1.02	0.0008	conserved hypothetical protein
Rv1801	PPE	1.02	0.0427	PPE-family protein
Rv0042c		1.02	0.0048	conserved hypothetical protein
Rv0062	celA	1.01	0.0068	cellulase/endoglucanase
Rv1359		1.01	0.0061	putative transcriptional regulator

Rv2688c		1.01	0.0021	similar to transport ATP-binding proteins
Rv1680		1.01	0.0214	hypothetical protein
Rv3809c	glf	1.01	0.0191	UDP-galactopyranose mutase
Rv3061c	fadE22	1.01	0.0363	acyl-CoA dehydrogenase
Rv2557		1.01	0.0183	conserved hypothetical protein
Rv0827c		1.01	0.0186	transcriptional regulator (ArsR family)
Rv2267c		1.01	0.0223	hypothetical protein
Rv0592		1.01	0.0015	part of mce2 operon
Rv2893		1.01	0.0054	similar to alkanal monooxygenase alpha chain
Rv2004c		1.01	0.0080	hypothetical protein
Rv0269c		1.01	0.0448	conserved hypothetical protein
Rv0210		1.01	0.0069	hypothetical protein
Rv2045c	lipT	1.01	0.0058	probable carboxylesterase
Rv0416		1.01	0.0062	conserved hypothetical protein
Rv1500		1.01	0.0264	similarity to B. subtilis glycosyltransferase
Rv3538	ufaA2	1.01	0.0060	unknown fatty acid methyltransferase
Rv3164c	moxR3	1.00	0.0000	transcriptional regulator, MoxR homologue
Rv2486	echA14	1.00	0.0195	enoyl-CoA hydratase/isomerase superfamily
Rv2282c		1.00	0.0004	transcriptional regulator (LysR family)
Rv3266c	rmlD	1.00	0.0117	dTDP-4-dehydrorhamnose reductase
Rv0158		1.00	0.0376	transcriptional regulator (TetR/AcrR family)
Rv0151c	PE	1.00	0.0055	PE-family protein
Rv3402c		1.00	0.0200	possible involved in LPS synthesis
Rv0121c		1.00	0.0001	hypothetical protein
Rv2240c		1.00	0.0036	conserved hypothetical protein
Rv1112		1.00	0.0056	conserved hypothetical protein
Rv1578c		1.00	0.0164	phiRV1 phage related protein
Rv3380c		1.00	0.0158	possible IS6110 transposase
Rv2038c		1.00	0.0064	probable ABC sugar transporter
Rv0457c		1.00	0.0006	probable peptidase
Rv0897c		1.00	0.0002	possible oxidoreductase
Rv1253	deaD	1.00	0.0420	ATP-dependent DNA/RNA helicase
Rv3247c	tmk	0.99	0.0015	thymidylate kinase
Rv2089c	pepE	0.99	0.0245	cytoplasmic peptidase
Rv0328		0.99	0.0075	transcriptional regulator (TetR/AcrR family)
Rv0406c		0.99	0.0127	conserved hypothetical protein
Rv3108		0.99	0.0021	hypothetical protein
Rv2820c		0.99	0.0002	hypothetical protein
Rv1557	mmpL6	0.99	0.0098	conserved large membrane protein
Rv2616		0.99	0.0187	hypothetical protein
Rv1456c		0.99	0.0001	probable membrane protein
Rv2236c	cobD	0.99	0.0016	cobinamide synthase
Rv2769c	PE	0.99	0.0103	PE-family protein
Rv3562	fadE31	0.99	0.0002	acyl-CoA dehydrogenase
Rv0116c		0.99	0.0023	conserved hypothetical protein
Rv0118c	oxcA	0.99	0.0232	oxalyl-CoA decarboxylase
Rv1089	PE	0.99	0.0207	PE-family protein
Rv1260		0.98	0.0007	probable oxidoreductase
Rv3892c	PPE	0.98	0.0047	PPE-family protein
Rv2263		0.98	0.0130	possible oxidoreductase
Rv1756c		0.98	0.0000	possible IS6110 transposase
Rv3326		0.98	0.0257	possible IS6110 transposase

Rv2654c		0.98	0.0151	phiRV2 phage related protein
Rv2545		0.98	0.0194	conserved hypothetical protein
Rv1576c		0.98	0.0001	phiRV1 phage related protein
Rv1662	pks8	0.98	0.0244	polyketide synthase
Rv1276c		0.98	0.0123	hypothetical protein
Rv0029		0.98	0.0006	conserved hypothetical protein
Rv3066		0.98	0.0001	putative transcriptional regulator
Rv3473c	bpoA	0.98	0.0075	probable non-heme bromoperoxidase
Rv1715	fadB3	0.97	0.0152	3-hydroxyacyl-CoA dehydrogenase
Rv0077c		0.97	0.0463	probable oxidoreductase
Rv3529c		0.97	0.0209	conserved hypothetical protein
Rv2548		0.97	0.0190	conserved hypothetical protein
Rv0375c		0.97	0.0115	putative CO dehydrogenase gene cluster
Rv2353c	PPE	0.97	0.0271	PPE-family protein
Rv2307c		0.97	0.0022	conserved hypothetical protein
Rv1115		0.97	0.0236	hypothetical protein
Rv2695		0.97	0.0274	conserved hypothetical protein
Rv2359	furB	0.97	0.0157	ferric uptake regulatory protein
Rv3670	ephE	0.96	0.0022	probable epoxide hydrolase
Rv0007		0.96	0.0491	conserved hypothetical protein
Rv3758c	proV	0.96	0.0003	osmoprotection ABC transporter
Rv1910c		0.96	0.0180	probable secreted protein
Rv3505	fadE27	0.95	0.0056	acyl-CoA dehydrogenase
Rv0100		0.95	0.0233	hypothetical protein
Rv0435c		0.95	0.0001	ATPase of AAA-family
Rv1354c		0.95	0.0201	conserved hypothetical protein
Rv1145		0.95	0.0384	probable drug transporter
Rv0265c	fecB2	0.95	0.0385	iron transport protein FeIII dicitrate transporter
Rv1946c	lppG	0.95	0.0311	lipoprotein
Rv1550	fadD11	0.95	0.0020	acyl-CoA synthase, N-term
Rv1000		0.95	0.0339	hypothetical protein
Rv1132		0.95	0.0044	possible transporter
Rv0764c		0.95	0.0051	possible lanosterol 14-demethylase cytochrome P450
Rv2972c		0.95	0.0469	hypothetical protein
Rv2379c	mbtF	0.95	0.0316	mycobactin/exochelin synthesis (lysine ligation)
Rv2320c	rocE	0.95	0.0114	arginine/ornithine transporter
Rv1758		0.95	0.0102	partial cutinase
Rv2856	nicT	0.94	0.0178	probable nickel transport protein
Rv2810c		0.94	0.0129	probable truncated transposase
Rv2242		0.94	0.0012	hypothetical protein
Rv0254c	cobU	0.94	0.0381	cobinamide kinase
Rv1968		0.94	0.0210	part of mce3 operon
Rv1066		0.94	0.0001	conserved hypothetical protein
Rv1937		0.94	0.0482	similar to ring-hydroxylating dioxygenases
Rv1666c		0.94	0.0036	Probable cytochrome p450
Rv0551c	fadD8	0.94	0.0001	acyl-CoA synthase
Rv1935c	echA13	0.94	0.0323	enoyl-CoA hydratase/isomerase superfamily
Rv1640c	lysX	0.94	0.0059	C-term lysyl-tRNA synthase
Rv2514c		0.94	0.0320	hypothetical protein
Rv2770c	PPE	0.94	0.0229	PPE-family protein
Rv2484c		0.94	0.0001	conserved hypothetical protein
Rv1894c		0.93	0.0032	some similarity to dioxygenases

Rv1536	ileS	0.93	0.0029	isoleucyl-tRNA synthase
Rv2804c		0.93	0.0040	hypothetical protein
Rv0752c	fadE9	0.93	0.0073	acyl-CoA dehydrogenase
Rv1868		0.93	0.0000	conserved hypothetical protein
Rv0122		0.93	0.0006	hypothetical protein
Rv0117	oxyS	0.93	0.0094	transcriptional regulator (LysR family)
Rv0132c		0.93	0.0033	putative oxidoreductase
Rv3169		0.93	0.0063	conserved hypothetical protein
Rv0561c		0.93	0.0026	similar to squalene monooxygenase
Rv3614c		0.93	0.0191	conserved hypothetical protein
Rv2918c	glnD	0.93	0.0070	uridylyltransferase
Rv0808	purF	0.93	0.0337	amidophosphoribosyltransferase
Rv3382c	lytB	0.93	0.0001	LytB protein homologue
Rv2064	cobG	0.93	0.0204	percorrin reductase
Rv1431		0.93	0.0315	possible transporter
Rv2279		0.92	0.0121	possible IS6110 transposase
Rv0340		0.92	0.0089	hypothetical protein
Rv2543	lppA	0.92	0.0078	lipoprotein
Rv0494		0.92	0.0059	transcriptional regulator (GntR family)
Rv1862	adhA	0.92	0.0178	alcohol dehydrogenase (Zn)
Rv1539	lspA	0.92	0.0274	lipoprotein signal peptidase
Rv3832c		0.92	0.0044	hypothetical protein
Rv0938		0.92	0.0051	conserved hypothetical protein
Rv1682		0.92	0.0026	conserved hypothetical protein
Rv2655c		0.91	0.0288	phiRV2 phage related protein
Rv3126c		0.91	0.0211	hypothetical protein
Rv0693	pqqE	0.91	0.0282	coenzyme pqq synthesis protein E
Rv0293c		0.91	0.0181	conserved hypothetical protein
Rv0078		0.91	0.0144	transcriptional regulator (TetR/AcrR family)
Rv3618		0.91	0.0016	similar bacterial luciferase alpha chains
Rv0998		0.91	0.0303	hypothetical protein
Rv1888c		0.90	0.0067	hypothetical protein
Rv2296		0.90	0.0006	halokane dehalogenase
Rv2133c		0.90	0.0227	conserved hypothetical protein
Rv1090		0.90	0.0010	probable inactivated cellulase/endoglucanase
Rv0776c		0.90	0.0001	conserved hypothetical protein
Rv0696		0.90	0.0402	glycosyltransferase
Rv1086		0.90	0.0002	conserved hypothetical protein
Rv0913c		0.90	0.0232	probable dioxygenase
Rv3756c	proZ	0.90	0.0001	transport system permease
Rv0277c		0.90	0.0350	conserved hypothetical protein
Rv3507	PE_PGRS	0.89	0.0016	PE_PGRS-family protein
Rv0307c		0.89	0.0317	hypothetical protein
Rv1804c		0.89	0.0024	conserved hypothetical protein
Rv1114		0.89	0.0074	conserved hypothetical protein
Rv2818c		0.89	0.0027	hypothetical protein
Rv0794c	lpdB	0.89	0.0233	dihydrolipoamide dehydrogenase
Rv2474c		0.89	0.0019	hypothetical protein
Rv1268c		0.89	0.0023	hypothetical protein
Rv2803c		0.89	0.0003	hypothetical protein
Rv3238c		0.89	0.0005	unknown, possible membrane protein
Rv1567c		0.89	0.0185	hypothetical protein

Rv1249c		0.89	0.0117	possible membrane protein
Rv1528c	papA4	0.88	0.0065	PKS-associated protein, unknown function
Rv3762c		0.88	0.0105	probable alkyl sulfatase
Rv0770		0.88	0.0056	similar to 3-hydroxyisobutyrate dehydrogenase
Rv3637		0.88	0.0089	hypothetical protein
Rv1250		0.88	0.0006	probable drug efflux protein
Rv3055		0.88	0.0001	putative transcriptional regulator
Rv2828c		0.88	0.0027	conserved hypothetical protein
Rv0180c		0.88	0.0092	probable membrane protein
Rv3434c		0.88	0.0061	conserved hypothetical protein
Rv0124	PE_PGRS	0.88	0.0080	PE_PGRS-family protein
Rv1136		0.88	0.0135	probable carnitine racemase
Rv1137c		0.88	0.0079	hypothetical protein
Rv2618		0.87	0.0004	conserved hypothetical protein
Rv1928c		0.87	0.0241	short-chain alcohol dehydrogenase family
Rv2761c		0.87	0.0089	hypothetical protein
Rv3773c		0.87	0.0287	conserved hypothetical protein
Rv2980		0.87	0.0001	hypothetical protein
Rv2814c		0.87	0.0018	possible IS6110 transposase
Rv3856c		0.87	0.0498	conserved hypothetical protein
Rv0389	purT	0.87	0.0072	phosphoribosylglycinamide formyltransferase II
Rv2393		0.87	0.0107	conserved hypothetical protein
Rv2636		0.87	0.0254	hypothetical protein
Rv0629c	recD	0.87	0.0143	exodeoxyribonuclease V
Rv2023c		0.87	0.0028	hypothetical protein
Rv0030		0.87	0.0224	hypothetical protein
Rv2990c		0.87	0.0073	hypothetical protein
Rv1075c		0.87	0.0302	conserved hypothetical protein
Rv3481c		0.87	0.0102	possible membrane protein
Rv3018c	PPE	0.87	0.0092	PPE-family protein
Rv0731c		0.87	0.0002	conserved hypothetical protein
Rv1787	PPE	0.86	0.0290	PPE-family protein
Rv3273		0.86	0.0015	C-term similar to carbonic anhydrase
Rv2406c		0.86	0.0278	conserved hypothetical protein
Rv0727c	fucA	0.86	0.0165	L-fuculose phosphate aldolase
Rv0914c		0.86	0.0002	lipid transfer protein
Rv2262c		0.86	0.0078	apolipoprotein N-acyltransferase-b
Rv1773c		0.86	0.0508	transcriptional regulator (IclR family)
Rv1394c		0.86	0.0159	possible cytochrome p450
Rv1672c		0.86	0.0002	probable ABC transporter
Rv2365c		0.86	0.0085	conserved hypothetical protein
Rv2482c	plsB2	0.86	0.0070	glycerol-3-phosphate acyltransferase
Rv3056	dinP	0.86	0.0234	DNA-damage-inducible protein
Rv1687c		0.85	0.0449	probable ABC transporter
Rv2277c		0.85	0.0236	possible glycerolphosphodiesterase
Rv2270	lppN	0.85	0.0011	possible lipoprotein
Rv3698		0.85	0.0003	hypothetical protein
Rv1564c	glgX	0.85	0.0458	probable glycogen debranching enzyme
Rv2361c		0.85	0.0413	conserved hypothetical protein
Rv2669		0.85	0.0109	putative transcriptional regulator
Rv1068c	PE_PGRS	0.85	0.0040	PE_PGRS-family protein
Rv1470	trxA	0.84	0.0030	thioredoxin

Rv3185		0.84	0.0008	possible IS6110 transposase
Rv1975		0.84	0.0014	hypothetical protein
Rv3026c		0.84	0.0006	some similarity to acyltransferase Q59601
Rv0411c	glnH	0.84	0.0074	putative glutamine binding protein
Rv0420c		0.84	0.0115	hypothetical protein
Rv0378		0.84	0.0309	conserved hypothetical protein
Rv1634		0.84	0.0160	probable drug efflux protein
Rv0107c	ctpI	0.84	0.0002	probable magnesium transport ATPase
Rv0738		0.84	0.0006	possible membrane protein
Rv2640c		0.84	0.0013	transcriptional regulator (ArsR family)
Rv0236c		0.84	0.0286	possible membrane protein
Rv0399c	lpqK	0.84	0.0389	possible PBP
Rv1618	tesB1	0.84	0.0508	thioesterase II
Rv0939		0.84	0.0171	probable dehydrase
Rv1205		0.84	0.0074	conserved hypothetical protein
Rv1376		0.84	0.0164	conserved hypothetical protein
Rv3121		0.84	0.0014	probable cytochrome p450
Rv0115		0.83	0.0001	conserved hypothetical protein
Rv3445c		0.83	0.0506	hypothetical protein
Rv0573c		0.83	0.0056	conserved hypothetical protein
Rv1573		0.83	0.0194	phiRV1 phage related protein
Rv3475		0.83	0.0041	possible IS6110 transposase
Rv0386		0.83	0.0224	transcriptional regulator (LuxR/UhpA family)
Rv0130		0.83	0.0060	conserved hypothetical protein
Rv0930	pstA1	0.83	0.0087	PstA component of phosphate uptake
Rv0266c		0.83	0.0168	conserved hypothetical protein
Rv1273c		0.83	0.0106	ABC transporter
Rv0917	betP	0.83	0.0014	glycine betaine transport
Rv1499		0.83	0.0007	hypothetical protein
Rv3615c		0.82	0.0426	conserved hypothetical protein
Rv0380c		0.82	0.0002	some similarity with methyltransferases
Rv0772	purD	0.82	0.0163	phosphoribosylamine-glycine ligase
Rv3439c		0.82	0.0502	conserved hypothetical protein
Rv3834c	serS	0.82	0.0037	seryl-tRNA synthase
Rv0604	lpqO	0.82	0.0113	lipoprotein
Rv3346c		0.82	0.0220	conserved hypothetical protein
Rv0370c		0.82	0.0000	putative CO dehydrogenase gene cluster
Rv3433c		0.82	0.0001	conserved hypothetical protein
Rv2253		0.82	0.0217	hypothetical protein
Rv1958c		0.82	0.0018	hypothetical protein
Rv2314c		0.82	0.0497	conserved hypothetical protein
Rv1585c		0.82	0.0061	phiRV1 phage related protein
Rv1561		0.82	0.0422	conserved hypothetical protein
Rv1674c		0.82	0.0276	putative transcriptional regulator
Rv2825c		0.81	0.0093	conserved hypothetical protein
Rv0252	nirB	0.81	0.0177	nitrite reductase flavoprotein
Rv0868c	moaD2	0.81	0.0010	molybdopterin converting factor subunit 1
Rv2651c		0.81	0.0015	phiRV2 prohead protease
Rv1673c		0.81	0.0013	conserved hypothetical protein
Rv1722		0.81	0.0241	possible biotin carboxylase
Rv2658c		0.81	0.0000	phiRV2 phage related protein
Rv2796c	lppV	0.81	0.0060	lipoprotein

Rv0202c	mmpL11	0.81	0.0169	conserved large membrane protein
Rv2451		0.81	0.0359	hypothetical protein
Rv3542c		0.81	0.0221	hypothetical protein
Rv1084		0.81	0.0042	conserved hypothetical protein
Rv1188		0.81	0.0159	probable dehydrogenase
Rv1474c		0.81	0.0000	transcriptional regulator (TetR/AcrR family)
Rv1489c		0.81	0.0100	hypothetical protein
Rv1670		0.81	0.0151	conserved hypothetical protein
Rv3743c		0.81	0.0245	probable cation-transporting ATPase
Rv1119c		0.80	0.0401	hypothetical protein
Rv0875c		0.80	0.0306	possible exported protein
Rv0128		0.80	0.0405	hypothetical protein
Rv0816c	thiX	0.80	0.0457	equivalent to M. leprae ThiX
Rv3253c		0.80	0.0324	probable cationic amino acid transport
Rv2268c		0.80	0.0123	Probable cytochrome P-450
Rv0274		0.80	0.0000	hypothetical protein
Rv3544c	fadE28	0.80	0.0042	acyl-CoA dehydrogenase
Rv2867c		0.80	0.0411	hypothetical protein
Rv0212c	nadR	0.80	0.0030	similar to E.coli NadR
Rv1553	frdB	0.80	0.0129	fumarate reductase iron sulphur protein
Rv0454		0.80	0.0021	conserved hypothetical protein
Rv3861		0.80	0.0269	hypothetical protein
Rv3068c	pgmA	0.80	0.0041	phosphoglucomutase
Rv1534		0.80	0.0213	transcriptional regulator (TetR/AcrR family)
Rv3784	epiB	0.79	0.0294	probable UDP-galactose 4-epimerase
Rv3328c		0.79	0.0007	similar to SigI, ECF family
Rv2315c		0.79	0.0060	conserved hypothetical protein
Rv1545		0.79	0.0294	hypothetical protein
Rv2266		0.79	0.0054	Probable cytochrome P-450
Rv1122	gnd2	0.79	0.0002	6-phosphogluconate dehydrogenase (Gram +)
Rv3236c	kefB	0.79	0.0047	probable glutathione-regulated potassium-efflux
Rv0886	fprB	0.79	0.0003	ferredoxin, ferredoxin-NADP reductase
Rv1878	glnA3	0.79	0.0182	probable glutamine synthase
Rv3300c		0.79	0.0179	probable deaminase, riboflavin synthesis
Rv0735	sigL	0.79	0.0454	sigma-70 factors ECF subfamily
Rv0319	pcp	0.78	0.0022	pyrrolidone-carboxylate peptidase
Rv3563	fadE32	0.78	0.0086	acyl-CoA dehydrogenase
Rv0391	metZ	0.78	0.0052	o-succinylhomoserine sulfhydrylase
Rv0487		0.78	0.0037	conserved hypothetical protein
Rv3540c	ltp2	0.78	0.0394	non-specific lipid transport protein
Rv1166	lpqW	0.78	0.0493	lipoprotein
Rv0616c		0.78	0.0215	hypothetical protein
Rv2100		0.78	0.0003	PE_PGRS-family protein
Rv1264		0.78	0.0053	similar to adenylate cyclases
Rv2364c	bex	0.78	0.0447	GTP-binding protein of Era/ThdF family
Rv0531		0.78	0.0185	unknown, membrane protein.
Rv2377c	mbtH	0.78	0.0136	mycobactin/exochelin synthesis
Rv3594		0.78	0.0423	hypothetical protein
Rv2892c	PPE	0.78	0.0282	PPE-family protein
Rv3251c	rubA	0.78	0.0031	rubredoxin A
Rv3286c	sigF	0.78	0.0327	ECF subfamily sigma subunit
Rv1524		0.78	0.0049	possible rhamnosyl/glycosyl transferase

Rv3561	fadD3	0.77	0.0199	acyl-CoA synthase
Rv3732		0.77	0.0477	conserved hypothetical protein
Rv2288		0.77	0.0004	hypothetical protein
Rv0024		0.77	0.0340	putative p60 homologue
Rv0960		0.77	0.0124	conserved hypothetical protein
Rv1351		0.77	0.0053	hypothetical protein
Rv1330c		0.77	0.0025	conserved hypothetical protein
Rv0138		0.77	0.0316	conserved hypothetical protein
Rv2381c	mbtD	0.77	0.0001	mycobactin/exochelin synthesis (polyketide
Rv3082c	virS	0.77	0.0002	putative virulence regulating protein (AraC/XylS
Rv1718		0.77	0.0153	conserved hypothetical protein
Rv1227c		0.77	0.0000	probable transmembrane protein
Rv0390		0.77	0.0246	hypothetical protein
Rv3437		0.77	0.0419	hypothetical protein
Rv2378c	mbtG	0.77	0.0046	mycobactin/exochelin synthesis (lysine hydroxylase)
Rv3123		0.77	0.0012	hypothetical protein
Rv1667c		0.77	0.0001	Probable ABC transporter
Rv3650	PE	0.76	0.0078	PE-family protein
Rv0214	fadD4	0.76	0.0489	acyl-CoA synthase
Rv1118c		0.76	0.0102	hypothetical protein
Rv0022c		0.76	0.0301	conserved hypothetical protein
Rv0940c		0.76	0.0004	probable monooxygenase
Rv0836c		0.76	0.0108	hypothetical protein
Rv3766		0.76	0.0000	hypothetical protein
Rv2405		0.76	0.0222	hypothetical protein
Rv0574c		0.76	0.0091	conserved hypothetical protein
Rv1681	moeX	0.76	0.0188	weak similarity to E. coli MoaA
Rv0486		0.76	0.0214	conserved hypothetical protein
Rv1918c	PPE	0.76	0.0031	PPE-family protein
Rv3500c		0.76	0.0130	part of mce4 operon
Rv0362	mgtE	0.76	0.0032	putative magnesium ion transporter
Rv1951c		0.76	0.0029	conserved hypothetical protein
Rv0174		0.76	0.0001	part of mce1 operon
Rv0336		0.76	0.0288	conserved hypothetical protein
Rv2808		0.75	0.0365	hypothetical protein
Rv3378c		0.75	0.0005	hypothetical protein
Rv2676c		0.75	0.0089	conserved hypothetical protein
Rv3566c	nhoA	0.75	0.0040	N-hydroxyarylamine o-acetyltransferase
Rv1163	narJ	0.75	0.0368	nitrate reductase [delta] chain
Rv1426c	lipO	0.75	0.0033	probable esterase
Rv0482	murB	0.75	0.0003	UDP-N-acetylenolpyruvoylglucosamine reductase
Rv0924c	nramp	0.75	0.0035	transmembrane protein belonging to Nramp family
Rv1332		0.75	0.0002	putative transcriptional regulator
Rv0610c		0.75	0.0395	possible monooxygenase
Rv2424c		0.75	0.0039	hypothetical protein
Rv3706c		-0.76	0.0286	hypothetical protein
Rv3587c		-0.76	0.0355	hypothetical protein
Rv0058	dnaB	-0.77	0.0281	DNA helicase (contains intein)
Rv2703	sigA	-0.79	0.0269	RNA polymerase sigma factor (aka MysA, RpoV)
Rv3260c	whiB2	-0.79	0.0405	WhiB transcriptional activator homologue
Rv2156c	murX	-0.80	0.0000	phospho-N-acetylmuramoyl-pentapeptide transferase
Rv3484	cpsA	-0.80	0.0285	cpsA,CpsA : Q50160

Rv2697c	dut	-0.81	0.0102	deoxyuridine triphosphatase
Rv2445c	ndkA	-0.81	0.0416	nucleoside diphosphate kinase
Rv3224		-0.83	0.0087	putative oxidoreductases
Rv0951	sucC	-0.83	0.0398	succinyl-CoA synthase [beta] chain
Rv3458c	rpsD	-0.84	0.0056	30S ribosomal protein S4
Rv0129c	fbpC2	-0.85	0.0082	antigen 85C, mycolytransferase
Rv0848	cysM3	-0.86	0.0340	putative cysteine synthase
Rv2876		-0.86	0.0138	hypothetical protein
Rv0682	rpsL	-0.90	0.0422	30S ribosomal protein S12
Rv3442c	rpsI	-0.93	0.0184	30S ribosomal protein S9
Rv2441c	rpmA	-0.94	0.0070	50S ribosomal protein L27
Rv0722	rpmD	-0.94	0.0378	50S ribosomal protein L30
Rv3628	ppa	-0.94	0.0041	probable inorganic pyrophosphatase
Rv1779c		-0.96	0.0140	possible integral membrane protein
Rv0056	rplI	-1.02	0.0368	50S ribosomal protein L9
Rv3248c	sahH	-1.05	0.0004	adenosylhomocysteinase
Rv0054	ssb	-1.07	0.0440	single strand binding protein
Rv3846	sodA	-1.12	0.0039	superoxide dismutase
Rv0867c		-1.13	0.0394	probable exported protein
Rv0716	rplE	-1.16	0.0380	50S ribosomal protein L5
Rv3804c	fbpA	-1.16	0.0126	antigen 85A, mycolyltransferase
Rv2450c		-1.20	0.0045	conserved hypothetical protein
Rv3924c	rpmH	-1.21	0.0367	50S ribosomal protein L34
Rv3460c	rpsM	-1.28	0.0017	30S ribosomal protein S13
Rv2243	fabD	-1.31	0.0414	malonyl CoA-[ACP] transacylase
Rv3417c	groEL1	-1.33	0.0276	60 kD chaperonin 1
Rv3461c	rpmJ	-1.36	0.0090	50S ribosomal protein L36
Rv0700	rpsJ	-1.39	0.0328	30S ribosomal protein S10
Rv0701	rplC	-1.47	0.0163	50S ribosomal protein L3
Rv3443c	rplM	-1.59	0.0199	50S ribosomal protein L13
Rv3418c	groES	-1.97	0.0018	10 kD chaperone
Rv2429	ahpD	-2.44	0.0189	member of AhpC/TSA family
Rv2428	ahpC	-2.47	0.0125	alkyl hydroperoxide reductase

APPENDIX D

APPENDIX D: Complete data set of differentially regulated ORFs (>1-fold) with p-values of ≤ 0.5 for Tn mutant *rv3660*

Tn mutant for *rv3660*

Locus	Gene	*Avg log_2 expression*	*p value*	Gene product
Rv1982c		2.68	0.0118	conserved hypothetical protein
Rv2493		1.90	0.0212	conserved hypothetical protein
Rv1813c		1.83	0.0302	conserved hypothetical protein
Rv2160c		1.74	0.0126	hypothetical protein
Rv2846c	efpA	1.50	0.0027	putative efflux protein
Rv0750		1.42	0.0385	conserved hypothetical protein
Rv1611	trpC	1.38	0.0198	indole-3-glycerol phosphate synthase
Rv1772		1.24	0.0001	hypothetical protein
Rv0341		1.21	0.0445	conserved hypothetical protein
Rv2247	accD6	1.16	0.0000	acetyl/propionyl CoA carboxylase [beta] subunit
Rv3413c		1.14	0.0287	hypothetical protein
Rv2246	kasB	1.14	0.0034	[beta]-ketoacyl-ACP synthase (meromycolate
Rv3295		1.12	0.0058	transcriptional regulator (TetR/AcrR family)
Rv3551		1.10	0.0126	possible glutaconate CoA-transferase
Rv2245	kasA	1.10	0.0344	[beta]-ketoacyl-ACP synthase (meromycolate
Rv1152		1.08	0.0003	transcriptional regulator (GntR family)
Rv2246		1.08	0.0046	[beta]-ketoacyl-ACP synthase (meromycolate
Rv3212		1.07	0.0062	hypothetical protein
Rv3241c		1.04	0.0468	member of S30AE ribosomal protein family
Rv1548c	PPE	1.03	0.0000	PPE-family protein
Rv2721c		1.00	0.0002	*conserved hypothetical protein*
Rv3414c	sigD	0.99	0.0363	ECF subfamily sigma subunit
Rv0655		0.98	0.0457	ABC transporter
Rv3871		0.98	0.0230	conserved hypothetical protein
Rv3171c	hpx	0.97	0.0006	probable non-heme haloperoxidase
Rv1135c	PPE	0.96	0.0015	PPE-family protein
Rv1738		0.96	0.0165	conserved hypothetical protein
Rv0847	lpqS	0.95	0.0178	lipoprotein
Rv3869		0.95	0.0012	conserved hypothetical protein
Rv3874		0.95	0.0034	conserved hypothetical protein
Rv1925	fadD31	0.94	0.0003	acyl-CoA synthase
Rv2444c	rne	0.93	0.0115	similar at C-term to ribonuclease E
Rv1595	nadB	0.93	0.0474	L-aspartate oxidase
Rv2494		0.92	0.0026	conserved hypothetical protein
Rv0442c	PPE	0.91	0.0003	PPE-family protein
Rv1398c		0.91	0.0054	conserved hypothetical protein
Rv3160c		0.91	0.0049	putative transcriptional regulator
Rv1594	nadA	0.90	0.0166	quinolinate synthase
Rv3875	esat6	0.90	0.0033	early secretory antigen target
Rv3870		0.90	0.0119	conserved hypothetical protein

Rv3533c	PPE	0.89	0.0047	PPE-family protein
Rv0083		0.88	0.0076	probable oxidoreductase subunit
Rv0047c		0.87	0.0003	conserved hypothetical protein
Rv1331		0.84	0.0046	conserved hypothetical protein
Rv1884c		0.84	0.0017	conserved hypothetical protein
Rv3130c		0.84	0.0132	conserved hypothetical protein
Rv2327		0.84	0.0204	conserved hypothetical protein
Rv3151	nuoG	0.83	0.0162	NADH dehydrogenase chain G
Rv3161c		0.83	0.0306	putative dioxygenasesdiooxygenases
Rv0872c	PE_PGRS	0.83	0.0023	PE_PGRS-family protein
Rv3878		0.83	0.0001	hypothetical protein
Rv2115c		0.83	0.0316	ATPase of AAA-family
Rv0443		0.82	0.0008	hypothetical protein
Rv3153	nuoI	0.81	0.0089	NADH dehydrogenase chain I
Rv3854c		0.81	0.0106	probable monooxygenase
Rv1731	gabD1	0.81	0.0309	succinate-semialdehyde dehydrogenase
Rv1996		0.80	0.0252	conserved hypothetical protein
Rv2576c		0.79	0.0373	hypothetical protein
Rv2926c		0.78	0.0149	hypothetical protein
Rv3917c	parA	0.78	0.0138	chromosome partitioning; DNA binding
Rv0878c	PPE	0.77	0.0006	PPE-family protein
Rv2359	furB	0.76	0.0184	ferric uptake regulatory protein
Rv0870c		0.76	0.0261	unknown hydrophobic protein
Rv2145c	wag31	0.75	0.0161	antigen 84 (aka wag31)
Rv1906c		0.75	0.0017	conserved hypothetical protein
Rv2369c		0.74	0.0000	hypothetical protein
Rv1797		0.74	0.0002	conserved hypothetical protein
Rv2557		0.74	0.0145	conserved hypothetical protein
Rv2244	acpM	0.74	0.0046	acyl carrier protein (meromycolate extension)
Rv3799c	accD4	0.73	0.0082	acetyl/propionyl CoA carboxylase [beta] subunit
Rv2990c		0.73	0.0054	hypothetical protein
Rv0694	lldD1	0.73	0.0491	L-lactate dehydrogenase (cytochrome)
Rv2963		0.73	0.0141	integral membrane protein
Rv1163	narJ	0.73	0.0220	nitrate reductase [delta] chain
Rv2352c	PPE	0.73	0.0004	PPE-family protein
Rv3683		0.73	0.0013	conserved hypothetical protein
Rv0509	hemA	0.73	0.0446	glutamyl-tRNA reductase
Rv0692		0.70	0.0019	hypothetical protein
Rv3655c		0.69	0.0355	hypothetical protein
Rv0848	cysM3	0.69	0.0120	putative cysteine synthase
Rv3018c		0.69	0.0077	PPE-family protein
Rv3149	nuoE	0.69	0.0213	NADH dehydrogenase chain E
Rv1593c		0.68	0.0011	conserved hypothetical protein
Rv1600	hisC	0.68	0.0007	histidinol-phosphate aminotransferase
Rv2134c		0.68	0.0007	conserved hypothetical protein
Rv0170		0.67	0.0197	part of mce1 operon
Rv1892		0.67	0.0040	hypothetical protein
Rv1916	aceAb	0.66	0.0022	isocitrate lyase, [beta] module
Rv2867c		0.65	0.0276	hypothetical protein
Rv1999c		0.65	0.0011	unknown membrane protein
Rv3154	nuoJ	0.65	0.0321	NADH dehydrogenase chain J
Rv1565c		0.65	0.0130	Unknown membrane protein

Rv1576c		0.65	0.0263	phiRV1 possible prohead protease
Rv3865		0.64	0.0175	conserved hypothetical protein
Rv2377c	mbtH	0.64	0.0121	mycobactin/exochelin synthesis
Rv2007c	fdxA	0.64	0.0258	ferredoxin
Rv2161c		0.64	0.0234	similar to alkanal monooxygenase beta chain
Rv3269		0.62	0.0004	probable heat shock protein
Rv1775		0.62	0.0397	conserved hypothetical protein
Rv1416	ribH	0.62	0.0009	riboflavin synthase [beta] chain
Rv2030c		0.62	0.0035	conserved hypothetical protein
Rv0860	fadB	0.62	0.0296	hypothetical protein
Rv2166c		0.62	0.0370	conserved hypothetical protein
Rv1995		0.62	0.0075	hypothetical protein
Rv0898c		0.62	0.0029	hypothetical protein
Rv0340		0.61	0.0148	hypothetical protein
Rv2198c	mmpS3	0.60	0.0038	conserved small membrane protein
Rv0931c	pknD	0.60	0.0132	serine-threonine protein kinase
Rv2725c	hflX	0.60	0.0230	GTP-binding protein
Rv0174		0.60	0.0228	part of mce1 operon
Rv0558		0.59	0.0000	ubiquinone/menaquinone biosynthesis methyltransferase
Rv1164	narI	0.59	0.0108	nitrate reductase [gamma] chain
Rv3209		0.58	0.0005	conserved hypothetical protein
Rv1161	narG	0.58	0.0276	nitrate reductase [alpha] subunit
Rv1893		0.58	0.0198	hypothetical protein
Rv3270	ctpC	0.58	0.0064	cation transport ATPase
Rv1793		0.58	0.0033	conserved hypothetical protein
Rv2809		0.58	0.0227	hypothetical protein
Rv3918c	parB	0.58	0.0023	possibly involved in chromosome partitioning
Rv3139	fadE24	0.58	0.0121	acyl-CoA dehydrogenase
Rv3872	PE	0.58	0.0000	PE-family protein
Rv2124c		0.57	0.0122	5-methyltetrahydrofolate-homocysteine
Rv2347c		0.57	0.0016	conserved hypothetical protein
Rv0486		0.56	0.0268	conserved hypothetical protein
Rv3142c		0.56	0.0012	hypothetical protein
Rv2841c	nusA	0.56	0.0187	transcription termination factor
Rv0578c	PE_PGRS	0.55	0.0315	PE_PGRS-family protein
Rv3371		0.55	0.0185	conserved hypothetical protein
Rv2927c		0.55	0.0055	thioesterase
Rv2325c		0.55	0.0141	conserved hypothetical protein
Rv1680		0.54	0.0047	hypothetical protein
Rv1110	lytB'	0.54	0.0249	very similar to LytB
Rv3583c		0.54	0.0004	putative transcriptional regulator
Rv3020c	PE	0.54	0.0163	PE-family protein
Rv1843c	guaB1	0.54	0.0032	inosine-5'-monophosphate dehydrogenase
Rv0896	gltA2	0.54	0.0274	citrate synthase 1
Rv3651		0.53	0.0397	hypothetical protein
Rv1699	pyrG	0.53	0.0018	CTP synthase
Rv1038c		0.53	0.0000	PPE-family protein
Rv0475		0.53	0.0205	possible exported protein
Rv2070c	cobK	0.53	0.0331	precorrin reductase
Rv2507		0.53	0.0462	probable membrane spanning protein
Rv0025		0.53	0.0265	conserved hypothetical protein
Rv2901c		0.53	0.0067	hypothetical protein

Rv1257c		0.53	0.0324	similar to many dehydrogenases
Rv1197		0.53	0.0125	conserved hypothetical protein
Rv3876		0.53	0.0030	conserved hypothetical protein
Rv0559c		0.53	0.0006	possible exported
Rv3211	rhlE	0.53	0.0013	probable ATP-dependent RNA helicase
Rv2457c	clpX	0.52	0.0162	ATP-dependent Clp protease ATP-binding subunit ClpX
Rv3873	PPE	0.52	0.0000	PPE-family protein
Rv3150	nuoF	0.52	0.0204	NADH dehydrogenase chain F
Rv2622		0.52	0.0053	similar to SAM-dependent methyltransferase
Rv2368c	phoH	0.52	0.0136	ATP-binding pho regulon component
Rv2783c	gpsI	0.52	0.0452	pppGpp synthase and polyribonucleotide phosphorylase
Rv0755c	PPE	0.52	0.0005	PPE-family protein
Rv2152c	murC	0.52	0.0081	UDP-N-acetyl-muramate-alanine ligase
Rv1596	nadC	0.51	0.0036	nicotinate-nucleotide pyrophosphatase
Rv1846c		0.51	0.0058	putative transcriptional regulator
Rv3173c		0.51	0.0023	transcriptional regulator (TetR/AcrR family)
Rv1522c	mmpL12	0.51	0.0197	conserved large membrane protein
Rv0171		0.51	0.0222	part of mce1 operon
Rv2711	ideR	0.51	0.0087	iron dependent repressor, IdeR
Rv3127		0.51	0.0000	conserved hypothetical protein
Rv2405		0.51	0.0005	hypothetical protein
Rv1273c		0.51	0.0368	ABC transporter
Rv3145	nuoA	0.51	0.0076	NADH dehydrogenase chain A
Rv0782	ptrBa	0.50	0.0504	protease II, [alpha] subunit
Rv2558		0.50	0.0287	conserved hypothetical protein
Rv3684		0.50	0.0084	Probable lyase, cysteine metabolism
Rv1891		0.50	0.0116	hypothetical protein
Rv3146	nuoB	0.50	0.0412	NADH dehydrogenase chain B
Rv2094c		0.50	0.0232	conserved hypothetical protein
Rv1229c	mrp	0.50	0.0453	similar to MRP/NBP35 ATP-binding proteins
Rv2931	ppsA	0.50	0.0078	phenolpthiocerol synthesis (pksB)
Rv3856c		0.50	0.0253	conserved hypothetical protein
Rv0880		-0.50	0.0174	conserved hypothetical protein
Rv0332		-0.50	0.0009	hypothetical protein
Rv3443c	rplM	-0.50	0.0003	50S ribosomal protein L13
Rv1498c		-0.50	0.0184	methyltransferase
Rv2703	sigA	-0.50	0.0380	RNA polymerase sigma factor (aka MysA, RpoV)
Rv1886c	fbpB	-0.50	0.0001	antigen 85B, mycolyltransferase
Rv0058	dnaB	-0.51	0.0225	DNA helicase (contains intein)
Rv1172c	PE	-0.51	0.0021	PE-family protein
Rv1779c		-0.51	0.0110	possible integral membrane protein
Rv0702	rplD	-0.51	0.0000	50S ribosomal protein L4
Rv2688c		-0.51	0.0330	similar to transport ATP-binding proteins
Rv2590	fadD9	-0.51	0.0423	acyl-CoA synthase
Rv1872c	lldD2	-0.52	0.0271	L-lactate dehydrogenase
Rv3720		-0.52	0.0421	C-term similar to cyclopropane fatty acid synthases
Rv0504c		-0.52	0.0060	conserved hypothetical protein
Rv3458c	rpsD	-0.52	0.0003	30S ribosomal protein S4
Rv3416	whiB3	-0.52	0.0123	WhiB transcriptional activator homologue
Rv3911		-0.52	0.0065	hypothetical protein
Rv0350	dnaK	-0.52	0.0340	70 kD heat shock protein, chromosome replication
Rv0333		-0.52	0.0060	hypothetical protein

Rv2735c		-0.52	0.0408	hypothetical protein
Rv0951	sucC	-0.52	0.0389	succinyl-CoA synthase [beta] chain
Rv3013		-0.53	0.0418	conserved hypothetical protein
Rv2971		-0.53	0.0418	oxidoreductase of Aldo/keto reductase family
Rv3634c	rmlB2	-0.54	0.0002	dTDP-glucose 4,6-dehydratase
Rv0636		-0.54	0.0136	hypothetical protein
Rv1251c		-0.54	0.0355	hypothetical protein
Rv0404	fadD30	-0.54	0.0113	acyl-CoA synthase
Rv1871c		-0.55	0.0158	hypothetical protein
Rv3376		-0.55	0.0009	conserved hypothetical protein
Rv1099c		-0.55	0.0509	conserved hypothetical protein
Rv0402c	mmpL1	-0.56	0.0025	conserved large membrane protein
Rv0384c	clpB	-0.56	0.0023	heat shock protein
Rv2422		-0.56	0.0284	hypothetical protein
Rv3575c		-0.56	0.0202	transcriptional regulator (LacI family)
Rv3260c	whiB2	-0.57	0.0269	WhiB transcriptional activator homologue
Rv3849		-0.57	0.0009	hypothetical protein
Rv1870c		-0.57	0.0014	hypothetical protein
Rv0302		-0.57	0.0462	transcriptional regulator (TetR/AcrR family)
Rv3460c	rpsM	-0.57	0.0371	30S ribosomal protein S13
Rv1471	trxB	-0.57	0.0165	thioredoxin reductase
Rv2079		-0.57	0.0003	hypothetical protein
Rv3598c	lysS	-0.57	0.0086	lysyl-tRNA synthase
Rv3455c	truA	-0.58	0.0428	probable pseudouridylate synthase
Rv3462c	infA	-0.58	0.0214	initiation factor IF-1
Rv3585	radA	-0.58	0.0062	probable DNA repair RadA homologue
Rv3117	cysA3	-0.58	0.0142	thiosulfate sulfurtransferase
Rv2392	cysH	-0.58	0.0103	3'-phosphoadenylylsulfate (PAPS) reductase
Rv0056	rplI	-0.58	0.0061	50S ribosomal protein L9
Rv0720	rplR	-0.59	0.0011	50S ribosomal protein L18
Rv3825c	pks2	-0.59	0.0459	polyketide synthase
Rv3459c	rpsK	-0.59	0.0116	30S ribosomal protein S11
Rv3794	embA	-0.60	0.0000	involved in arabinogalactan synthesis
Rv0430		-0.60	0.0071	hypothetical protein
Rv1150		-0.61	0.0005	hypothetical protein
Rv2563		-0.61	0.0264	possible membrane protein
Rv1388		-0.61	0.0217	integration host factor
Rv0825c		-0.62	0.0326	conserved hypothetical protein
Rv0925c		-0.62	0.0399	hypothetical protein
Rv0718	rpsH	-0.62	0.0035	30S ribosomal protein S8
Rv2190c		-0.62	0.0163	putative p60 homologue
Rv2478c		-0.63	0.0002	hypothetical protein
Rv1307	atpH	-0.63	0.0436	ATP synthase [delta] chain
Rv1303		-0.63	0.0250	conserved hypothetical protein
Rv2156c	murX	-0.64	0.0138	phospho-N-acetylmuramoyl-pentapeptide transferase
Rv2518c	lppS	-0.64	0.0246	lipoprotein
Rv3846	sodA	-0.64	0.0476	superoxide dismutase
Rv3826	fadD23	-0.64	0.0065	acyl-CoA synthase
Rv3424c		-0.64	0.0067	hypothetical protein
Rv1304	atpB	-0.64	0.0011	ATP synthase a chain
Rv3101c	ftsX	-0.64	0.0003	membrane protein
Rv3461c	rpmJ	-0.65	0.0342	50S ribosomal protein L36

Rv0519c		-0.65	0.0109	conserved hypothetical protein
Rv2890c	rpsB	-0.66	0.0006	30S ribosomal protein S2
Rv0732	secY	-0.66	0.0217	SecY subunit of preprotein translocase
Rv2069	sigC	-0.66	0.0434	ECF subfamily sigma subunit
Rv2081c		-0.66	0.0266	hypothetical protein
Rv0715	rplX	-0.67	0.0041	50S ribosomal protein L24
Rv3716c		-0.67	0.0004	conserved hypothetical protein
Rv3924c	rpmH	-0.67	0.0033	50S ribosomal protein L34
Rv1306	atpF	-0.67	0.0020	ATP synthase b chain
Rv3820c	papA2	-0.68	0.0081	PKS-associated protein, unknown function
Rv0921		-0.68	0.0004	resolvase
Rv2105		-0.68	0.0200	possible IS6110 transposase
Rv2168c		-0.69	0.0130	possible IS6110 transposase
Rv1179c		-0.69	0.0258	hypothetical protein
Rv2876		-0.69	0.0254	hypothetical protein
Rv2564	glnQ	-0.70	0.0289	probable ATP-binding transport protein
Rv0251c	hsp	-0.70	0.0192	possible heat shock protein
Rv1192		-0.71	0.0056	hypothetical protein
Rv1309	atpG	-0.71	0.0458	ATP synthase [gamma] chain
Rv0637		-0.71	0.0377	conserved hypothetical protein
Rv0054	ssb	-0.72	0.0414	single strand binding protein
Rv1196	PPE	-0.72	0.0131	PPE-family protein
Rv3029c	fixA	-0.73	0.0094	electron transfer flavoprotein [beta] subunit
Rv0352	dnaJ	-0.73	0.0411	acts with GrpE to stimulate DnaK ATPase
Rv3184		-0.76	0.0007	possible IS6110 transposase
Rv3420c	rimI	-0.77	0.0001	ribosomal protein S18 acetyltransferase
Rv2719c		-0.77	0.0225	conserved hypothetical protein
Rv2450c		-0.77	0.0195	conserved hypothetical protein
Rv3584	lpqE	-0.78	0.0491	lipoprotein
Rv0164		-0.79	0.0005	conserved hypothetical protein
Rv1945		-0.79	0.0020	REP-family protein
Rv0467	aceA	-0.80	0.0187	isocitrate lyase
Rv2354		-0.81	0.0076	possible IS6110 transposase
Rv0714	rplN	-0.82	0.0185	50S ribosomal protein L14
Rv3788		-0.82	0.0033	hypothetical protein
Rv3616c		-0.83	0.0014	conserved hypothetical protein
Rv0795		-0.83	0.0388	possible IS6110 transposase
Rv3614c		-0.83	0.0126	conserved hypothetical protein
Rv3824c	papA1	-0.84	0.0267	PKS-associated protein, unknown function
Rv2124c	metH	-0.84	0.0005	5-methyltetrahydrofolate-homocysteine
Rv3628	ppa	-0.84	0.0003	probable inorganic pyrophosphatase
Rv3646c	topA	-0.85	0.0251	DNA topoisomerase
Rv0716	rplE	-0.86	0.0043	50S ribosomal protein L5
Rv0867c		-0.86	0.0040	probable exported protein
Rv2952		-0.87	0.0233	glycosyltransferase
Rv2276		-0.87	0.0045	Probable cytochrome P-450
Rv0682	rpsL	-0.88	0.0000	30S ribosomal protein S12
Rv2480c		-0.88	0.0008	possible IS6110 transposase
Rv3186		-0.88	0.0025	possible IS6110 transposase
Rv0053	rpsF	-0.89	0.0371	30S ribosomal protein S6
Rv1130		-0.89	0.0365	conserved hypothetical protein
Rv3136	PPE	-0.90	0.0000	PPE-family protein

Rv0184		-0.91	0.0000	conserved hypothetical protein
Rv0055	rpsR	-0.91	0.0281	30S ribosomal protein S18
Rv1096		-0.91	0.0257	carbohydrate degrading enzyme
Rv0841c		-0.92	0.0501	hypothetical protein
Rv2648		-0.92	0.0197	possible IS6110 transposase
Rv0351	grpE	-0.93	0.0345	stimulates DnaK ATPase activity
Rv2625c		-0.94	0.0169	conserved hypothetical protein
Rv0945		-0.95	0.0030	ketoacyl reductase
Rv2466c		-0.97	0.0389	conserved hypothetical protein
Rv2950c	fadD29	-0.99	0.0230	acyl-CoA synthase
Rv3767c		-1.00	0.0470	conserved hypothetical protein
Rv2412	rpsT	-1.01	0.0278	30S ribosomal protein S20
Rv0196		-1.02	0.0421	transcriptional regulator (TetR/AcrR family)
Rv3524		-1.03	0.0245	possible membrane sensor protein
Rv2278		-1.04	0.0001	possible IS6110 transposase
Rv3804c	fbpA	-1.06	0.0146	antigen 85A, mycolyltransferase
Rv1078	pra	-1.13	0.0005	MLPRAG (64.8% id) proline rich Ag
Rv2376c		-1.13	0.0357	conserved hypothetical protein
Rv2034		-1.17	0.0014	transcriptional regulator (ArsR family)
Rv3417c	groEL1	-1.25	0.0436	60 kD chaperonin 1
Rv3750c		-1.33	0.0020	excisionase
Rv2220	glnA1	-1.50	0.0001	glutamine synthase class I
Rv0440	groEL2	-1.53	0.0286	60 kD chaperonin 2
Rv3822		-1.54	0.0277	conserved hypothetical protein
Rv3418c	groES	-1.71	0.0157	10 kD chaperone
Rv3686c		-1.77	0.0014	conserved hypothetical protein
Rv2429	ahpD	-2.21	0.0197	member of AhpC/TSA family
Rv3478	PPE	-2.27	0.0369	PPE-family protein
Rv2428	ahpC	-2.46	0.0053	alkyl hydroperoxide reductase
Rv3408		-2.96	0.0097	conserved hypothetical protein

APPENDIX E

APPENDIX E: Significantly differentially regulated ORFs(>2-fold, p value<0.05) from overexpression *of rv2216*

ID	Name	Gene product	Avg Log_2 expression	p vlaue
Rv2216		conserved hypothetical protein	10.59	0.042
Rv3577		hypothetical protein	5.13	0.012
Rv2370c		conserved hypothetical protein	4.56	0.001
Rv2892c	PPE	*PPE-family protein*	4.54	0.051
Rv0765c		short-chain alcohol dehydrogenase family	3.71	0.000
Rv3189		conserved hypothetical protein	3.66	0.000
Rv0791c		possible monooxygenasemonoxygenase	3.63	0.015
Rv1367c		probable penicillin binding protein	3.56	0.017
Rv1551	plsB1	glycerol-3-phosphate acyltransferase	3.45	0.009
Rv0851c		Short-chain dehydrogenases/reductase	3.41	0.000
Rv0136		cytochrome p450	3.20	0.005
Rv1997	ctpF	probable cation transport ATPase	3.03	0.000
Rv3229c	desA3	acyl-[ACP] desaturase	3.03	0.007
Rv2032		conserved hypothetical protein	3.01	0.000
Rv1584c		phiRV1 phage related protein	2.90	0.001
Rv3131		conserved hypothetical protein	2.88	0.011
Rv2662		hypothetical protein	2.86	0.000
Rv2624c		conserved hypothetical protein	2.85	0.000
Rv3841	bfrB	bacterioferritin	2.83	0.001
Rv0381c		hypothetical protein	2.76	0.001
Rv2016		hypothetical protein	2.76	0.033
Rv3060c		transcriptional regulator (GntR family)	2.75	0.008
Rv2659c		phiRV2 integrase	2.75	0.025
Rv3127		conserved hypothetical protein	2.70	0.001
Rv2230c		conserved hypothetical protein	2.65	0.005
Rv0140		conserved hypothetical protein	2.65	0.044
Rv3287c	rsbW	anti-sigma B factor	2.62	0.003
Rv0342		conserved hypothetical protein	2.61	0.030
Rv0588		part of mce2 operon	2.56	0.009
Rv2626c		conserved hypothetical protein	2.55	0.033
Rv0936	pstA2	PstA component of phosphate uptake	2.51	0.028
Rv0135c		putative transcriptional regulator	2.46	0.021
Rv0563	htpX	probable (transmembrane) heat shock protein	2.41	0.045
Rv0570	nrdZ	ribonucleotide reductase, class II	2.40	0.005
Rv2304c		hypothetical protein	2.34	0.000
Rv2630		hypothetical protein	2.30	0.052
Rv1581c		phiRV1 phage related protein	2.27	0.042
Rv1585c		phiRV1 phage related protein	2.26	0.001
Rv2629		hypothetical protein	2.20	0.008
Rv3732		conserved hypothetical protein	2.19	0.013
Rv0589	mce2	cell invasion protein	2.16	0.002
Rv0064		possible membrane protein	2.12	0.007
Rv3854c		probable monooxygenase	2.11	0.011
Rv3244c	lpqB	lipoprotein	2.09	0.031
Rv1471	trxB	thioredoxin reductase	2.07	0.044

Rv3855	putative transcriptional regulator	2.07	0.024
Rv3288c	conserved hypothetical protein	2.07	0.000
Rv0678	hypothetical protein	1.95	0.018
Rv3097c PE	PE-family protein	1.92	0.021
Rv2466c	conserved hypothetical protein	1.91	0.011
Rv3550 echA20	enoyl-CoA hydratase/isomerase superfamily	1.91	0.001
Rv1063c	conserved hypothetical protein	1.88	0.010
Rv0024	putative p60 homologue	1.87	0.010
Rv1043c	hypothetical protein	1.86	0.022
Rv3354	conserved hypothetical protein	1.85	0.043
Rv1286 cysN	ATP:sulphurylase subunit 1	1.85	0.003
Rv3130c	conserved hypothetical protein	1.83	0.003
Rv2775	hypothetical protein	1.83	0.038
Rv0411c glnH	putative glutamine binding protein	1.82	0.047
Rv1896c	conserved hypothetical protein	1.82	0.039
Rv2913c	probable D-amino acid aminohydrolase	1.81	0.000
Rv3327	hypothetical protein	1.78	0.041
Rv3133c	two-component response regulator	1.78	0.004
Rv0671 lpqP	probable esterase	1.76	0.000
Rv2675c	putative methyltransferase	1.74	0.035
Rv2033c	hypothetical protein	1.73	0.022
Rv0926c	conserved hypothetical protein	1.69	0.000
Rv0410c pknG	serine-threonine protein kinase	1.67	0.019
Rv3445c	hypothetical protein	1.66	0.009
Rv1580c	phiRV1 phage related protein	1.64	0.004
Rv0839	conserved hypothetical protein	1.64	0.008
Rv1653 argJ	glutamate N-acetyltransferase	1.63	0.004
Rv1599 hisD	histidinol dehydrogenase	1.63	0.015
Rv1854c ndh	probable NADH dehydrogenase	1.59	0.014
Rv2242	hypothetical protein	1.59	0.053
Rv2973c recG	ATP-dependent DNA helicase	1.59	0.054
Rv2918c glnD	uridylyltransferase	1.57	0.045
Rv3334	transcriptional regulator (MerR family)	1.57	0.012
Rv2031c hspX	14kD antigen, heat shock protein Hsp20 family	1.56	0.013
Rv1129c	transcriptional regulator (PbsX/Xre family)	1.55	0.002
Rv3551	possible glutaconate CoA-transferase	1.54	0.039
Rv1134	hypothetical protein	1.54	0.008
Rv3159c PPE	PPE-family protein	1.51	0.018
Rv2254c	hypothetical protein	1.51	0.014
Rv3561 fadD3	acyl-CoA synthase	1.50	0.019
Rv3463	probable neuraminidase	1.49	0.000
Rv2860c glnA4	proable glutamine synthase	1.49	0.006
Rv0769	similar to 7-alpha-hydroxysteroid dehydrogenase	1.49	0.042
Rv0181c	conserved hypothetical protein	1.48	0.016
Rv3543c fadE29	acyl-CoA dehydrogenase	1.48	0.012
Rv2052c	hypothetical protein	1.46	0.012
Rv2030c	conserved hypothetical protein	1.46	0.027
Rv3548c	short-chain alcohol dehydrogenase family	1.46	0.007
Rv1235 lpqY	possible role in sugar transport	1.45	0.042
Rv1786	Probable ferredoxin	1.45	0.003
Rv0412c	unknown probable membrane protein	1.45	0.048
Rv2735c	hypothetical protein	1.44	0.034
Rv2877c	possible mercury resistance transport system	1.43	0.006
Rv3290c lat	lysine-[epsilon] aminotransferase	1.42	0.016
Rv1809 PPE	PPE-family protein	1.40	0.007
Rv1621c cydD	ABC transporter	1.39	0.055
Rv3552	hypothetical protein	1.38	0.015

Rv3169	conserved hypothetical protein	1.38	0.015
Rv1601 hisB	imidazole glycerol-phosphate dehydratase	1.37	0.027
Rv3270 ctpC	cation transport ATPase	1.37	0.006
Rv1918c PPE	PPE-family protein	1.36	0.004
Rv2372c	conserved hypothetical protein	1.35	0.038
Rv0743c	hypothetical protein	1.33	0.020
Rv1724c	hypothetical protein	1.32	0.002
Rv2532c	hypothetical protein	1.32	0.018
Rv2497c pdhA	pyruvate dehydrogenase E1 component [alpha] subunit	1.32	0.001
Rv3347c PPE	PPE-family protein	1.29	0.041
Rv2784c lppU	lipoprotein	1.28	0.047
Rv3544c fadE28	acyl-CoA dehydrogenase	1.27	0.034
Rv2153c murG	transferase in peptidoglycan synthesis	1.27	0.000
Rv2501c accA1	acetyl/propionyl-CoA carboxylase, [alpha] subunit	1.26	0.042
Rv1034c	hypothetical protein	1.25	0.003
Rv1813c	conserved hypothetical protein	1.25	0.000
Rv1579c	phiRV1 phage related protein	1.25	0.038
Rv1874	hypothetical protein	1.24	0.009
Rv2235	hypothetical protein	1.24	0.035
Rv1982c	conserved hypothetical protein	1.24	0.021
Rv0855 far	fatty acyl-CoA racemase	1.24	0.011
Rv1957	hypothetical protein	1.23	0.002
Rv2465c rpi	phosphopentose isomerase	1.23	0.035
Rv2135c	conserved hypothetical protein	1.23	0.002
Rv2550c	hypothetical protein	1.22	0.003
Rv1128c	REP-family protein	1.20	0.007
Rv2028c	conserved hypothetical protein	1.19	0.002
Rv2524c fas	fatty acid synthase	1.18	0.007
Rv2483c	possible transferase	1.17	0.009
Rv0786c	conserved hypothetical protein	1.15	0.028
Rv2364c bex	GTP-binding protein of Era/ThdF family	1.15	0.032
Rv1396c PE_PGRS	PE_PGRS-family protein	1.14	0.053
Rv1875	conserved hypothetical protein	1.14	0.047
Rv1627c	lipid carrier protein	1.14	0.001
Rv3289c	hypothetical protein	1.13	0.007
Rv2870c	conserved hypothetical protein	1.13	0.000
Rv2989	transcriptional regulator (IclR family)	1.13	0.032
Rv2147c	hypothetical protein	1.12	0.031
Rv3088	conserved hypothetical protein	1.12	0.045
Rv2495c pdhC	dihydrolipoamide acetyltransferase	1.11	0.007
Rv1620c cydC	ABC transporter	1.11	0.000
Rv2218 lipA	lipoate biosynthesis protein A	1.11	0.002
Rv3245c mtrB	sensor histidine kinase	1.10	0.008
Rv1334	conserved hypothetical protein	1.10	0.001
Rv1536 ileS	isoleucyl-tRNA synthase	1.09	0.012
Rv0624	conserved hypothetical protein	1.08	0.000
Rv1224	conserved hypothetical protein	1.08	0.010
Rv3685c	Probable cytochrome P-450	1.07	0.018
Rv1082	similar to S. lincolnensis lmbE	1.07	0.027
Rv0749	conserved hypothetical protein	1.07	0.003
Rv3869	conserved hypothetical protein	1.06	0.024
Rv0921	resolvase	1.06	0.028
Rv2920c amt	putative ammonium transporter	1.06	0.012
Rv2825c	conserved hypothetical protein	1.03	0.040
Rv3545c	cytochrome p450	1.03	0.048
Rv1733c	possible membrane protein	1.03	0.000
Rv1593c	conserved hypothetical protein	1.03	0.023

Rv3553	similar to dioxygenasesdiooxygenases	1.03	0.044
Rv1152	transcriptional regulator (GntR family)	1.02	0.000
Rv0660c	conserved hypothetical protein	1.02	0.013
Rv0190	conserved hypothetical protein	1.02	0.014
Rv1102c	conserved hypothetical protein	1.02	0.000
Rv1603 hisA	phosphoribosylformimino-5-aminoimidazole	1.02	0.039
Rv3560c fadE30	acyl-CoA dehydrogenase	1.01	0.015
Rv2499c	putative aldehyde dehydrogenase	1.01	0.036
Rv3047c	hypothetical protein	1.01	0.001
Rv0464c	conserved hypothetical protein	1.00	0.036
Rv3532 PPE	PPE-family protein	1.0	0.000
Rv3752c	probable cytidine/deoxycytidylate deaminase	1.0	0.047
Rv2399c cysT	sulphate transport system permease protein	1.0	0.000
Rv1329c dinG	probable ATP-dependent helicase	1.0	0.019
Rv0724 sppA	protease IV, signal peptide peptidase	1.0	0.033
Rv2212	conserved hypothetical protein	1.0	0.052
Rv1035c	hypothetical protein	1.0	0.022
Rv0633c	hypothetical protein	1.0	0.015
Rv1600 hisC	histidinol-phosphate aminotransferase	1.0	0.042
Rv2246 kasB	[beta]-ketoacyl-ACP synthase (meromycolate	-1.0	0.053
Rv0168	part of mce1 operon	-1.0	0.009
Rv0662c	hypothetical protein	-1.0	0.032
Rv2412 rpsT	30S ribosomal protein S20	-1.0	0.030
Rv3234c	conserved hypothetical protein	-1.0	0.015
Rv3043c ctaD	cytochrome c oxidase polypeptide I	-1.04	0.038
Rv2969c	possible transmembrane domain	-1.04	0.010
Rv1209	conserved hypothetical protein	-1.05	0.039
Rv2188c	conserved hypothetical protein	-1.06	0.030
Rv2721c	conserved hypothetical protein	-1.06	0.002
Rv2901c	hypothetical protein	-1.06	0.002
Rv1346 fadE14	acyl-CoA dehydrogenase	-1.07	0.035
Rv3148 nuoD	NADH dehydrogenase chain D	-1.07	0.000
Rv2528c mrr	restriction system protein	-1.07	0.003
Rv0814c sseC2	thiosulfate sulfurtransferase	-1.07	0.052
Rv1886c fbpB	antigen 85B, mycolyltransferase	-1.08	0.022
Rv1308 atpA	ATP synthase [alpha] chain	-1.08	0.004
Rv3483c	conserved hypothetical protein	-1.09	0.004
Rv3808c	hypothetical protein	-1.10	0.019
Rv0709 rpmC	50S ribosomal protein L29	-1.10	0.026
Rv3219 whiB1	WhiB transcriptional activator homologue	-1.10	0.012
Rv3703c	conserved hypothetical protein	-1.10	0.001
Rv1343c	conserved hypothetical protein	-1.11	0.001
Rv1919c	weak similarity to pollen antigens	-1.11	0.035
Rv3924c rpmH	50S ribosomal protein L34	-1.11	0.049
Rv0237 lpqI	beta-hexosaminidase precursorBETA-HEXOSAMINIDASE A	-1.11	0.007
Rv3029c fixA	electron transfer flavoprotein [beta] subunit	-1.11	0.009
Rv2371 PE	PE-family protein	-1.12	0.004
Rv1040c PE	PE-family protein	-1.13	0.030
Rv3778c	conserved hypothetical protein	-1.13	0.000
Rv3883c	probable secreted protease	-1.14	0.001
Rv2612c pgsA	CDP-diacylglycerol-glycerol-3-phosphate	-1.14	0.006
Rv1312	conserved hypothetical protein	-1.16	0.016
Rv3864	conserved hypothetical protein	-1.16	0.039
Rv3884c	conserved hypothetical protein	-1.17	0.045
Rv3319 sdhB	succinate dehydrogenase B	-1.18	0.010
Rv0745	conserved hypothetical protein	-1.18	0.007
Rv1302 rfe	hypothetical protein	-1.19	0.005

Rv3622c	PE	PE-family protein	-1.20	0.002
Rv2346c		conserved hypothetical protein	-1.20	0.007
Rv2952		glycosyltransferase	-1.21	0.001
Rv2831	echA16	enoyl-CoA hydratase/isomerase superfamily	-1.22	0.041
Rv1435c		conserved hypothetical protein	-1.22	0.002
Rv2953		conserved hypothetical protein	-1.23	0.015
Rv1887		hypothetical protein	-1.24	0.030
Rv0694	lldD1	L-lactate dehydrogenase (cytochrome)	-1.24	0.015
Rv1704c	cycA	transport of D-alanine, D-serine and glycine	-1.24	0.003
Rv2244	acpM	acyl carrier protein (meromycolate extension)	-1.25	0.027
Rv0514		possible membrane protein	-1.25	0.013
Rv3597c	lsr2	conserved hypothetical protein	-1.25	0.001
Rv1436	gap	glyceraldehyde 3-phosphate dehydrogenase	-1.25	0.032
Rv2459		probable drug efflux protein	-1.27	0.004
Rv3763		19 kDKD	-1.27	0.000
Rv2127	ansP	L-asparagine permease	-1.29	0.051
Rv1793		conserved hypothetical protein	-1.29	0.009
Rv3150	nuoF	NADH dehydrogenase chain F	-1.30	0.000
Rv3403c		hypothetical protein	-1.30	0.003
Rv1932	tpx	thiol peroxidase	-1.30	0.033
Rv3146	nuoB	NADH dehydrogenase chain B	-1.32	0.037
Rv3116	moeB	molybdopterin biosynthesis	-1.33	0.012
Rv1792		conserved hypothetical protein	-1.36	0.003
Rv2948c	fadD22	acyl-CoA synthase	-1.36	0.010
Rv1477		putative exported p60 protein homologue	-1.36	0.047
Rv3922c		possible hemolysin	-1.37	0.012
Rv3331	sugI	probable sugar transport protein	-1.38	0.009
Rv3619c		conserved hypothetical protein	-1.40	0.002
Rv2685	arsB	probable arsenical pump	-1.42	0.004
Rv3648c	cspA	cold shock protein, transcriptional regulator	-1.43	0.021
Rv1712	cmk	cytidylate kinase	-1.45	0.050
Rv1386	PE	PE-family protein	-1.45	0.053
Rv1198		conserved hypothetical protein	-1.45	0.031
Rv1613	trpA	tryptophan synthase [alpha] chain	-1.48	0.007
Rv2986c	hupB	DNA-binding protein II	-1.52	0.003
Rv3077	atsF	proable arylsulfatase	-1.56	0.050
Rv2940c	mas	mycocerosic acid synthase	-1.59	0.009
Rv0652	rplL	50S ribosomal protein L7/L12	-1.64	0.010
Rv1078	pra	conserved hypothetical protein	-1.71	0.040
Rv2951c		putative oxidoreductase	-1.74	0.010
Rv3478	PPE	PPE-family protein	-1.77	0.003
Rv2190c		putative p60 homologue	-1.83	0.052
Rv3425	PPE	PPE-family protein	-1.84	0.014
Rv0282		conserved hypothetical protein	-1.85	0.007
Rv0284		conserved hypothetical protein	-1.87	0.001
Rv1037c		conserved hypothetical protein	-1.89	0.042
Rv3307	deoD	probable purine nucleoside phosphorylase	-1.93	0.000
Rv0286	PPE	PPE-family protein	-1.94	0.006
Rv3093c		hypothetical protein	-1.95	0.051
Rv0290		unknown hydrophobic protein	-2.04	0.013
Rv3156	nuoL	NADH dehydrogenase chain L	-2.06	0.004
Rv3346c		conserved hypothetical protein	-2.06	0.001
Rv2450c		conserved hypothetical protein	-2.07	0.048
Rv0283		conserved hypothetical protein	-2.07	0.005
Rv0285	PE	PE-family protein	-2.09	0.006
Rv3155	nuoK	NADH dehydrogenase chain K	-2.09	0.029
Rv2947c	pks15	polyketide synthase	-2.37	0.008

Rv0287	Rv0287	conserved hypothetical protein	-2.45	0.004
Rv0288		conserved hypothetical protein	-2.46	0.003
Rv3019c		similar to Esat6	-2.74	0.001
Rv2386c	trpE2	anthranilate synthase component I	-2.74	0.013