DISTRIBUTION AGREEMENT

In presenting this dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I agree that the Library of the University shall make it available for inspection and circulation in accordance with regulations, governing materials of this type. I agree that permission to copy from, or to publish, this dissertation may be granted by the professor under whose direction it was written, or, in his absence, by the Dean of the Graduate School when such copying or publication is solely for scholarly purposes and does not involve potential financial gain. It is understood that copying from, or publication of, this dissertation which involves potential financial gain will not be allowed without written permission.

____
Mary Analise Zaunbrecher

Low-Level Kanamycin Resistance in *Mycobacterium tuberculosis*:
Molecular Mechanisms and Clinical Implications

By

Mary Analise Zaunbrecher
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences,
Program in Microbiology and Molecular Genetics

____
Thomas M. Shinnick, PhD
Advisor

____
Gordon G. Churchward, PhD
Committee Member

____
Daniel Kalman, PhD
Committee Member

____
William Shafer
Committee Member, PhD

____
Charles Moran, Jr., PhD
Committee Member

Accepted:

____
Dean of the Graduate School

____
Date

Low-Level Kanamycin Resistance in *Mycobacterium tuberculosis*:
Molecular Mechanisms and Clinical Implications

By

Mary Analise Zaunbrecher

B.S., University of Louisiana at Lafayette, 2004

Advisor: Thomas M. Shinnick, PhD.

An Abstract of
A dissertation submitted to the Faculty of the Graduate
School of Emory University in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Program in Microbiology and Molecular Genetics
Graduate Division of Biological and Biomedical Sciences

Abstract

Low-Level Kanamycin Resistance in *Mycobacterium tuberculosis*:
Molecular Mechanisms and Clinical Implications

Mary Analise Zaunbrecher

The aminoglycosides kanamycin (KAN) and amikacin (AMK) are required for the treatment of multidrug-resistant tuberculosis (MDR-TB) and resistance to these drugs is a growing concern. Knowledge of the mechanism(s) responsible for KAN resistance is limited. High-level resistance has been attributed to mutations in the 16S rRNA gene, *rrs*. However, in 30-80% of KAN resistant clinical isolates, low-level resistance is observed which cannot be ascribed to any known mechanism. This work investigates the molecular basis of low-level KAN resistance through either the utilization of a cosmid library or whole genome sequencing analysis of spontaneous low-level KAN resistant mutants. Mutations in the -10 and -35 regions promoter region of *eis (Rv2416c)*, a gene encoding a previously uncharacterized aminoglycoside acetyltransferase, were found to confer low-level KAN resistance. These mutations led to a 20- to 180-fold increase in the amount of *eis* leaderless mRNA transcript with a corresponding increase in protein expression. *In vitro* acetyltransferase assays confirmed that the Eis protein acetylates and inactivates KAN and AMK. 80% of clinical isolates that exhibit low-level mono-KAN resistance harbor *eis* promoter mutations. Experiments presented here demonstrate that low-level KAN resistance is also conferred by mutations in the promoter region of the transcriptional activator, *whiB7* that cause a 23- to 145-fold increase in *whiB7* transcripts. qRT-PCR assays demonstrate that increased *whiB7* expression enhances expression of genes in the WhiB7 regulon including *eis* and the *tap (Rv1258c)* efflux pump. The increased expression of *eis* confers KAN resistance, and increased expression of *tap* confers cross-resistance to streptomycin (STR), presumably by enhanced efflux of STR. Overall, this study identifies the molecular basis for the involvement of *eis* in KAN resistance, *tap* in STR resistance, and *whiB7* in KAN and STR cross-resistance. Together, these data provide a means to develop rapid diagnostics of drug resistant strains and ultimately may affect how treatment is designed for MDR- and XDR-TB cases.

Low-Level Kanamycin Resistance in *Mycobacterium tuberculosis*:
Molecular Mechanisms and Clinical Implications

By

Mary Analise Zaunbrecher

B.S., University of Louisiana at Lafayette, 2004

Advisor: Thomas M. Shinnick, PhD.

A dissertation submitted to the Faculty of the Graduate
School of Emory University in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Program in Microbiology and Molecular Genetics
Graduate Division of Biological and Biomedical Sciences

"If the importance of a disease for mankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera and the like." Robert Koch, 1905

Acknowledgments

I would like to sincerely thank my family for their unending support as I have pursued my PhD, especially Laura who shared my experience of the good and bad parts of graduate school and helped me to persevere. Also, to my mother, whose guidance I will always cherish and whose early days of work with Leprosy paved the way for my interest in mycobacteria. Many thanks go to Dr. Don Ennis, who introduced me to scientific research and who encouraged me to pursue an advanced degree. I sincerely hope our relationship as colleagues will continue to grow throughout the duration of our scientific careers. To my lab mates in the Mycobacteriology Laboratory Branch at the CDC, especially Melissa, Patricia, Melisa, and Seidu, thank you for friendship, encouragement, and support. It has been an absolute pleasure learning from you. Thank you to Dr. Jamie Posey for teaching me countless molecular techniques, for acting as a mentor to me through the years, and for teaching me what it takes to succeed as a scientist. I would particularly like to thank my committee member Dr. Daniel Kalman, who has been an honored mentor, confidant, colleague, and friend. Most of all, I appreciate the support you have given me and your confidence in me. Thank you for always pushing me to aim higher, for your advice on subjects as wide ranging as writing a paper introduction to finding a job, for going out of your way for me, for challenging me, and for all that you have taught me. Thank you to my thesis advisor, Dr. Thomas Shinnick, for your guidance on countless aspects of research, writing, and tuberculosis. I would also like to thank my Committee Members Dr. William Shafer, Dr. Charlie Moran, and Dr. Gordon Churchward for their support. To my friends, I could not have done it without you! Thank you for the everyday encouragement and for providing a balance in my life and an outlet from science. Especially Ruth, who shares my passion for pursuing a cure to TB and who has spent way too many hours talking TB with me, thank you. Finally, I would like to thank Patrick, whose steady support has been instrumental in my completion of this degree. I am so thankful to have such wonderful people in my life.

Table of Contents

Chapter 1. Background……………………………………………………….... 1

Background References…………………………………………....... 38

Background Figures……………………………………………… 54

Chapter 2. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*……………..…………………………. 57

Chapter 2 References……………………………………………….. 79

Chapter 2 Tables/Figures…………………………………………… 83

Chapter 2 Supplemental Tables/Figures……………………………. 90

Chapter 3. Mutations in the *whiB7* promoter lead to cross resistance to kanamycin and streptomycin in *Mycobacterium tuberculosis*……… 101

Chapter 3 References……………………………………………….. 122

Chapter 3 Tables/Figures…………………………………………… 127

Chapter 3 Supplemental Tables/Figures……………………………. 135

Chapter 4. Kanamycin resistant *Mycobacterium tuberculosis* harboring mutations in the *eis* promoter display enhanced growth in macrophages……………………………………………………… 144

Chapter 4 References……………………………………………….. 158

Chapter 4 Tables/Figures…………………………………………… 161

Chapter 5. Conclusion……………………………………………………... 167

Conclusion References……………………………………………. 173

List of Tables

Chapter 2.

Table 1 *eis* mutations and aminoglycoside resistance levels……….………. 85

Table 2 Distribution of *eis* mutations in clinical isolates………………..…...… 86

Table S1 Strains used in this study……………………………………………. 94

Table S2 Plasmids and phage used in this study……………………………… 95

Table S3 Primer sequences…………………………………………………… 96

Chapter 3

Table 1 Spontaneous mutant survey and derivatives……………………… 129

Table 2 Effect of the *whiB7* ΔC-125 mutation on the emergence of high level streptomycin resistant mutants…………………………….…... 130

Table S1 Strains used in this study……………………………………………. 139

Table S2 Plasmids and phage used in this study……………………………… 140

Table S3 Primer sequences…………………………………………………… 141

Chapter 4

Table 1 Strains and phage used in this study………………………………… 162

List of Figures

Chapter 1.

Figure 1 Structure of kanamycin and amikacin………………………………. 55

Figure 2 Structure of streptomcyin…………………………………………… 56

Chapter 2

Figure 1 Characterization of *eis* promoter and expression…………………… 87

Figure 2 Analysis of *eis* expression and acetyltransferase activity…………... 88

Figure 3 Eis acetylates kanamycin and amikacin, kinetic analysis…………... 89

Figure S1 *sigA* transcript levels in H37Rv and K204………………………….. 97

Figure S2 Immunoblot analysis of cell filtrate proteins from wild type strains and *eis* mutants…………………………………………………… 98

Figure S3 Promoter truncation constructs……………………………………… 99

Figure S4 Disk diffusion assays for kanamycin, amikacin, and streptomycin… 100

Chapter 3.

Figure 1 *whiB7* promoter and mutations…………………………………... 131

Figure 2 *whiB7* mutations confer increased expression of *whiB7*, *eis*, and *tap*………………………………………………………………. 132

Figure 3 K301 derivatives on kanamycin and streptomycin…………………. 133

Figure 4 *whiB7* is necessary for basal level *eis* and *tap* expression………….. 134

Figure S1 qRT-PCR analysis of *eis* in K204 and K204Δ*whiB7*………………. 142

Figure S2 Acetyltransferase activity of clinical isolates with unexplained KAN resistance…………………………………………………….. 143

Chapter 4

Figure 1 Analysis of *eis* expression………………………………………….. 163

Figure 2 Comparison of intracellular growth………………………………… 164

Figure 3 *In vitro* growth curves………………………………………………… 165

Figure 4 Amikacin intracellular survival assays……………………………… 166

Chapter 1

BACKGROUND

Part I: Tuberculosis

Background & Epidemiology

Mycobacterium tuberculosis, the etiologic agent of tuberculosis (TB), has been evolving with humans for thousands of years and is arguably one of the world's most successful pathogens[1, 2]. TB is known by many names; the white plague for the paleness of an infected person's skin, consumption or wasting disease for the deterioration of an individual's health and lungs, and even Koch's disease in honor of Robert Koch who first identified the mycobacterium in 1884[3, 4].

Throughout history, tuberculosis has led to high levels of morbidity and mortality[1]. Some of the earliest evidence of this ancient disease dates back to 3000-2400 B.C.E. with the identification of acid fast bacilli and distinctive tubercule spinal caries in mummified remains[5, 6]. During the Middle Ages, 25% of deaths in Western Europe were attributed to TB[7]. Tuberculosis was also common in Europe and the United States during the Industrial Revolution due to an increase in urbanization, crowded living conditions, and a general decline in public health practices[8].

Today, *Mycobacterium tuberculosis* (MTB) is estimated to reside in a third of the world's population[9]. As a result of faltering control programs and an unprecedented international disease burden, tuberculosis was declared a global health emergency by the World Health Organization in 1993[10]. In the past few decades, the escalating HIV epidemic and emergence of drug resistant MTB strains have further complicated the treatment and control of TB[9]. Consequently, there is renewed interest in TB research to understand MTB pathogenesis and identify new methods of detection and treatment.

Tuberculosis in the United States

In 1900, TB was responsible for 194 out of every 100,000 deaths in the United States[11]. TB remained a leading cause of death until the 1950's when disease rates began to decline as the first anti-TB antibiotics became available[11]. By 1984, TB control efforts appeared to be working as the number of cases decreased to 9.4 cases per 100,000[12]. As the TB case load reduced in the 1970's and 1980's, the federal government began to decrease monetary support for TB research and control programs[12]. However, complacency regarding TB control contributed to a resurgence of the disease by 1985[13]. Over the next 7 years the number of TB cases increased by 13% each year. The resurgence was exacerbated by the concurrent emergence of the HIV epidemic and outbreaks of drug resistant TB strains in health care facilities[14]. Once federal funding increased in 1992 in response to the TB crisis, rates began to decline again, demonstrating that effective domestic TB programs are essential for disease control[12]. Initially, the average annual decline from 1991 to 2001 was measured at 7.3%[12]. After a slight stagnation in 2002, TB incidence in the Unites States has been steadily declining since 2004 with a reduction in cases each year between 3.8-4.2%[15, 16]. In 2009, the greatest single year decrease in TB cases was recorded with a drop of 11.4%[17]. Only 11,540 TB cases were reported (3.8 cases per 100,000), with the majority of cases occurring in foreign born immigrants[17]. In spite of the reduced number of TB cases, improvement in TB control and treatment is still needed to reach the goal set by TB Elimination of an annual incidence of <1 case per 1,000,000 population[12, 18].

Tuberculosis Worldwide

In 2007, the WHO reported over 9 million new cases of TB, and 1.7 million individuals succumbed to the disease[9]. Greater than 95% of TB cases occur in developing nations, exemplifying the disproportionate amount of global disease burden that falls on poor populations[9]. The pervasiveness of TB on the African continent is particularly disconcerting. The fifteen countries with the highest rates of TB incidence includes thirteen African nations[9]. Incidence rates in sub-Saharan Africa are 2 to 5 times higher than the average for high burden countries and range from 311-948 cases per 100,000[9]. In terms of the total number of cases, African nations account for 35% of the global TB burden. Southeast Asia and the Western Pacific nations comprise another 55% of TB cases[9]. India and China maintain the highest disease prevalence since both countries report over 1,000,000 new cases of TB annually.

Despite an enormous number of TB cases this year, the overall global per capita incidence rates of TB have been declining since 2004. However, as certain areas continue to struggle with TB control, accomplishing the goal set by the STOP TB partnership to half the number TB cases by 2015 seems increasingly difficult[19]. Improvement may still be possible with increased diligence and commitment to TB control.

Drug resistant *M. tuberculosis*

Drug resistant MTB strains present a significant challenging to controlling TB worldwide and pose a serious threat to accomplishing TB elimination. Multidrug resistant (MDR) TB strains are resistant to at least the two most effective first line anti-TB drugs, isoniazid (INH) and rifampin (RIF), but may also harbor additional resistances to other drugs[20]. Primary drug resistance occurs when a patient becomes infected with a drug resistant strain of MTB but has no previous history of TB infection or treatment. This commonly occurs when a patient is directly and repeatedly exposed to someone infected with MDR TB or lives in an area with high rates of MDR TB[14]. Secondary resistance arises in the host over the course of antibiotic treatment and is the result of treatment failure due to patient noncompliance or inadequate or inappropriate use of anti-TB drugs[14, 21]. Previous but ineffective TB treatment is the main risk factor for MDR TB and accounts for the majority of cases in the world[22-24].

Rates of MDR TB continue to rise[9]. In 2008, the WHO documented the highest proportion of MDR TB ever recorded at 5% of all TB cases[20]. That means of the 9.4 million new TB cases this year, 511,000 were classified as MDR[25]. These drug resistant strains are found globally in both developing and industrialized nations. China, India, and the Russian Federation account for the major global burden collectively harboring 62% of all MDR TB cases[21, 26]. Several areas of the world harbor an exceedingly high prevalence of MDR TB. A particularly troubling circumstance is the Former Soviet Union, where nine countries report MDR TB rates as high as 25%[20]. Additional hot spot regions include Latvia, Estonia, Thailand, South Africa, Bangladesh and the

Philippines[20]. These areas act as a reservoir of these dangerous strains where direct transmission of drug resistant strains remains a public health threat[20].

Many times, outbreaks occur in hospitals. From 1990-1992, eight nosocomial MDR TB outbreaks were investigated by the CDC in US hospitals that involved over 200 patients and 100 health care workers[14]. The largest nosocomial outbreak in Europe occurred in two Italian hospitals where 116 cases were reported in a largely HIV positive population[27].

Currently, identifying and diagnosing MDR TB primarily relies on the phenotypic analysis of an isolate[28]. Conventional drug susceptibility tests for *M. tuberculosis* are performed by agar proportion using a number of different possible media containing a pre-determined concentration of antibiotic, referred to as the critical concentration. An isolate is considered clinically resistant if at least 1% of colonies are able to grow on the critical concentration of a drug[28]. According to the CLSI standards, MDR TB strains are characterized by growth on 2 μg/ml rifampin and 0.125 μg/ml isoniazid[28]. However, a major disadvantage to this type of phenotypic testing is the amount of time required to receive results as the assay takes 30 days or longer. For patients receiving the wrong treatment regimen, this could be equivalent to not being treated or only receiving one or two effective drugs instead of four. Improper treatment can select for the development of additional resistances, and increases the chances of transmitting drug resistant strains.

Treating MDR-TB requires an increased duration of therapy with second line antibiotics, which are more toxic, less well tolerated, and less effective against MTB[14]. Second line drugs include the fluoroquinolones, the injectable aminoglycosides streptomycin, amikacin, and kanamycin, and the cyclic peptide, capreomycin among

others[29]. As cases of MDR TB increased, the WHO-sponsored Green Light Committee set to the task of supplying quality second line drugs globally to high burden areas and ensuring proper usage of drugs to prevent the development of further drug resistance[29].

Regardless, in 2006 the emergence of extensively drug resistant or XDR TB was described[30]. XDR isolates are MDR TB strains that have acquired additional resistances to a fluoroquinolone and at least one of the injectable antibiotics: kanamycin, amikacin, or capreomycin[30]. These strains leave few treatment options available to patients and have resulted in mortality rates similar to those seen in the pre-antibiotic era[9]. Fifty-eight countries have now reported XDR TB and a recent report calculated a global estimate that 7% of MDR strains are actually XDR, but this number varied greatly between different parts of the world and correlated with the strength of TB control programs in those areas[31]. The danger posed by XDR TB is exemplified by an outbreak in a hospital in KwaZulu Natal, South Africa[32]. Of the fifty-three HIV positive patients infected with the deadly strain, only one survived. Most patients quickly succumbed to the infection, dying an average of sixteen days after sputum collection[32].

Identifying XDR TB strains is difficult because drug susceptibility testing must be performed with second line antibiotics. For many second line drugs there is still no consensus on the best way to perform and interpret susceptibility tests. Guidelines have been proposed for susceptibility testing of some drugs being used to treat MDR TB including the injectable antibiotics and fluoroquinolones[28]. Even so, many developing countries lack the capacity and resources to perform susceptibility tests on second line drugs contributing to very poor patient outcomes.

Using molecular methods to detect resistance is an intriguing alternative, as it offers much more rapid results and thereby, appropriate adjustments to therapy. Several molecular tests are already on the market for identification of MDR TB such as the InnoLiPA hybridization assay that detects *rpoB* mutations that confer rifampin resistance. Developing such molecular methods requires understanding the mechanisms that cause resistance to the anti-TB drugs and the ability to distinguish which mutations are associated with resistance and which are not. New genetic methods have been recently introduced into the TB field that will help with addressing these questions including mycobacteriophage mediated systems of allelic exchange and recombineering[33-35]. As more information becomes available on which mutations confer antibiotic resistance, rapid molecular tests can be developed that accurately determine clinical resistance leading to more effective, individualized treatment regimens.

Sound TB control programs and public health practices are necessary to prevent further spread and creation of additional resistance[19, 31]. Drug resistant TB strains not only represent a threat to public health but are also difficult and expensive to diagnose and treat. The WHO estimates that the cost of MDR and XDR TB will reach $11 billion over the next five years[36]. The emergence of XDR TB illustrates the need to find novel and more affordable anti-TB drugs and to understand the mechanisms of resistance for the drugs already in use.

Tuberculosis risk factors

The chances of an individual contracting or developing TB disease are influenced by various risk factors. The most significant risk factor for TB is infection with HIV. Nearly 1.37 million of all new TB cases this year will be in HIV positive individuals, accounting for 15% all TB cases worldwide[9]. Countries with the highest burden of TB cases also struggle to control the HIV epidemic and consequently harbor high rates of TB and HIV co-infection. The highest rates are reported in the African nations of Zimbabwe (69%) and South Africa (73%)[9]. The synergy of these diseases makes co-infection a particularly deadly combination. Once co-infected, the risk for developing active TB disease increases to 10% for every year the individual is alive[37]. Additionally, once the disease begins progressing the infection will not be adequately controlled by the compromised immune system[37].

Other factors contribute to disease including poor nutrition, alcohol or drug use, and lack of medical care or sanitary living conditions[1, 38-40]. Certain subsets of the population are considered at higher risk such as the elderly, health care workers, and those in overcrowded situations such as prisons or homeless shelters[41]. Location can be a factor since residence in a country with a high burden of TB increases the risk of exposure and infection with MTB[39]. Similarly, frequent travel to countries with high rates of TB can be a risk factor[9]. There is some suggestion that genetic traits may increase susceptibility to TB. For example, it has been demonstrated many times that there are disparities in rates of TB incidence among racial and ethnic minority groups[15]. Data also suggests that susceptibility to TB may be heritable[42]. One study found a correlation between polymorphisms in the IL12β cytokine receptor and TB

susceptibility[42]. However, careful tests will need to be performed with larger group numbers to attest whether single gene polymorphisms significantly contribute to TB susceptibility.

Tuberculosis Disease

TB disease does not manifest in every individual that becomes infected[40]. In fact, only about 5-10% of those infected will develop active TB in their lifetime[40]. In greater than 90% of people, the immune system effectively prevents progression to active disease and the bacteria remain in a state of dormancy or latency[40]. In a latent tuberculosis infection (LTBI), the bacteria are thought to be in a metabolically inactive state and may remain that way for the lifetime of the individual[14, 40]. In a small percentage (5%) of latently infected individuals reactivation of the disease can occur later in life[37, 40]. The exact mechanism(s) of reactivation are not clear[37]. Reactivation can occur spontaneously or be induced by immunosuppressive events such as infection with HIV or the use of immune suppressing drugs[37]. Persons that develop active disease are estimated to pass the infection to up to 15 other people annually and are major contributors of disease transmission[43]. If untreated, active TB infections are fatal in 50% of cases and are a major cause of the high mortality rates associated with TB[37].

An active case of TB is characterized by fever and fatigue, and the person may have unexplained weight loss or loss of appetite and night sweats[44]. Typically, a productive cough occurs that may contain blood[44]. The infection is acquired by the aerosol route[40]. When an infected individual coughs, aerosolized droplets containing the bacterium are produced which can remain airborne for several hours[40, 45]. These aerosol droplets range from 3-5 μm and carry up to 10 bacilli[46]. Notably, it is estimated that a single aerosolized bacillus is sufficient to initiate infection[40]. Therefore, when an infectious droplet is inhaled by another individual, the disease can be transmitted[45]. Upon inhalation, the bacilli traverse into the alveoli of the lung where they encounter

alveolar macrophages and become phagocytosed[40]. In most cases, the alveolar macrophage is in an inactive or poorly activated state and engulfment of the bacilli into a phagosomal compartment provides a niche for the bacilli to replicate[40]. MTB block phagosomal maturation and fusion with lysosomes and instead continue slowly replicating in the phagosomal compartment of the macrophage until the cell bursts or undergoes apoptosis, releasing the bacilli and cellular contents into the surrounding tissue[40]. The presence of large numbers of bacteria and cellular debris recruits more macrophages and mononuclear cells to the primary lesion through the peripheral blood where they serve as new host cells for MTB replication[40]. The cycle of replication and re-infection of macrophages continues until an adaptive immune response develops and generates MTB-antigen specific lymphocytes[37, 40]. The recruitment of additional monocytes and lymphocytes to the site of infection forms the characteristic lesion of a tuberculosis infection, the granuloma[40]. The initial lesion becomes surrounded by a population of unactivated macrophages and foamy giant cells interspersed with lymphocytes[40]. Specific sets of cytokines produced by mononuclear cells and $CD4^+$ and $CD8^+$ T cells contribute to the development and architecture of the granuloma[40]. However, the fate of an individual granulomatous lesion varies. It is hypothesized that some granulomas lead to sterilization of the tissue and localized resolution of the disease[37, 40]. The majority of granulomas progress to fibrotic capsules that become calcified over time and perhaps harbor a population of nonreplicating or latent mycobacteria[40]. These fibrotic lesions wall off the mycobacteria from the surrounding tissue and infected individuals are likely asymptomatic and unable to transmit the disease[40]. Finally, some granulomas including old fibrotic lesions, become caseous and

necrotic and produce an environment conducive for MTB growth[40]. Individuals with compromised immune systems, who are at a higher risk for reactivation, are also more likely to develop caseous granulomas[37]. The high bacterial numbers present in a caseous granuloma can cause nearby tissue to become necrotic as well, leading to cavity formation[37, 40]. Ultimately, these lesions provide a vessel for MTB to spread within the lungs and enter the airways, leading to transmission of the disease[40].

***Mycobacterium tuberculosis* characteristics**

The biological characteristics of MTB contribute to the success of the pathogen to cause disease for thousands of years[47]. *Mycobacterium tuberculosis* is a member of the *Mycobacteriaceae* family which includes other disease causing pathogens including *M. leprae*, the causative agent for leprosy[47]. Mycobacteria are aerobic, nonmotile rods of approximately 2-4 µm in length that have a slow generation time of 18-24 hours[47]. The genome is guanine and cytosine rich (about 60%) and a large proportion of the annotated ~4000 genes are dedicated to lipid synthesis and metabolism[48, 49]. Unlike faster growing mycobacterial species, MTB only has a single copy of the 16S ribosomal RNA gene, *rrs,* which may contribute to the slow growth[48].

Although the mycobacterial cell wall contains peptidoglycan, the remaining ultrastructure greatly differs from gram positive and gram negative organisms[50]. Covalently linked to the peptidoglycan are arabinogalactan sugar moieties that serve as a scaffold for linking long chain fatty acids called mycolic acids to the cell surface[50]. Additional glycolipids and peptides are interspersed among the mycolic acids including phosphatidyl-innositol mannosides (PIM) and lipo-arabinomannan[50, 51]. The waxy, lipid rich cell wall prevents the decolorization caused by acid-alcohol in a traditional gram stain, leading to the characterization of mycobacteria as acid-fast organisms[47]. The mycolic acids become partially permeabilized by phenol present in the carbol-fuschin dye and retain a red color in an acid-fast stain, also known as the Ziehl-Neelsen stain[47].

About 60% of the mycobacterial cell wall is comprised of mycolic acids which affect the pathogenesis of MTB[50, 52]. Their hydrophobic nature influences and limits the

permeability of the mycobacterial cell wall, contributing to the high amount of intrinsic antibiotic resistance observed in mycobacteria[52].

TB diagnosis

In most parts of the world, the diagnosis of TB infections relies on sputum microscopy. This method involves direct Ziehl-Neelson staining of a clinical sputum sample followed by microscopic examination for acid-fast bacteria[53]. There are many limitations to sputum microscopy including low sensitivity and the inability to distinguish between pathogenic mycobacteria and environmental contaminants of non-tuberculosis mycobacteria (NTMs)[53]. However, it is still the best and only method available in many developing nations.

Many diagnostic labs in developing countries are now starting to use basic PCR technology to more accurately determine TB cases[54]. Several Nucleic Acid Amplification Tests (NAATs) that amplify MTB specific 16S ribosomal RNA sequences have now been made available to developing countries including the Amplified *Mycobacterium tuberculosis* Direct (ATBD) test and Amplicor (Roche Diagnostics)[54]. The Centers for Disease Control has released guidelines on how to properly use and interpret such tests[55].

A useful diagnostic for MTB infections is the Tuberculin Skin Test (TST). The TST, also known as the Mantoux test, involves the intradermal injection of tuberculin into the dorsal side of the forearm of an individual[56]. Tuberculin is composed of mycobacterial degradation products and purified protein derivatives (PPD)[56]. Test results are read 48-72 hours later allowing sufficient time for the development of a delayed-type hypersensitivity immune response[56]. A positive result is indicated by the induration of lymphocytes and phagocytic cells to the site of injection causing a red swollen bump to appear[56]. The TST is the most common test used in the United States today to determine

TB exposure[53, 56]. However, there are many limitations and complications associated with the TST. Persons immunized with the BCG vaccine will produce a false positive skin test due to BCG specific $CD4^+$ T-cells cross-reacting with tuberculin[53, 56]. This prevents skin testing from being used to diagnose MTB exposure in most foreign born populations. Additionally, an improperly administered test or a test given to an immuno-compromised individual can produce false negative results[53]. These and other complications warranted the design and implementation of more sophisticated TB tests.

Serological tests address many of the issues with skin testing and are commercially available in kits like Quanterferon-GOLD and T SPOT-TB[53]. In serologic tests, blood collected from a suspected TB patient is mixed with the specific MTB antigens, CFP10 and ESAT6[53]. After incubation for 16 hours, MTB-specific T-cells in the patient's blood are identified by measuring the production of interferon-gamma[53]. Because BCG lacks CFP10 and ESAT6 antigens, this test is specific to MTB without cross-reactivity, making it applicable to a larger percentage of the population and less subject to human error during results interpretation[53]. However, because the tests are only available as kits, most low-resource countries lack adequate funding to purchase serologic tests[53]. Also, because blood is required, processing of samples is time sensitive and may not be reliable in developing countries[53].

TB treatment

Few treatment options were available for TB patients prior to the advent of antibiotic therapy[57]. Based on folklore or superstition, patients would practice bleeding, starvation diets, or exercise in an attempt to be cured[58]. In the nineteenth century and early twentieth century, collapse therapy was widely practiced[58]. Extrapleural pneumothorax or plombage, in which air or foreign objects such as Lucite balls or gauze were inserted into extrapleural space, was among the many methods physicians used to attempt to collapse infected lung tissue[58]. Unfortunately, these methods frequently led to infection or the objects migrated away from the insertion site[58]. Many patients resorted to surgical intervention. Physicians would perform a thoracoplasty which removed part of the ribs in order to collapse infected lobes of the lung[58]. Pulmonary resection, which directly removed infected lung nodules provided slightly better patient outcomes and is sometimes used today for patients infected with drug resistant forms of MTB[58]. However, the invasiveness and complications associated with these procedures caused many TB patients to search for alternative treatment methods.

In the 1800's, the belief that fresh air and bed rest could improve TB symptoms assisted in the startup of the sanatorium movement that lasted approximately 100 years[59]. Sanatoriums served a dual purpose in TB treatment and control; patients were removed from the general population to prevent transmission and were provided with a healthy diet to assist in recovery[59]. Poor improvement rates and continued disease relapses eventually contributed to the closing of sanatoriums, especially after Robert Koch's discovery in 1882 that tuberculosis is caused by a microorganism[59].

The introduction of chemotherapy eradicated the need for collapse therapy, made surgical resection safer, and marked the beginning of a new era in TB treatment. In 1944, Selman Waksman's graduate student, Alfred Schatz and collaborators discovered that streptomycin (STR), purified from *Streptomyces griseus,* effectively killed MTB in guinea pigs with relatively low toxicity[60, 61]. The next year, the first human patient to receive streptomycin rapidly recovered from their illness[62]. In 1946, the British Medical Research Council (BMC) conducted the first clinical trials testing the efficacy of streptomycin[63]. Patients initially marked significant clinical and radiological improvement[63]. However, after six months of treatment 65% remained sputum positive and had developed high-level streptomycin resistance[63]. The high occurrence of streptomycin resistance in patients questioned whether the drug could consistently be used treat TB[63]. To inhibit streptomycin resistance development, the BMC conducted trials that added p-aminosalicyclic acid (PAS) to the TB treatment regimen[64]. The results found that when PAS is administered concurrently with STR, the emergence of streptomycin resistance was reduced from 70% to 9%[64]. This study demonstrated that combination drug therapy was more effective at preventing drug resistance than a single drug alone and set the standard for the multidrug therapy used to treat TB today[64].

Over the next two decades, new anti-tuberculosis drugs were introduced including isoniazid (INH) in 1952, pyrazinamide (PZA) in 1954, ethambutol (EMB) in 1962, and rifampin (RIF) in 1963[57]. Collectively, these four compounds are the first line drugs used for treating pansusceptible TB infections. The World Health Organization (WHO) currently recommends four treatment regimens for pansusceptible strains of MTB that generally include two months of combined therapy with isoniazid, rifampin,

pyrazinamide, and ethambutol followed by a continuation phase of four to seven months of treatment with isoniazid and rifampin[66]. Excellent cure rates are obtained when this standard course of treatment is followed correctly[29, 66]. Unfortunately, treatment failures and TB relapses continue to occur.

The WHO suggests that certain guidelines be followed when designing treatment regimens for MDR or XDR cases[29]. Generally, four to seven drugs should be included with certain or almost certain effectiveness against a patient's particular strain[29]. Standardized MDR TB treatment regimens vary in different countries depending on drug availability and funding[29]. Certain factors should be considered when designing an individualized treatment regimen such as drug susceptibility profiles and whether the drug is commonly used in the geographic area or is commonly associated with cross resistance[29]. Additionally, therapy should include drugs with the highest potency first before relying on more experimental antibiotics[29, 66]. Directly observed therapy should be conducted throughout treatment to detect any adverse reactions to second line agents or failure to improve[29].

DOTS and DOTS PLUS

A major goal proposed by the Stop TB Strategy is to increase case detection worldwide to 70% and cure rates of these cases to 85% in order to half the number of TB cases by 2015[19]. To meet this goal, the WHO developed and implemented the Directly Observed Therapy-Short Course (DOTS) program in 1991 to supply high quality drugs to TB patients and monitor their treatment[19]. Under the DOTS strategy, TB patients are identified through sputum microscopy and then tracked by health care workers or volunteers[19]. The health care workers provide drugs to TB patients and ensure the entire treatment course is completed. DOTS has been very advantageous to TB control since that patients who follow through with the standard regimen have excellent cure rates of >95%[19]. Additionally, by identifying more cases of TB and starting correct treatment quickly, an infected person will transmit less disease and prevent the development of drug-resistance[19]. With an average cost of ~$11 per patient for anti-TB drugs, DOTS established one of the most affordable public health care systems in the world[19]. It is estimated that 8 million deaths have been averted since the implementation of DOTS[67].

The DOTS program has met considerable success. The 182 countries that have adopted DOTS have increased detection rates, created more effective patient monitoring, and have developed systems for local program evaluation[9]. In 2009, fifty three countries reported treatment success rates of over 85%, meeting the goal set in 1991 for the first time[9].

A major obstacle to treating MDR TB and to TB eradication is access to second line drugs in resource-poor settings[68]. DOTS-Plus is an extension of the DOTS program designed to deal with the challenges of the emerging MDR TB epidemic[68]. One of the

initiatives of DOTS-Plus was the formation of the Green Light Committee (GLC), a working group that carefully controls access to high quality second line drugs and supervises their use[29]. Also, by partnering with pharmaceutical companies, the GLC provides second line drugs at very low cost[29]. If successful, this program should help prevent the emergence of and further transmission of drug resistant strains of MTB.

Anti-tuberculosis Drugs

Isoniazid (INH) is a synthetic derivative of nicotinamide[69]. When administered, INH is in a pro-drug form and must be activated by the catalase-peroxidase enzyme (*katG*) of *M. tuberculosis* before exhibiting antibacterial properties[69]. Upon activation, INH forms an adduct with NADH which binds to and inhibits InhA, an enoyl-ACP reductase essential for the synthesis of mycolic acids[69]. Resistance to INH occurs at a relatively high frequency (10^{-6}) and can be due to several mechanisms including mutations in the *katG*, *inhA, ndh,* or *mshA* loci[69, 70].

Rifampin (RIF) is a semi-synthetic bactericidal antibiotic that efficiently binds to the RNA polymerase β subunit (*rpoB*) and inhibits transcription[69]. The interaction between RIF and RpoB occurs at a specific binding pocket and >95% of RIF resistant bacteria harbor mutations in this 81 bp region termed the Rifampin Resistance Determining Region (RRDR)[69]. The addition of RIF to anti-TB therapy effectively shortened the duration of treatment from over a year to 6 months due to its potent sterilizing capability[71].

Pyrazinamide (PZA), another nicotinamide derivative, is a particularly important antituberculosis drug for the sterilization of an infected individual[69]. Because PZA efficiently targets non-replicating mycobacteria, the persister populations thought to be responsible for disease reactivation are eliminated[69]. Like INH, PZA is also administered as a pro-drug and must be activated by the mycobacterial enzyme pyrazinamidase, or *pncA*[69]. The mechanism of action of PZA is rather unique; activity is only maintained

under acidic pH and there is thought not to be a specific cellular target of PZA, but rather that the accumulation of pyranazoic acid acidifies the cytoplasm[69]. Increasing concentrations of protons in the cytoplasm damages the cell membrane or perhaps interferes with proton motive force and the ability of the cell to make energy[69]. Resistance is due to mutation in the activating enzyme, *pncA* which account for up to 100 % of all PZA resistant strains[72].

Ethambutol [65] is a bacteriostatic drug that inhibits the production of arabinogalactan and arabinomannan, leading to the disruption of cell wall synthesis[25, 69]. The arabinosyl-transferase, *embB,* is the target of EMB and mutations in codon 306 of this gene are responsible for the majority of ethambutol resistance[25, 69]. Several other mutations in the *embBAC* operon confer modest increases in EMB resistance[73]

New anti-TB drugs

New drugs are needed to shorten the current length of TB therapy, treat drug resistant strains of TB, and to improve treatment of latent TB cases[74]. Few anti-TB drugs have been developed since the 1950's partly because developing drugs for use primarily in developing countries offers poor profit margins to pharmaceutical companies[75].

One promising new class of drugs is the diarylquinolones (DARQs)[76]. DARQs target ATP synthases of *M. tuberculosis* and are capable of inhibiting both dormant and actively replicating cells[76]. The recently published results of Phase 2 clinical trials reported that including the DARQ compound TMC207 in treatment of MDR TB strains reduced bacterial counts in sputum faster than placebo and saw faster conversion to

sputum negative cultures[76]. Adding TMC207 to a treatment regimen encourages the possibility of decreasing treatment duration which should improve patient compliance and inhibit the emergence of drug resistance[76].

Other drugs are currently entering into clinical trials that also have novel mechanisms of action of action against MTB. The ethambutol analog, SQ109, is thought to target cell wall synthesis and has shown excellent synergy with rifampin[75]. The compound PA-824 is a bicyclic-nitroimidazo-furan prodrug that potently inhibits both cell wall and protein synthesis in mycobacteria[75]. Because PA-824 shows efficacy against replicating and non-replicating mycobacteria, it may be a good candidate for treating latent MTB infections[75]. Linezolid is a novel synthetic antibiotic belonging to the oxazolidinones class of protein synthesis inhibitors[77]. Linezolid is thought to bind to the 23S rRNA, but linezolid-resistant clinical isolates lack cross resistance with other 23S binding drugs like macrolides and chloramphenicol, suggesting a novel binding site or mechanism of action[77]. Studies examining the efficacy of linezolid against pulmonary TB patients found only moderate early bactericidal activity against *M. tuberculosis*, about half as effective as isoniazid in the first 2 days of treatment[78]. Another proposed approach to treating drug resistant *M. tuberculosis* has been to combine a traditional β-lactam antibiotic with a β-lactamase inhibitor such as clavulanate[65, 79]. Most of these compounds are years away from being incorporated into treatment regimens for drug resistant tuberculosis and will likely only be reserved for the most severe or untreatable cases, however, it is encouraging to see a renewed interest in developing the novel anti-TB drugs which are desperately needed[75].

Part II: Aminoglycosides

Properties of aminoglycosides

The aminoglycosides kanamycin, amikacin, and streptomycin are used as second line drugs in TB treatment and reserved to treat MDR TB infections. An assortment of aminoglycoside antibiotics are naturally produced by members of the genus *Streptomyces* and *Micromonospora*[80, 81]. Their structure is highly polar and consists of a core 2-deoxystreptamine ring that is mono- or di-substituted with amino sugar moieties (Fig 1)[81]. Streptomycin is an exception as it contains a central five-membered streptidine ring (Fig 2)[80]. A nearly infinite number of synthetic derivatives can be generated by substituting different side chains onto any position of the core structure or amino sugar rings[80]. Numerous synthetic modifications have increased the variety of aminoglycoside compounds produced and serve to reduce toxicity of the drugs, increase potency, and attempt to escape bacterial resistance mechanisms[80, 82]. Additionally, the broad specificity of aminoglycosides has led to their use in treatment against many types of bacterial infections like *Staphylococcus aureus* and several aerobic gram negatives including *Klebsiella pnuemoniae*[83]. Combining aminoglycosides with β-lactam antibiotics has shown a synergistic effect against intrinsically resistant bacteria like *Pseudomonas aeruginosa*, which has a relatively impermeable cell wall[80]. There are several disadvantages to using aminoglycosides for treatment. The hydrophilic nature of these drugs results is poor oral absorption, meaning the majority of compounds must be administered intravenously or by subcutaneous injection[84]. Also, due to possible renal- or oto-toxicity, the drugs can only be given either once a day or several times a week to

reduce side effects[84, 85]. Patients may experience permanent hearing loss or sensitivity in their extremities after prolonged exposure to aminoglycosides[85].

Aminoglycoside Mechanism of Action

Aminoglycosides inhibit protein translation by binding the A site of the 30S ribosomal subunit[80, 86]. X-ray crystallography and NMR studies that examined aminoglycoside and 30S ribosomal subunit interactions determined that the most important molecular contacts occur at the major groove of a triple adenine pocket in helix 44 of the 16S rRNA at positions A1408, A1492, and A1493 (*E. coli* numbering)[86]. Hydrogen bonds that form between the aminoglycoside and the ribosome cause a conformational shift of adenines A1492 and A1493 that stalls translocation of peptidyl-tRNA from the A-site and hinders protein production[86]. Streptomycin binds in a slightly different area of the 30S ribosome and makes additional interactions with the 530 loop of the 16S rRNA and the ribosomal S12 protein, *rpsL*[80]. Interaction of streptomycin with the ribosome not only inhibits tRNA translocation, but also causes miscoding of mRNA-tRNA pairs and the production of toxic protein intermediates that accumulate in the cytoplasm and membrane and poison the cell[87].

The specificity of aminoglycosides for bacterial rRNA is largely determined by the nucleotide in position 1408 (*E. coli* numbering, A1401 in MTB)[88]. Whereas bacterial rRNA sequence has an adenine at position 1408 that allows for efficient aminoglycoside binding, eukaryotic ribosomes have a conserved guanine residue in the homologous position on the rRNA that prevent aminoglycosides from having strong activity against human cells[88]. However, some weak activity targets mitochondrial rRNA which accounts for the ototoxicity and nephrotoxicity associated with aminoglycoside treatment[88].

Aminoglycoside Resistance

Resistance to aminoglycosides occurs through a variety of mechanisms and can either be intrinsic or acquired[82, 89]. Intrinsic resistance is due to the natural properties of an organism through either an impermeable cell wall, anaerobic metabolism, or efficient efflux of a drug[84, 90, 91]. Resistance is acquired through either horizontal transfer of a plasmid carrying resistance conferring genes or through spontaneous mutations[81]. Spontaneous mutations in the 16S ribosomal RNA sequence block aminoglycoside binding[81]. Mutations can also occur at different loci in the chromosome that may lead to slight cell wall modifications that decrease permeability[92, 93]. Resistance plasmids (or R plasmids) carry different types of enzymes that confer high levels of aminoglycoside resistance[81]. rRNA methyltransferases, such as *armA* of *Klebsiella pneumoniae*, modify critical positions on the rRNA that prevent efficient aminoglycoside interaction with the ribosome[51, 94]. The most common resistance mechanism is expression of an aminoglycoside modifying enzyme[80, 95]. Three types of modifying enzymes are found in a diverse set of bacteria: the phosphotransferases, acetyltransferases, and nucleotidyltransferases[80, 95]. These enzymes require a co-factor to covalently transfer a side chain onto an aminoglycoside[95]. Consequently, the modified drugs have considerably lower affinity for ribosomes and can no longer bind to the 16S rRNA to inhibit protein synthesis[95]. Many times a residue essential for intermolecular hydrogen bonding with the ribosome has been altered[95]. Modifying enzymes can be rather promiscuous or nonspecific and can modify several different structurally similar aminoglycosides, conferring cross resistance between them[95]. Therefore the enzymes are classified based on the pattern of resistance they confer[80]. The majority of the structural

genes for modifying enzymes are found on multidrug resistance R plasmids, transposons, or integrons, although a few cases of aminoglycoside resistance due to upregulation of a chromosomally encoded modifying enzyme have been documented[81, 95, 96].

Aminoglycoside Use in TB treatment

Aminoglycosides are currently reserved for use against MDR strains of TB[29]. Streptomycin, amikacin, or kanamycin is administered as part of a multidrug regimen including other second line antibiotics like the fluoroquinolones and capreomycin[29]. Determining which aminoglycoside antibiotic is used depends on the individual resistances of the TB strain, what drugs are available in the geographic region of the patient, and patient tolerance[29]. The three drugs are delivered at a dose of 15 mg/kg by subcutaneous injection into muscle tissue 3 to 5 times a week for the duration of therapy, which can last 24 months[21]. The peak serum concentration reaches approximately 30 mg/kg about 1 - 4 hours after injection[97]. Patients receiving aminoglycosides should be monitored for adverse side effects, including renal toxicity and nerve damage which could lead to permanent hearing loss[98].

Aminoglycoside Resistance in *M. tuberculosis*

Streptomycin resistance

Streptomycin was the first antibiotic used to treat tuberculosis infections[57]. However, because streptomycin was given to patients as mono-drug therapy, resistant strains quickly emerged[63]. Prevalence of streptomycin resistance has reduced the utility of including streptomycin in a drug regimen and the once first-line antibiotic is now reserved for cases of MDR or areas where streptomycin is the only choice[29].

Resistance to streptomycin is observed over a range of minimal inhibitory concentrations (MIC) from high (growth at ≥10μg/ml) to low (growth on 2 μg/ml)[100]. The Clinical Laboratory Standards Institute (CLSI) document recommends drug susceptibility testing be performed at two concentration levels (2 μg/ml and 10 μg/ml) which allows for distinction between high and low level resistant strains and may influence the treatment prescribed by physicians[28].

High level streptomycin resistance is conferred by point mutations in either the *rrs* or *rpsL* (S12 protein) loci and arises at a frequency of 10^{-8} [89, 101, 102]. Although many SNPs are found in the *rpsL* gene, only a few are actually associated with streptomycin resistance[69, 102, 103]. The recent introduction of recombineering and specialized transducing phages into mycobacterial research has facilitated the understanding and identification of which mutations are involved in drug resistance[34]. For example, the introduction of the K43R *rpsL* allele into a pansusceptible strain by recombineering confirmed that this mutation is sufficient at conferring streptomycin resistance[34]. Streptomycin resistant isolates may also harbor mutations in the 530 loop of the 16S rRNA, such as the A514C or C517T alleles (formerly annotated as A513C and

C516T)[103, 104]. Although no functional genetics have been performed with the *rrs* mutations, the correlation between these mutations and streptomycin resistance is very high[103, 104].

Recently, one of the loci responsible for low level streptomycin resistance was described[105]. Through comparative genomic sequencing of streptomycin resistant and susceptible strains, the *gidB* locus was identified[105]. *gidB* encodes a 7-methylguanosine (m7G) methyltransferase that is thought to methylate the G527 in the 530 loop of the 16S rRNA at the streptomycin binding site[105]. Since a *gidB* knockout is resistant to streptomycin, this implies that the methylation is important for proper binding of streptomycin to the ribosome[105]. Additionally, mutations in *gidB* were found in a third of low level streptomycin resistant clinical isolates surveyed and arise at a relatively high spontaneous mutation frequency (2.8×10^{-6})[105]. Because cells that harbor *gidB* mutations are 10^3 times more likely to develop high level streptomycin resistance due to *rrs* or *rpsL* mutations, this mechanism may help explain why resistance emerged so quickly upon the introduction of streptomycin as an anti-tuberculosis drug[105].

It has also been proposed that efflux pumps could mediate low level resistance to streptomycin as well as other antibiotics[102, 106]. Treatment of *M. tuberculosis* strains with the efflux pump inhibitors CCCP or verapamil was found to decrease the MIC of streptomycin in strains with or without mutations known to confer streptomycin resistance[102]. Therefore, it is possible that other uncharacterized mechanisms of streptomycin resistance exist due to increased expression of efflux pumps.

Kanamycin resistance

If a patient is diagnosed with MDR TB and has no prior history of treatment with aminoglycosides, kanamycin may be a viable treatment option[29]. In guidelines set by the Clinical Laboratory Standards Institute, resistance to kanamycin is defined as growth on 7H10 agar containing the critical concentration of drug; 5 µg/ml[28]. However, this recommendation does not account for the poorly understood fact that more than one type of kanamycin resistance exists.

Resistance to kanamycin is either high (MIC ≥80 µg/ml) or low (MIC < 80 µg/ml) level[107]. Strains with high level resistance usually harbor *rrs* mutations[107]. Specifically, the *rrs* alleles A1401G and G1484T confer the majority of cases of high level KAN resistance and occur at a frequency of approximately $1x10^{-8}$ in laboratory strains[107]. High level kanamycin resistant strains are also cross resistant to other ribosome binding antibiotics including capreomycin and amikacin (discussed more below)[107]. The role of the A1401G in kanamycin resistance was first demonstrated in *M. smegmatis* by the introduction of the A1389G allele (equivalent to A1401G in MTB) into a kanamycin susceptible isolate[108]. Many reports document that clinical isolates harboring A1401G and G1484T mutations display high level kanamycin resistance, although no functional genetics have been performed for the G1484T allele[99, 107-110].

Low level kanamycin resistance is less well understood, but appears to be the result of a distinct, but uncharacterized mechanism(s) of resistance[107]. This phenotype arises at a much higher frequency in the lab than high level resistance ($\sim 1x10^{-6}$) suggesting that multiple chromosomal targets are involved[107]. Additionally, in contrast to high level resistant strains, low level resistant strains display a range of MICs from 5 – 40

µg/ml, lack *rrs* mutations, and are susceptible to amikacin[99],[107]. A proportion of these strains can be explained by harboring the *rrs* C1402T allele, which confers a kanamycin MIC of around 10 µg/ml[107]. Although low level resistance has been reported in 30- 80% of kanamycin resistant clinical isolates, the mechanism responsible for this phenotype remains unexplained[99, 109, 110].

Amikacin resistance

Some physicians may incorporate amikacin into an MDR TB treatment regimen instead of kanamycin depending on geographic location and which drug is readily available[29, 111]. Amikacin is a semi-synthetic derivative of kanamycin with increased potency against *M tuberculosis*[29, 80]. Amikacin resistant clinical isolates almost always harbor point mutations in *rrs*[107, 112, 113]. Both the A1401G and G1484T alleles are associated with high level amikacin resistance (MICs ≥ 64 µg/ml) and are cross resistant to kanamycin, whereas low level amikacin resistance is rarely observed[107]. Currently the Clinical Laboratory Standards Institute (CLSI) document recommends using kanamycin as the class representative for both amikacin and kanamycin, although complete cross resistance between the two drugs does not always occur[28].

Cross resistance between ribosome binding antibiotics

The aminoglycosides, kanamycin and amikacin, share a similar binding site on the ribosome with the cyclic peptides capreomycin and viomycin[88, 114]. Therefore, it is not surprising that cross resistance is observed between them[107]. Capreomycin is a cyclic peptide antibiotic used for treating MDR TB strains and shares a similar structure to

viomycin[115]. A study that compared spontaneous mutants generated on the four drugs singularly or in combination found clear associations between specific *rrs* mutations and the MIC conferred on each antibiotic[107]. This study demonstrated that a C1402T mutation is associated with high level resistance to CAP and VIO and low level resistance to KAN, but susceptibility to AMK[107]. In contrast, an *rrs* G1484T mutation confers high level resistance to all four antibiotics[107]. One area that is still not fully understood is the association between an A1401G allele and CAP resistance. The A1401G mutation consistently confers high level resistance to KAN and AMK, but resistance to CAP is detected in only about 50% of strains[107]. Functional genetics and careful studies that investigate the CAP MIC in a clean genetic background are still required to fully understand the contribution of the A1401G allele in CAP resistance.

Analysis of spontaneous mutants generated on single antibiotics revealed important implications for treatment selection for MDR TB strains and the prevention of further antibiotic resistance[107]. Selection of mutants on CAP or VIO alone typically resulted in *tlyA* mutations that do not confer cross resistance with the aminoglycosides[107]. Selection on KAN alone usually resulted in mono-KAN resistant mutants that were susceptible to AMK and CAP[107]. Importantly, this study documented that the generation of spontaneous mutants to amikacin almost exclusively led to mutations in the 16S ribosomal RNA (namely A1401G and G1484T) that conferred cross-resistance to high concentrations of both KAN and CAP[107].

The implication of this work is that treatment with amikacin likely selects for mutants with cross-resistance to capreomycin and kanamycin and eliminates the prospect of treatment with any of these injectable antibiotics. However, treatment with kanamycin

may select for mutants in unidentified loci that may retain the option of treatment with either amikacin or capreomycin. Taken together, these findings suggest that a general hierarchy of the injectable antibiotics be considered when treating drug resistant TB cases to prevent cross resistance development. Specifically, capreomycin should be used for treatment first, then kanamycin, and amikacin last. Molecular tests that could quickly distinguish the mechanism of aminoglycoside resistance in a clinical isolate could rapidly provide data on what treatment options are possible.

REFERENCES

1. Russell, D. G. Mycobacterium tuberculosis: here today, and here tomorrow. Nat Rev Mol Cell Biol 2, 569-77 (2001).
2. Donoghue, H. D. Human tuberculosis--an ancient disease, as elucidated by ancient microbial biomolecules. Microbes Infect 11, 1156-62 (2009).
3. Sacchettini, J. C., Rubin, E. J. & Freundlich, J. S. Drugs versus bugs: in pursuit of the persistent predator Mycobacterium tuberculosis. Nat Rev Microbiol 6, 41-52 (2008).
4. Bhansali, S. K. Abdominal tuberculosis. Experiences with 300 cases. Am J Gastroenterol 67, 324-37 (1977).
5. Zink, A. R. et al. Characterization of Mycobacterium tuberculosis complex DNAs from Egyptian mummies by spoligotyping. J Clin Microbiol 41, 359-67 (2003).
6. Allison, M. J., Mendoza, D. & Pezzia, A. Documentation of a case of tuberculosis in Pre-Columbian America. Am Rev Respir Dis 107, 985-91 (1973).
7. Chalke, H. D. Some historical aspects of tuberculosis. Public Health 74, 83-95 (1959).
8. Murray, J. F. Mycobacterium tuberculosis and the cause of consumption: from discovery to fact. Am J Respir Crit Care Med 169, 1086-8 (2004).
9. World Health Organization. Global tuberculosis control - epidemiology, strategy, financing. World Report 2009. WHO/HTM/TB/2009.411 (2009).
10. World Health Organization. World TB day 2003 highlights report. WHO/HTM/STB/2003.26 (2003).
11. Control of infectious diseases. MMWR Morb Mortal Wkly Rep 48, 621-9 (1999).

12. Navin, T. R., McNabb, S. J. & Crawford, J. T. The continued threat of tuberculosis. Emerg Infect Dis 8, 1187 (2002).

13. Cantwell, M. F., Snider, D. E., Jr., Cauthen, G. M. & Onorato, I. M. Epidemiology of tuberculosis in the United States, 1985 through 1992. Jama 272, 535-9 (1994).

14. Humma, L. M. Prevention and treatment of drug-resistant tuberculosis. Am J Health Syst Pharm 53, 2291-8; quiz 2335-6 (1996).

15. Trends in tuberculosis--United States, 1998-2003. MMWR Morb Mortal Wkly Rep 53, 209-14 (2004).

16. Trends in tuberculosis--United States, 2007. MMWR Morb Mortal Wkly Rep 57, 281-5 (2008).

17. Vetting, M. W., et al. Structure and functions of the GNAT superfamily of acetyltransferases. Arch Biochem Biophys 433, 212-26 (2005).

18. Trends in tuberculosis--United States, 2004. MMWR Morb Mortal Wkly Rep 54, 245-9 (2005).

19. World Health Organization. The Stop TB Strategy. WHO/HTM/TB/2006.368 (2006).

20. World Health Organization. Anti-Tuberculosis drug resistance in the world, Report No. 4. WHO/HTM/TB/2008.394 (2008).

21. Yew, W. W. & Leung, C. C. Management of multidrug-resistant tuberculosis: Update 2007. Respirology 13, 21-46 (2008).

22. Faustini, A., Hall, A. J. & Perucci, C. A. Risk factors for multidrug resistant tuberculosis in Europe: a systematic review. Thorax 61, 158-63 (2006).

23. Espinal, M. A. et al. Determinants of drug-resistant tuberculosis: analysis of 11 countries. Int J Tuberc Lung Dis 5, 887-93 (2001).

24. Sotgiu, G. et al. Epidemiology and clinical management of XDR-TB: a systematic review by TBNET. Eur Respir J 33, 871-81 (2009).

25. Starks, A. M., Gumusboga, A., Plikaytis, B. B., Shinnick, T. M. & Posey, J. E. Mutations at embB codon 306 are an important molecular indicator of ethambutol resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 53, 1061-6 (2009).

26. Zignol, M. et al. Global incidence of multidrug-resistant tuberculosis. J Infect Dis 194, 479-85 (2006).

27. Moro, M. L. et al. An outbreak of multidrug-resistant tuberculosis involving HIV-infected patients of two hospitals in Milan, Italy. Italian Multidrug-Resistant Tuberculosis Outbreak Study Group. Aids 12, 1095-102 (1998).

28. National Committee for Clinical laboratory Standards. Susceptibilty Testing of *Mycobacteria, Norcardiae,* and other aerobic actinomycetes. Approved Standard M24-A.

(Wayne, Pa, 2003).

29. World Health Organization. Treatment of tuberculosis guidelines. WHO/HTM/TB/2009.420 (2010).

30. Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs--worldwide, 2000-2004. MMWR Morb Mortal Wkly Rep 55, 301-5 (2006).

31. World Health Organization. The global MDR-TB & XDR-TB responce plan. WHO/HTM/TB/2007.387 (2007).

32. Gandhi, N. R. et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet 368, 1575-80 (2006).

33. Bardarov, S. et al. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in Mycobacterium tuberculosis, M. bovis BCG and M. smegmatis. Microbiology 148, 3007-17 (2002).

34. van Kessel, J. C. & Hatfull, G. F. Efficient point mutagenesis in mycobacteria using single-stranded DNA recombineering: characterization of antimycobacterial drug targets. Mol Microbiol 67, 1094-107 (2008).

35. van Kessel, J. C. & Hatfull, G. F. Mycobacterial recombineering. Methods Mol Biol 435, 203-15 (2008).

36. world Health Organization. The Ministerial Meeting of High M/XDR-TB Burden Countries Meeting Report. WHO/HTM/TB/2009.415 (2009).

37. Kaufmann, S. H. How can immunology contribute to the control of tuberculosis? Nat Rev Immunol 1, 20-30 (2001).

38. Buskin, S. E., Gale, J. L., Weiss, N. S. & Nolan, C. M. Tuberculosis risk factors in adults in King County, Washington, 1988 through 1990. Am J Public Health 84, 1750-6 (1994).

39. Murray, C. J. & Lopez, A. D. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. Lancet 349, 1436-42 (1997).

40. Russell, D. G. Who puts the tubercle in tuberculosis? Nat Rev Microbiol 5, 39-47 (2007).

41. Tuberculosis transmission in a homeless shelter population--New York, 2000-2003. MMWR Morb Mortal Wkly Rep 54, 149-52 (2005).

42. Tso, H. W., Lau, Y. L., Tam, C. M., Wong, H. S. & Chiang, A. K. Associations between IL12B polymorphisms and tuberculosis in the Hong Kong Chinese population. J Infect Dis 190, 913-9 (2004).

43. Kaufmann, S. H. Is the development of a new tuberculosis vaccine possible? Nat Med 6, 955-60 (2000).

44. Miller, L. G. et al. A population-based survey of tuberculosis symptoms: how atypical are atypical presentations? Clin Infect Dis 30, 293-9 (2000).

45. Behr, M. A. et al. Transmission of Mycobacterium tuberculosis from patients smear-negative for acid-fast bacilli. Lancet 353, 444-9 (1999).

46. Cole, E. C. & Cook, C. E. Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies. Am J Infect Control 26, 453-64 (1998).

47. Wayne, L., Kubica GP. in Bergey's Manual of Systematic Bacteriology, vol 2, (ed. Sneath, P. H. A.) p. 1435-1454.

(Baltimore, 1986).

48. Cole, S. T. et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393, 537-44 (1998).

49. Camus, J. C., Pryor, M. J., Medigue, C. & Cole, S. T. Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv. Microbiology 148, 2967-73 (2002).

50. Brennan, P. J. Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. Tuberculosis (Edinb) 83, 91-7 (2003).

51. Galimand, M., Sabtcheva, S., Courvalin, P. & Lambert, T. Worldwide disseminated armA aminoglycoside resistance methylase gene is borne by composite transposon Tn1548. Antimicrob Agents Chemother 49, 2949-53 (2005).

52. Nguyen, L. & Thompson, C. J. Foundations of antibiotic resistance in bacterial physiology: the mycobacterial paradigm. Trends Microbiol 14, 304-12 (2006).

53. Hauck, F. R., Neese, B. H., Panchal, A. S. & El-Amin, W. Identification and management of latent tuberculosis infection. Am Fam Physician 79, 879-86 (2009).

54. Dinnes, J. et al. A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. Health Technol Assess 11, 1-196 (2007).

55. Update: Nucleic acid amplification tests for tuberculosis. MMWR Morb Mortal Wkly Rep 49, 593-4 (2000).

56. Bates, J. H. in Tuberculosis (ed. William N and Stuart Garay) p. 865-871. (Little, Brown and Company, Boston, 1996).

57. Harris, W. H. in Tuberculosis (ed. William N. and Stuart M. Garay) p 745-761 (Little, Brown and Company, Boston, 1996).

58. Boyd, A. D., Bernard K. Crawford, Lawernce Glassman,. in Tuberculosis (ed. William N. and Stuart M. Garay) p 513-523 (Little, Brown and Company, Boston, 1996).

59. Davis, A. in Tuberculosis (ed. Garay, W. N. S. M.) p. 35-54. (Little, Brown and Company, Boston, 1996).

60. Schatz, A., Bugie, E. & Waksman, S. A. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. 1944. Clin Orthop Relat Res, 3-6 (2005).

61. Feldman, W. H., Hinshaw, H. C. & Mann, F. C. Studies in streptomycin in tuberculosis; the action of streptomycin in experimental tuberculosis. Trans Annu Meet Natl Tuberc Assoc 42, 64 (1947).

62. Pfuetze, K. H., Pyle, M. M., Hinshaw, H. C. & Feldman, W. H. The first clinical trial of streptomycin in human tuberculosis. Am Rev Tuberc 71, 752-4 (1955).

63. STREPTOMYCIN treatment of pulmonary tuberculosis. Br Med J 2, 769-82 (1948).

64. TREATMENT of pulmonary tuberculosis with streptomycin and para-aminosalicylic acid; a Medical Research Council investigation. Br Med J 2, 1073-85 (1950).

65. Hugonnet, J. E., Tremblay, L. W., Boshoff, H. I., Barry, C. E., 3rd & Blanchard, J. S. Meropenem-clavulanate is effective against extensively drug-resistant Mycobacterium tuberculosis. Science 323, 1215-8 (2009).

66. Treatment of tuberculosis. MMWR Recomm Rep 52, 1-77 (2003).

67. World Health Organization. Tuberculosis Facts 2009 Update. WHO/HTM/TB/2009.426 (2009).

68. Farmer, P. & Kim, J. Y. Community based approaches to the control of multidrug resistant tuberculosis: introducing "DOTS-plus". Bmj 317, 671-4 (1998).

69. Wade, M. M. & Zhang, Y. Mechanisms of drug resistance in Mycobacterium tuberculosis. Front Biosci 9, 975-94 (2004).

70. Vilcheze, C. et al. Mycothiol biosynthesis is essential for ethionamide susceptibility in Mycobacterium tuberculosis. Mol Microbiol 69, 1316-29 (2008).

71. Dickinson, J. M. & Mitchison, D. A. Experimental models to explain the high sterilizing activity of rifampin in the chemotherapy of tuberculosis. Am Rev Respir Dis 123, 367-71 (1981).

72. Scorpio, A. et al. Rapid differentiation of bovine and human tubercle bacilli based on a characteristic mutation in the bovine pyrazinamidase gene. J Clin Microbiol 35, 106-10 (1997).

73. Safi, H. et al. Allelic exchange and mutant selection demonstrate that common clinical embCAB gene mutations only modestly increase resistance to ethambutol in Mycobacterium tuberculosis. Antimicrob Agents Chemother 54, 103-8.

74. O'Brien, R. J. & Nunn, P. P. The need for new drugs against tuberculosis. Obstacles, opportunities, and next steps. Am J Respir Crit Care Med 163, 1055-8 (2001).

75. Rivers, E. C. & Mancera, R. L. New anti-tuberculosis drugs in clinical trials with novel mechanisms of action. Drug Discov Today 13, 1090-8 (2008).

76. Diacon, A. H. et al. The diarylquinoline TMC207 for multidrug-resistant tuberculosis. N Engl J Med 360, 2397-405 (2009).

77. Hillemann, D., Rusch-Gerdes, S. & Richter, E. In vitro-selected linezolid-resistant Mycobacterium tuberculosis mutants. Antimicrob Agents Chemother 52, 800-1 (2008).

78. Dietze, R. et al. Early and extended early bactericidal activity of linezolid in pulmonary tuberculosis. Am J Respir Crit Care Med 178, 1180-5 (2008).

79. Chambers, H. F., Kocagoz, T., Sipit, T., Turner, J. & Hopewell, P. C. Activity of amoxicillin/clavulanate in patients with tuberculosis. Clin Infect Dis 26, 874-7 (1998).

80. Magnet, S. & Blanchard, J. S. Molecular insights into aminoglycoside action and resistance. Chem Rev 105, 477-98 (2005).

81. Jana, S. & Deb, J. K. Molecular understanding of aminoglycoside action and resistance. Appl Microbiol Biotechnol 70, 140-50 (2006).

82. Shakil, S., Khan, R., Zarrilli, R. & Khan, A. U. Aminoglycosides versus bacteria--a description of the action, resistance mechanism, and nosocomial battleground. J Biomed Sci 15, 5-14 (2008).

83. Davies, J. & Wright, G. D. Bacterial resistance to aminoglycoside antibiotics. Trends Microbiol 5, 234-40 (1997).

84. Dworkin, R. J. Aminoglycosides for the treatment of gram-negative infections: therapeutic use, resistance and future outlook. Drug Resist Updat 2, 173-179 (1999).

85. Duggal, P. & Sarkar, M. Audiologic monitoring of multi-drug resistant tuberculosis patients on aminoglycoside treatment with long term follow-up. BMC Ear Nose Throat Disord 7, 5 (2007).

86. Fourmy, D., Recht, M. I. & Puglisi, J. D. Binding of neomycin-class aminoglycoside antibiotics to the A-site of 16 S rRNA. J Mol Biol 277, 347-62 (1998).

87. Melancon, P., Tapprich, W. E. & Brakier-Gingras, L. Single-base mutations at position 2661 of Escherichia coli 23S rRNA increase efficiency of translational proofreading. J Bacteriol 174, 7896-901 (1992).

88. Recht, M. I., Douthwaite, S. & Puglisi, J. D. Basis for prokaryotic specificity of action of aminoglycoside antibiotics. Embo J 18, 3133-8 (1999).

89. Walsh, C. Molecular mechanisms that confer antibacterial drug resistance. Nature 406, 775-81 (2000).

90. Poole, K. Efflux-mediated antimicrobial resistance. J Antimicrob Chemother 56, 20-51 (2005).

91. Mingeot-Leclercq, M. P., Glupczynski, Y. & Tulkens, P. M. Aminoglycosides: activity and resistance. Antimicrob Agents Chemother 43, 727-37 (1999).

92. Bryan, L. E. & Kwan, S. Aminoglycoside-resistant mutants of Pseudomonas aeruginosa deficient in cytochrome d, nitrite reductase, and aerobic transport. Antimicrob Agents Chemother 19, 958-64 (1981).

93. Schurek, K. N. et al. Novel genetic determinants of low-level aminoglycoside resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother 52, 4213-9 (2008).

94. Cundliffe, E. How antibiotic-producing organisms avoid suicide. Annu Rev Microbiol 43, 207-33 (1989).

95. Wright, G. D. Aminoglycoside-modifying enzymes. Curr Opin Microbiol 2, 499-503 (1999).

96. Hotta, K., Ishikawa, J., Ogata, T. & Mizuno, S. Secondary aminoglycoside resistance in aminoglycoside-producing strains of Streptomyces. Gene 115, 113-7 (1992).

97. Bastian, I. & Colebunders, R. Treatment and prevention of multidrug-resistant tuberculosis. Drugs 58, 633-61 (1999).

98. Peloquin, C. A., et al. Aminoglycoside toxicity: daily versus thrice-weekly dosing for treatment of mycobacterial diseases. Clin Infect Dis 38, 1538-44 (2004).

99. Kruuner, A., Jureen, P., Levina, K., Ghebremichael, S. & Hoffner, S. Discordant resistance to kanamycin and amikacin in drug-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother 47, 2971-3 (2003).

100. Cooksey, R. C., Morlock, G. P., McQueen, A., Glickman, S. E. & Crawford, J. T. Characterization of streptomycin resistance mechanisms among Mycobacterium tuberculosis isolates from patients in New York City. Antimicrob Agents Chemother 40, 1186-8 (1996).

101. Sreevatsan, S. et al. Characterization of rpsL and rrs mutations in streptomycin-resistant Mycobacterium tuberculosis isolates from diverse geographic localities. Antimicrob Agents Chemother 40, 1024-6 (1996).

102. Spies, F. S., da Silva, P. E., Ribeiro, M. O., Rossetti, M. L. & Zaha, A. Identification of mutations related to streptomycin resistance in clinical isolates of

Mycobacterium tuberculosis and possible involvement of efflux mechanism. Antimicrob Agents Chemother 52, 2947-9 (2008).

103. Via, L. E. et al. Polymorphisms associated with resistance and cross-resistance to aminoglycosides and capreomycin in Mycobacterium tuberculosis isolates from South Korean Patients with drug-resistant tuberculosis. J Clin Microbiol 48, 402-11.

104. Tracevska, T. et al. Characterisation of rpsL, rrs and embB mutations associated with streptomycin and ethambutol resistance in Mycobacterium tuberculosis. Res Microbiol 155, 830-4 (2004).

105. Okamoto, S. et al. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. Mol Microbiol 63, 1096-106 (2007).

106. Gupta, A. K. et al. Estimation of efflux mediated multi-drug resistance and its correlation with expression levels of two major efflux pumps in mycobacteria. J Commun Dis 38, 246-54 (2006).

107. Maus, C. E., Plikaytis, B. B. & Shinnick, T. M. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in Mycobacterium tuberculosis. Antimicrob Agents Chemother 49, 3192-7 (2005).

108. Taniguchi, H. et al. Molecular analysis of kanamycin and viomycin resistance in Mycobacterium smegmatis by use of the conjugation system. J Bacteriol 179, 4795-801 (1997).

109. Alangaden, G. J., et al, . Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 42, 1295-7 (1998).

110. Suzuki, Y. et al. Detection of kanamycin-resistant Mycobacterium tuberculosis by identifying mutations in the 16S rRNA gene. J Clin Microbiol 36, 1220-5 (1998).

111. American Thoracic Society. Centers for Disease Control and Prevention. and Infectious Diseases Society of America. in American Journal of Respiratory and Critical Care Medicine 603-62 (2003).

112. Jugheli, L. et al. High level of cross-resistance between kanamycin, amikacin, and capreomycin among Mycobacterium tuberculosis isolates from Georgia and a close relation with mutations in the rrs gene. Antimicrob Agents Chemother 53, 5064-8 (2009).

113. Feuerriegel, S. et al. Sequence analyses of just four genes to detect extensively drug-resistant Mycobacterium tuberculosis strains in multidrug-resistant tuberculosis patients undergoing treatment. Antimicrob Agents Chemother 53, 3353-6 (2009).

114. Johansen, S. K., Maus, C. E., Plikaytis, B. B. & Douthwaite, S. Capreomycin binds across the ribosomal subunit interface using tlyA-encoded 2'-O-methylations in 16S and 23S rRNAs. Mol Cell 23, 173-82 (2006).

115. Maus, C. E., Plikaytis, B. B. & Shinnick, T. M. Mutation of tlyA confers capreomycin resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 49, 571-7 (2005).

116. World Health Organization. in
Antituberculosis Drug Resistance in the World Report No. 4 (Geneva, Switzerland, 2008).

117. CDC. Emergence of *Mycobacterium tuberculosis* with extensive resistance to second line drugs-worldwide, 2000-2004. *MMWR* 55, 301-5 (2006).

118. Gandhi, N. R., et al,. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* 368, 1575-80 (2006).

119. Johnson, R., et al. Drug resistance in *Mycobacterium tuberculosis*. Curr Issues Mol Biol 8, 97-111 (2006).

120. Suzuki, Y., et al. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. J Clin Microbiol 36, 1220-5 (1998).

121. Taniguchi, H., et al. Molecular analysis of kanamycin and viomycin resistance in *Mycobacterium smegmatis* by use of the conjugation system. J Bacteriol 179, 4795-801 (1997).

122. NCCLS. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes; approved standard M24-A. (2003).

123. Tuberculosis Coalition for Technical Assistance. in The Hague: Tuberculosis Coalition for Technical Assistance, 2006. .

124. Samuel, L. P., et al. Expression, production and release of the Eis protein by *Mycobacterium tuberculosis* during infection of macrophages and its effect on cytokine secretion. Microbiology 153, 529-40 (2007).

125. Wu, S., et al. Activation of the eis gene in a W-Beijing strain of Mycobacterium tuberculosis correlates with increased SigA levels and enhanced intracellular growth. Microbiology 155, 1272-81 (2009).

126. Gomez M. and Smith I. in Molecular genetics of Mycobacteria (ed. Hatfull GF, J. W., Jr.) 111-29. (American Society for Microbiology Press, Washington, DC, 2000).

127. Roberts, E. A., Clark, A., McBeth, S. & Friedman, R. L. Molecular characterization of the *eis* promoter of *Mycobacterium tuberculosis*. J Bacteriol 186, 5410-7 (2004).

128. Jackson, M., et al. The *Mycobacterium tuberculosis* purine biosynthetic pathway: isolation and characterization of the *purC* and *purL* genes. Microbiology 142 (Pt 9), 2439-47 (1996).

129. Timm, J., et al.,. Transcription and expression analysis, using *lacZ* and *phoA* gene fusions, of *Mycobacterium fortuitum* beta-lactamase genes cloned from a natural isolate and a high-level beta-lactamase producer. Mol Microbiol 12, 491-504 (1994).

130. Dhandayuthapani, S., Mudd, M. & Deretic, V. Interactions of OxyR with the promoter region of the *oxyR* and *ahpC* genes from *Mycobacterium leprae* and *Mycobacterium tuberculosis*. *J Bacteriol* 179, 2401-9 (1997).

131. Mick, V., et al. Transcriptional analysis of and resistance level conferred by the aminoglycoside acetyltransferase gene *aac(2')-Id* from *Mycobacterium smegmatis*. J Antimicrob Chemother 61, 39-45 (2008).

132. Wei, J., et al. Identification of a *Mycobacterium tuberculosis* gene that enhances mycobacterial survival in macrophages. J Bacteriol 182, 377-84 (2000).

133. Lella, R. K. & Sharma, C. Eis (enhanced intracellular survival) protein of Mycobacterium tuberculosis disturbs the cross regulation of T-cells. J Biol Chem 282, 18671-5 (2007).

134. Dahl, J. L., Wei, J., Moulder, J. W., Laal, S. & Friedman, R. L. Subcellular localization of the Intracellular survival-enhancing Eis protein of *Mycobacterium tuberculosis*. *Infect Immun* 69, 4295-302 (2001).

135. Hamano, Y., Hoshino, Y., Nakamori, S. & Takagi, H. Overexpression and characterization of an aminoglycoside 6'-N-acetyltransferase with broad specificity from an epsilon-poly-L-lysine producer, *Streptomyces albulus* IFO14147. *J Biochem* 136, 517-24 (2004).

136. Bardarov, S., et al,. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis, M. bovis* BCG and *M. smegmatis*. *Microbiology* 148, 3007-17 (2002).

137. Kim, J., Carver, E. A. & Stubbs, L. Amplification and sequencing of end fragments from bacterial artificial chromosome clones by single-primer polymerase chain reaction. Anal Biochem 253, 272-5 (1997).

Figure Legends

Figure 1: Structure of streptomycin. Adapted from Jana and Deb, 2006.

Figure 2: Structure of kanamycin and amikacin. Adapted from Recht and Puglisi, 2001.

Figure 1

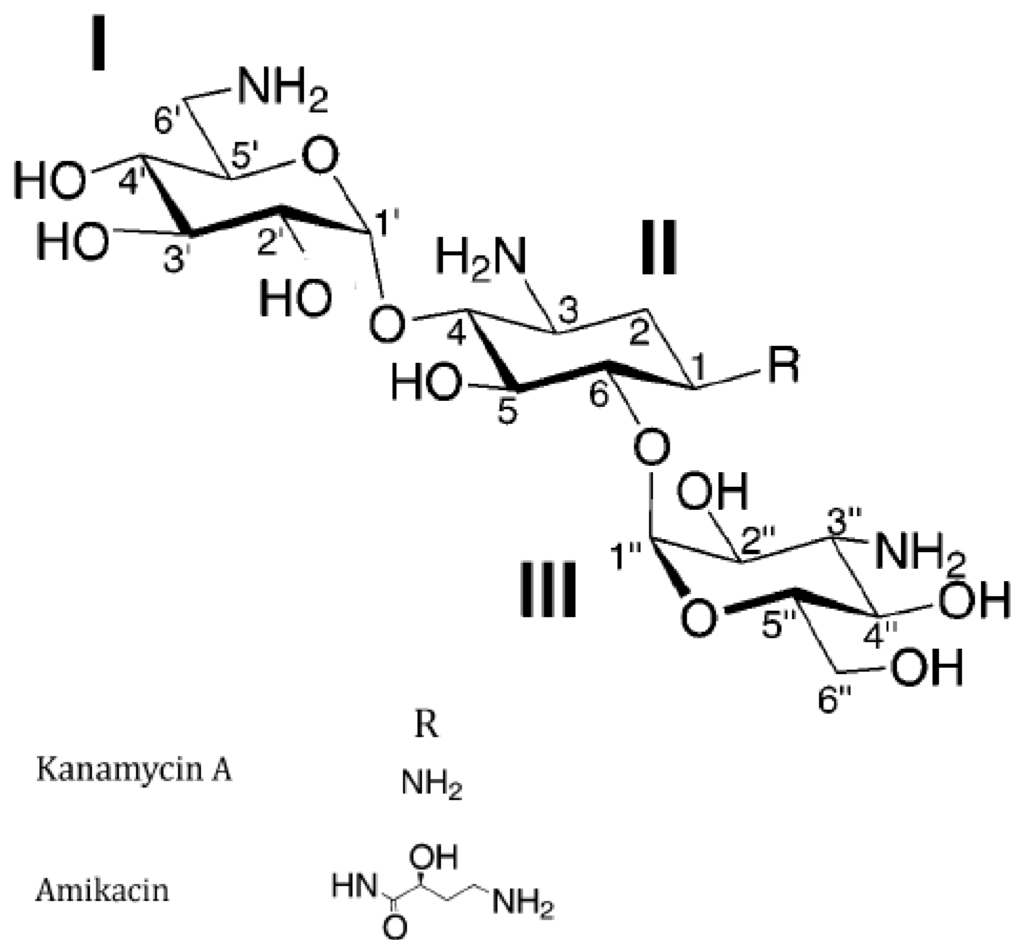
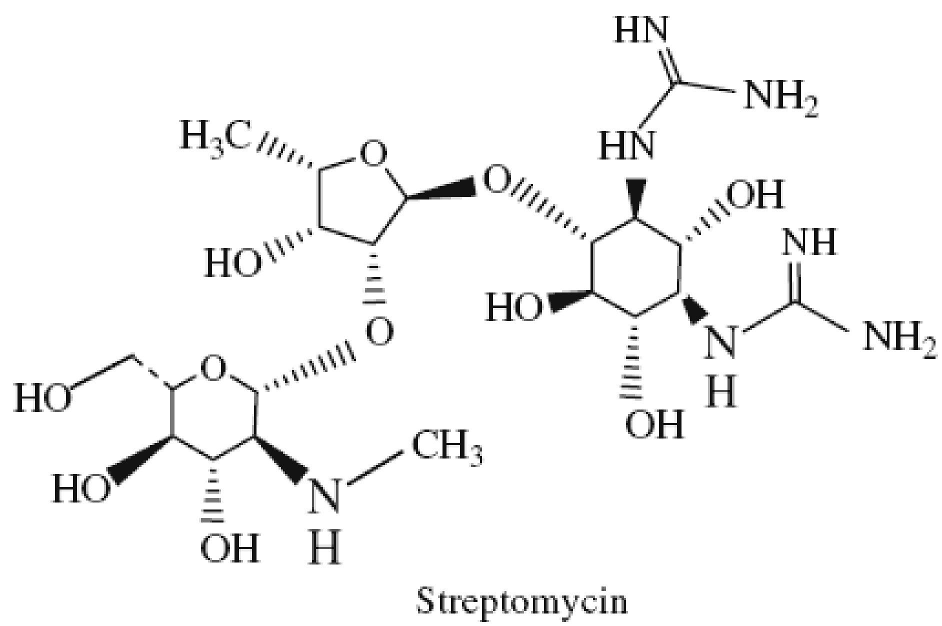


Figure 2



Streptomycin

Chapter 2

Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*

Published in Proceedings of the National Academy of Sciences
November 24, 2009 Vol 106, No. 47 p. 20004-9

This manuscript was written by Mary Analise Zaunbrecher, Thomas Shinnick and James Posey. Additional contributing authors include Beverly Metchock and David Sikes

Mary Analise Zaunbrecher was intellectually involved in the concept, experimental design, and implementation of this project from its conception as well as the analysis and interpretation of the results.

Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*

M. Analise Zaunbrecher[1,2], R. David Sikes, Jr[2], Beverly Metchock[2], Thomas M Shinnick[2] & James E Posey[2]

[1]Microbiology and Molecular Genetics Graduate Program, Department of Microbiology and Immunology, Emory University, Rollins Research Center, Suite 3001, 1510 Clifton Rd, Atlanta, GA, 30322 USA;

[2]Division of TB Elimination, National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention, Centers for Disease Control and Prevention, 1600 Clifton Rd, Mail Stop G35, Atlanta, GA, 30333 USA

ABSTRACT

The emergence of multidrug-resistant (MDR) tuberculosis (TB) highlights the urgent need to understand the mechanisms of resistance to the drugs used to treat this disease. The aminoglycosides kanamycin and amikacin are important bactericidal drugs used to treat MDR TB, and resistance to one or both of these drugs is a defining characteristic of extensively drug-resistant (XDR) TB. We identified mutations in the -10 and -35 promoter region of the *eis* gene which encodes a previously uncharacterized aminoglycoside acetyltransferase. These mutations led to a 20- to 180-fold increase in the amount of *eis* leaderless mRNA transcript with a corresponding increase in protein expression. Importantly, these promoter mutations conferred resistance to kanamycin (5μg/ml<MIC≤40μg/ml) but not to amikacin (MIC<4μg/mL). Additionally, 80% of clinical isolates examined in this study that exhibited low-level kanamycin resistance harbored *eis* promoter mutations. These results have important clinical implications in that clinical isolates determined to be resistant to kanamycin may not be cross resistant to amikacin as is often assumed. Molecular detection of *eis* mutations should distinguish strains resistant to kanamycin and those resistant to kanamycin and amikacin. This may help avoid excluding a potentially effective drug from a treatment regimen for drug-resistant TB.

INTRODUCTION

The World Health Organization estimates 9.2 million new cases of tuberculosis (TB) occur each year [116]. Despite intensive efforts to ensure proper drug dosages and patient compliance with drug regimens, multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* have emerged [117]. These strains cause extensive mortality in immuno-compromised individuals [118] and hinder the control and prevention of the disease. XDR and MDR TB infections cannot be adequately treated with the first-line anti-TB drugs and require expensive, prolonged treatment with second-line anti-TB drugs. The rapid determination of the resistance profile of an isolate can facilitate selection of an appropriate drug regimen and preclude development of additional drug resistances. Rapid detection of resistances is best achieved with molecular diagnostic approaches, particularly in developing countries where access to culture facilities is limited. Such strategies require a detailed understanding of the molecular basis for drug resistance. Although the mechanisms of resistance to first-line drugs such as isoniazid (INH) and rifampin (RIF) are well characterized, much less is known about such mechanisms for the second-line drugs [119].

An important second-line anti-TB drug is the aminoglycoside kanamycin (KAN), which binds to the 16S ribosomal RNA (rRNA) in the 30S ribosomal subunit and inhibits protein synthesis [80]. In other bacteria, characterized mechanisms of KAN resistance include altered efflux or influx of the drug, inactivation of the drug by enzymatic modification, and mutation or methylation of rRNA which disrupts binding of the drug to the ribosome [80]. In contrast, our understanding of the mechanism of KAN resistance in *M. tuberculosis* is limited. Mutations in the 16S rRNA gene, *rrs,* can cause high-level

resistance to KAN (MIC ≥ 80µg/mL) and some mutations can confer cross-resistance to other second-line drugs, including amikacin (AMK) and capreomycin (CAP) [107]. However, up to 80% of KAN-resistant (KAN^{R}) clinical isolates display low-level resistance to KAN (5 < MIC < 80µg/mL), do not contain *rrs* mutations, and do not exhibit cross-resistance [99, 109, 120, 121]. The molecular basis of this low-level resistance is unclear. We report here the discovery and characterization of novel mutations, common in clinical isolates of *M. tuberculosis*, which confer low-level resistance to KAN by causing overexpression of the enhanced intracellular survival protein, Eis.

RESULTS

C-14T mutation in *eis (Rv2416c)* confers kanamycin resistance and increases expression of *eis*

In a previous study [107], *M. tuberculosis* K204 (Table S1) was isolated as a spontaneous KAN^R mutant of *M. tuberculosis* H37Rv. Strain K204 is resistant to low levels of KAN (MIC of 25 μg/ml); susceptible to AMK (MIC ≤ 4 μg/mL), CAP (MIC ≤ 10 μg/mL), and viomycin (VIO; MIC ≤ 10 μg/mL); and harbors a wild type (WT) *rrs* gene. To identify the mutation that confers KAN resistance in this strain, a cosmid library constructed from K204 genomic DNA was introduced into the pansusceptible H37Rv strain, and five KAN^R transformants were isolated. Rapid Amplification of Transposon Ends (RATE) and sequence analysis of the transformants identified a common C to T transition located 14 bp upstream of the start codon of the *eis* gene (*Rv2416c*) encoding the enhanced intracellular survival protein (Fig 1A).

To evaluate the contribution of this gene to KAN resistance, we performed qRT-PCR assays using RNA isolated from H37Rv and K204 and compared the level of *eis* transcripts to that of the housekeeping gene *sigA* in each strain. The ratio of *eis* to *sigA* transcripts in K204 was approximately 180-fold higher than the ratio in H37Rv (Fig 2A). The increased amount of *eis* transcript in K204 was not due to differences in the level of expression of *sigA* between strains.

To determine if the C-14T mutation confers resistance to KAN and is responsible for the increased level of *eis* transcript in K204, we introduced the point mutation into the parent strain H37Rv by allelic exchange using the specialized-linkage transducing phage, phAlexC-14T (Table S2). We also used the specialized-linkage transduction system to

revert the mutant *eis* allele in K204 to the WT allele. Two representative strains containing either the C-14T *eis* allele (K315 and K317, Table S1) or WT *eis* allele (K214 and K215, Table S1) were chosen for further analysis. The introduction of the C-14T allele into H37Rv (K315 and K317) was sufficient to confer at least a 10-fold increase in resistance to KAN (MIC of 20-25 μg/mL, Table 1) and approximately 180-fold increase in *eis* transcript levels (Fig 2A). Conversely, the reversion of the C-14T mutant *eis* allele to the WT allele (K214 and K215) caused a decrease in the MIC of KAN (2 μg/mL, Table 1) and the amount of *eis* transcript to levels similar to that observed for H37Rv (Fig 2A). The increase in *eis* transcripts correlated with an increase in Eis protein, as determined by immunoblots on cell lysates generated from each strain. Eis was detected in cell lysates generated from strains harboring the C-14T allele (K204, K315 and K317) but could not be detected in cell lysates generated from strains harboring the WT allele (H37Rv, K214 and K215) (Fig. 1B). Thus, the C-14T allele is responsible for the increased *eis* transcripts, KAN MICs, and protein levels in the strains harboring this mutation.

Because single point mutations in another gene, *rrs*, can confer resistance to both AMK and KAN [107], we measured the MICs of the parental strains and allelic exchange derivatives to determine if the *eis* C-14T point mutation affected the resistance level of AMK. Strains harboring the C-14T *eis* allele exhibited a 6-fold increase in their MIC (from 0.5 μg/mL to 3 μg/mL, Table 1). Although the MIC for AMK increased, these strains are defined as susceptible to AMK in current testing and treatment guidelines [122, 123].

Transcription from the *eis* promoter produces a leaderless mRNA

Using RACE (Rapid Amplification of cDNA Ends) and primer extension analysis, the transcription start site in H37Rv and K204 was mapped to the first G of the start codon of *eis* (Fig 1B), indicating that transcription from both the WT and mutant *eis* promoter produces a leaderless mRNA transcript. We confirmed the location of the transcription start site with RT-PCR assays using primers that annealed either directly upstream (annealing at -20 to -1) or downstream of the GTG start codon (annealing at +1 to +20) of *eis* in conjunction with a reverse primer. When cDNA generated either from H37Rv or K204 was used as template, a PCR product was only detected when the primer overlapping the GTG start codon was present in the reaction (Fig. 1C). Identification of the transcription start site allowed us to define the -10 and -35 regions of the *eis* promoter and map the C-14T mutation to the -10 promoter region (Fig 1A).

To identify any *cis*-acting elements present in the promoter region, we complemented an *eis* deletion strain, H37RvΔ*eis* (Table S1), with constructs containing the entire open reading frame of *eis* and various derivatives of the mutant *eis* promoter (Fig S1). Constructs containing between 520 bp to 35 bp of the upstream promoter region exhibited similar levels of *eis* transcripts regardless of the amount of upstream sequence (Fig 2B). A promoter construct containing 20 bp of upstream sequence demonstrated somewhat decreased *eis* transcript levels when compared to the longer upstream regions but produced over 100-fold higher transcript levels than the WT promoter in H37Rv. Because the 20 bp promoter construct lacks the -35 region of the *eis* promoter, the data indicates that the -10 region of the promoter is able to initiate transcription. Finally, only minimal transcript levels were detected with a construct harboring only 10 bp of upstream sequence which lacks both the -10 and -35 promoter

regions (Fig 2B). Based on sequence homology and these results, the *eis* promoter seems to employ an extended -10 region (Fig 1A and Fig 2B).

Eis is an aminoglycoside acetyltransferase that acetylates and inactivates KAN and AMK.

Eis shares sequence homology and secondary structural characteristics with the GNAT-super family of acetyltransferases which includes the aminoglycoside N-acetyltransferases (AACs) [17, 124]. To determine if recombinant Eis protein exhibited acetyltransferase activity, we tested KAN, AMK, and streptomycin (SM) individually as substrates using a colorometric assay that quantifies the conversion of acetyl-CoA to CoA-SH. Reactions that lacked either Eis protein, acetyl-CoA, or any of the three substrates exhibited no acetyltransferase activity. Acetyltransferase activity was detected with KAN (specific activity of 447 nmol/min/mg) and AMK (98.4 nmol/min/mg), but not with SM (Fig 3A). Eis acetylated KAN at a rate 3.3-fold higher than AMK (366 nmol/min and 111 nmol/min, respectively); despite exhibiting similar binding affinities for both drugs, with an estimated K_m for KAN (154 µM) and AMK (112 µM) (Fig 3B). Additionally, Eis exhibited a 4-fold higher maximum acetylation rate of KAN (0.47 µmol/min) versus AMK (0.12 µmol/min). Finally, the V_{max}/K_m ratio for KAN was 3 fold higher than AMK, also indicating that Eis acetylated KAN more efficiently than AMK. This difference in substrate utilization by Eis likely explains the observed lack of cross resistance to KAN and AMK for isolates harboring *eis* mutations.

To determine if Eis modifies KAN and AMK, we analyzed the acetyltransferase assay products using thin layer chromatography (TLC). An increase of the migration of the acetylated products on a TLC plate confirmed that KAN (100% of substrate by

densitometric analysis) and AMK (56% of substrate) were modified by Eis (Fig 3C and 3D). When 20 µl of acetyltransferase reactions were spotted onto a lawn of *M. smegmatis* cells, reactions that lacked the Eis protein or acetyl-CoA produced zones of clearing of about 5.0 – 5.8 cm (Fig S2). Conversely, when complete reactions with KAN or AMK were spotted, no zone of inhibition was detected (Fig S2, lane 4) indicating inactivation of both antibiotics. Similar zones of inhibition were detected for SM in the presence or absence of Eis indicating that SM is not inactivated by Eis (Fig S2). If 40 µl reactions were spotted onto *M. smegmatis*, a zone of clearing was detected for AMK (3.0 cm), but not for KAN, consistent with the observation that AMK is acetylated less efficiently than KAN.

A colorimetric assay was used to quantify acetyltransferase activity in cell lysates of *eis* mutants. Activity was detected in cell lysates from strains harboring the C-14T allele, but not from strains harboring the WT *eis* allele (Fig 2E). The lysates exhibited about three times more activity when KAN was used as the substrate as compared with AMK. We also employed the *in vitro* acetyltransferase assay to analyze activity detected from cell lysates harboring the *eis* promoter truncation constructs and found the activity generally correlated with the amount of *eis* transcript produced in each strain (Fig 2F).

Clinical isolate analysis

We sequenced the *eis* and *rrs* alleles of KAN^R clinical isolates that did not exhibit any cross-resistance to AMK or CAP. Of the 42 isolates analyzed, 33 (79%) harbored a mutation within the promoter region of *eis* (Table 2). We observed the mutation identified in K204 (C-14T) and discovered two new mutations (G-37T and G-10A) that mapped within the -35 or -10 region of the *eis* promoter (Fig 1A and Table 2). Nine

(21%) of the KAN^R clinical isolates did not contain a mutation within *eis* or *rrs*. We also analyzed 16 KAN^S clinical isolates and did not find a mutation in either locus in 12 (75%) of the isolates (Table 2). However, four KAN^S isolates did contain a mutation (A-13G or C-12T) within the promoter region of *eis*.

Analysis of the level of *eis* expression in representative clinical isolates with each promoter mutation revealed a general correlation between *eis* transcript levels, KAN MICs, and acetyltransferase activity (Fig 2D,H, Table 1). Clinical isolates with the *eis* mutations C-14T, G-37T, and G-10A exhibited detectable acetyltransferase activity against KAN that correlated with what is considered clinically significant levels of KAN resistance (MIC $\geq$ 5 μg/mL)[122]. Several clinical isolates (KAN1 and KAN14) also exhibited detectable activity against AMK. However, these strains have AMK MIC $\leq$ 4 μg/mL, and therefore, are considered to be susceptible to AMK (Table 1) [122]. The clinical isolates harboring a C-12T mutation (KAN20 and KAN33) exhibited only slightly elevated levels of *eis* transcripts and a minimal amount of acetyltransferase activity (Fig 2D,H). Clinical isolates containing WT *eis* (KAN31 and KAN32) or the A-13G mutation (KAN39) demonstrated *eis* transcript levels similar to the pansusceptible H37Rv strain, had minimal or no detectable acetyltransferase activity, and were susceptible to KAN and AMK based on CLSI guidelines [122] (Fig 2D,H, Table 1).

There was some variation in observed MICs between strains with identical mutations. It is possible that clinical isolates harbor additional mutations at undefined genomic loci that may contribute to the KAN^R phenotype. To assess the contributions of individual *eis* mutations to KAN resistance in a clean genetic background, we complemented the knockout strain, H37RvΔ*eis,* with each mutation individually.

Generally, the transcript level, aminoglycoside resistance, and acetyltransferase activity were higher in complemented H37RvΔ*eis* strains than clinical isolates with the corresponding mutation (Fig 2C,G). However, the same hierarchy of mutations remained. Complementation of H37RvΔ*eis* with the C-14T promoter allele resulted in the highest *eis* transcript level, acetyltransferase activity, and MICs to KAN and AMK (Fig 2C,G, Table 1). Compared to the C-14T allele, the G-37T, G-10A, and C-12T promoter alleles demonstrate progressively lower *eis* transcript levels, acetyltransferase activity, and aminoglycoside resistance (Fig 2C,G, Table 1). Complementation of H37RvΔ*eis* with either the WT or A-13G promoter allele produced *eis* transcript levels, acetyltransferase activity, and KAN and AMK MICs similar to levels observed for the parental strain H37Rv (Fig 2C,G, Table 1).

DISCUSSION

Here we describe a previously unidentified mechanism of KAN^R in *M. tuberculosis* due to point mutations in the promoter region of the enhanced intracellular survival gene, *eis*. The KAN resistance conferred by *eis* promoter mutations is due to the significant increase in *eis* transcript levels and corresponding increase in the levels of an enzyme that acetylates and inactivates KAN. Importantly, although Eis was found to have some activity against AMK, *eis* mutant strains are susceptible to AMK (MIC ≤ 4 μg/mL) according to current testing guidelines [122] and treatment guidelines [123] suggesting the possibility that amikacin could be used to treat strains displaying the resistance pattern of *eis* mutants.

The lack of cross-resistance to AMK is explained by the finding that Eis utilizes KAN as substrate 3-fold more efficiently than AMK. The difference in substrate utilization is likely due to differences in the structures of KAN and AMK. AMK contains an L-hydroxyaminobuteroyl amide (L-HABA) group substitution in the N1 position of the deoxystreptamine ring [80] which may sterically hinder the acetylation by Eis.

Because the mutations in K204 and KAN^R clinical isolates cluster at the -10 region or -35 region of the *eis* promoter, it is likely that the increase in transcription is due to improving the strength of the existing promoter. Recently, it was shown that *sigA* binds to the promoter region and regulates expression of the *eis* gene [125]. Indeed, the -10 regions of the *eis* mutant promoters (C-14T, TACAGT; G-10A, CACAAT; C-12T, CATAGT) are more homologous with the SigA consensus sequence (TA(G/T)(A/G)AT) [126] than the WT promoter (CACAGT). Conversely, the A-13G promoter (CGCACT) shares less homology with the SigA consensus sequence and does not increase *eis*

transcript levels. Along similar lines, the -35 region of the WT *eis* (GTGCAC) promoter is similar to the consensus sequence proposed for group A mycobacterial promoters (TTGACn) [126] and the observed G-37T mutation (TTGCAC) improves the match with the SigA -35 consensus sequence. Although it seems likely that these mutations increase *sigA* binding to the *eis* promoter, studies are currently underway to clarify the mechanism of increased transcription at the mutant *eis* promoters and to identify any regulators that may be acting to facilitate the increase.

Our results demonstrate that the transcription start site of the *eis* promoter in K204 and H37Rv maps to the GTG start codon and produces a leaderless mRNA transcript (Fig 1). This differs from the published transcription start site for *eis* reported to be 33 bp upstream of the GTG start codon [127]. One possible reason for the discrepancy is that the transcription start site of *eis* was not mapped in *M. tuberculosis,* but rather from a multicopy plasmid in *M. smegmatis* and the avirulent derivative of H37Rv, *M. tuberculosis* H37Ra*,* which both differ significantly from H37Rv. A growing number of mycobacterial genes have been shown to produce leaderless mRNAs [128-130], including the aminoglycoside acetyltransferase gene *aac(2'-Id),* in *M. smegmatis* [131]. Interestingly, sequence analysis of the *eis* promoter indicated the presence of an extended -10 region motif (TGN) just upstream of the -10 region (Fig 1A). Our observation that the 20 bp of sequence upstream of the GTG start codon are sufficient to initiate transcription suggests that this motif does indeed act as an extended -10 region. The extended -10 motif is a characteristic shared with other leaderless mRNA producing mycobacterial promoters [128, 130, 131] and may imply a conserved regulatory mechanism for these poorly understood transcripts.

Analyses of the *eis* mutant strains revealed consistent correlations of *eis* mutations with *eis* transcription level, aminoglycoside MICs, and acetyltransferase activity. Our data suggest that *M. tuberculosis* strains harboring a C-14T, G-37T, or G-10A *eis* mutation are likely to be resistant to KAN, but susceptible to AMK in currently used laboratory tests. The higher levels of *eis* transcript observed in the H37RvΔ*eis* complemented strains when compared to clinical isolates with the same mutation is probably due to read-through transcription that can occur when genes are expressed at a non-native locus. This is supported by the fact that H37RvΔ*eis* complemented with the WT *eis* allele produces almost 3-fold greater *eis* transcript than the parental H37Rv strain (Fig 2C). Read-through transcription may also account for the increased level of *eis* expression in H37RvΔ*eis* complemented with the C-12T *eis* allele. Although the C-12T complement had an increased KAN MIC, no clinical isolates harboring the C-12T mutation were resistant to KAN (Table 1). This suggests that strains harboring a C-12T mutation may have an MIC close to the critical concentration used to test for KAN susceptibility. Our data also suggest that another mechanism of resistance to KAN is due to mutation(s) in as yet unidentified gene(s) since approximately 20% of our clinical isolates exhibited KAN resistance that could not be explained by *rrs* or *eis* mutations. For example, the clinical isolate KAN26, which harbors a G-10A mutation, expresses low, but detectable levels of acetyltransferase activity and has an MIC to KAN of 80 μg/mL that cannot be explained by the *eis* mutation alone.

The function of Eis in *M. tuberculosis* is unclear. The high K_m value of Eis with either KAN (154 μM) or AMK (112 μM) argues that neither antibiotic is a natural substrate of the protein. Eis was originally reported for its role in intracellular survival of

mycobacteria in macrophages [132]. Recently, activation of the *eis* gene in a W-Beijing strain of *M. tuberculosis* due to increased *sigA* expression levels has been linked to an enhanced survival phenotype in monocytes [125]. Additionally, purified recombinant Eis protein was shown to be sufficient in stimulating the expression of anti-inflammatory cytokines in macrophages [127] and disrupting the cross regulation of T-cells [133]. It is unclear how the acetylation function of Eis modulates both cytokine production and intracellular survival. Because Eis is secreted into host cell cytoplasm [134], one may speculate that Eis could be acetylating host factors during infection that could affect these different phenotypes.

Our findings have significant clinical and diagnostic implications. For example, the current CLSI guidelines [122] recommend using KAN as the class representative for both KAN and AMK resistance. That is, strains found to be susceptible (or resistant) to KAN would be assumed to be susceptible (or resistant) to AMK. While this appears to be mostly true for resistance due to mutations in the *rrs* gene, our data indicate that KAN^R strains due to *eis* mutations are AMK susceptible. Therefore, cross-resistance between KAN and AMK should not be assumed, and to avoid excluding a potentially effective drug from a regimen, susceptibility testing for KAN and AMK should be conducted individually.

In several research studies, low-level KAN^R strains differ from high-level KAN^R strains by both lacking *rrs* mutations and cross-resistance to other ribosome binding drugs, including AMK [99, 107, 109, 120, 121]. The prevalence of mutations in the *eis* promoter in 79% of the low-level KAN^R clinical isolates studied and absence from the KAN^S isolates studied suggests that these mutations account for much of the observed low-level

KAN resistance. Specifically, our data indicate that at the molecular level, KAN^R, AMK^S isolates are likely to harbor an *eis* mutation whereas KAN^R, AMK^R strains are likely to harbor an *rrs* mutation. Over all, our findings suggest that these mutations could serve as a molecular marker to identify KAN-resistant strains and distinguish low-level resistant isolates from high-level, cross-resistant isolates, leading to more appropriate treatment regimens for those infected with drug resistant strains.

MATERIALS & METHODS

Bacterial strains, media, and DNA manipulations. The strains and plasmids used in this study are listed in Table S1 and Table S2 and were cultivated as previously described [107]. Details of culture conditions, cloning and MIC procedures can be found in the Supplementary Text.

Expression and purification of Eis. The *eis* gene was amplified by PCR using primers AZ109 and AZ120 (Table S3), and the amplicon cloned into pET19b (Novagen) to create an N-terminal histidine tagged fusion protein. The His-Eis fusion was purified from the lysate of an IPTG-induced culture using a His-Bind NiNTA column (Novagen). The eluate containing the His-Eis protein was dialyzed against 50mM Tris HCl, pH 8.0 and concentrated using a spin column (Millipore). Purity was assessed by SDS-PAGE and the protein was quantified using the MicroBCA Protein Kit (Peirce).

Immunoblot analysis. Immunoblots were performed as described [124]. Briefly, 10µg of whole cell lysate were separated on 12% Bis-Tris SDS PAGE gels (Invitrogen) and transferred to PVDF membranes. Membranes were incubated with primary antibody specific to Eis (1:2500) or GroEL2 (1:50) for 1 hr in 1mM Phosphate Buffered Saline with Tween20 (PBS-Tw20) containing 0.25% skim milk, washed with PBS-Tw20 and then probed for 1 hr with anti-IgG secondary antibody (1:10,000) conjugated to horseradish peroxidase (HRP). The reactive bands were visualized using ECL detection reagents (Amersham). αEis antibodies were a gift from Richard Friedman of Arizona State University. αGroEL2 antibodies were obtained from Colorado State University.

Cell lysate collection and acetyltransferase assays. *M. tuberculosis* strains were grown to mid-log phase, washed, harvested, resuspended in 50mM Tris HCl pH 8.0, transferred

to tubes containing glass beads (Biospec Products, Inc), and lysed using a MiniBead Beater (BioCold Scientific). Acetyltransferase assays were carried out as described [135] using either purified His-Eis protein or cell lysate. To quantify acetylation, the degree of colorimetric change associated with either cell lysate or purified protein was subtracted from that of a control mixture that lacked antibiotic substrate. The reaction mixture (1mL) contained 0.1 mM acetyl-CoA (Sigma), 1 mM KAN or AMK, 1 mM DTNB (5,5'-dithio-bis(2-nitrobenzoate)) (Sigma), 50 mM Tris-HCl pH 8.0, and either 10 μg purified His-Eis protein or 50 μg cell lysate. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol TNB (2-nitro-5-benzoate) (14,151 $M^{-1}cm^{-1}$) per min at room temperature at A_{412}. Kinetic analysis: reaction conditions were the same as above except final KAN and AMK concentrations varied from 50 nM to 2 μM. Triplicate sets of reactions at each concentration were initiated with the addition of antibiotic substrate and Lineweaver-Burk plots were used to estimate the kinetic constants for each antibiotic.

Thin Layer Chromatography and Disk-Diffusion Assays

Acetyltransferase reactions for TLC analysis and disk-diffusion assays were performed in 20μL reaction volumes; 5μL portions of each reaction were spotted onto 0.25 mm silica gel TLC plates (Whatman) and developed with methanol:ammonium hydroxide (5:2). Aminoglycosides and acetylated products were detected by spraying the TLC plates with orcinol and charring at 110°C for 10 minutes. For disk-diffusion assays, the entire 20 μL acetyltransferase reaction was applied to a Whatman filter disk (Millipore) and placed on a lawn of *M. smegmatis* cells on Mueller-Hinton agar and incubated for 3 days at 37°C.

Quantitative RT-PCR. *M. tuberculosis* strains were grown to mid-log phase (OD_{600} 0.4-0.6) in Middlebrook 7H9 medium; bacteria were harvested and lysed using a FastPrep 120 (Bio 101 Savant); and RNA purified using an RNeasy kit (Qiagen). 1µg of DNAse-treated RNA from each *M. tuberculosis* strain was used to generate cDNA using the Promega Reverse Transcriptase System. Quantification of transcripts from 2 µL cDNA was performed by real-time PCR using Probes Master Mix (Roche) in a LightCycler480 detection system. Reactions to detect the *sigA* transcript used primers AZ139 and AZ140 and probe UPL#133 (Roche). Primers AZ141 and AZ142 and UPL#62 probe (Roche) were used to detect the *eis* transcript. The relative amounts of each PCR product was calculated from standard curves obtained from PCR with the same primers and probes and serially diluted K204 cDNA.

Primer Extension, RACE (Rapid Amplification of cDNA Ends) and RT-PCR. 50 µg of RNA from *M. tuberculosis* H37Rv or K204 bacteria were incubated with 100 pmole of 5'FAM labeled primer AZ143 at 70°C for 10 min, followed by 10 min on ice, 20 min at 58°C, and allowed to cool to room temperature for 15 min. cDNA was synthesized using the Promega Reverse Transcription System. The primer-extension products were purified using a Zymo DNA clean up kit, spiked with 0.5µL ROX markers (MapMarker1000, BioVentures), and analyzed on an ABI 3130xl sequencer using the Fragment Analysis module and PeakScanner software. RACE was performed using a 5'/3' RACE Kit, 2nd Generation (Roche). For K204 and H37Rv, 100 ng or 2 µg of RNA was used for cDNA synthesis respectively using primer AZ85 (12.5 µM) and 80 U transcriptor reverse transcriptase (Roche). cDNA was purified using a High Pure PCR Product Purification Kit (Roche). A homopolymeric A-tail was added to the 3'end of the

purified cDNA by incubation of the cDNA with recombinant terminal transferase (80 U) and dATP (200 nM) for 20 min at 37°C. 5 µL of poly-adenylated cDNA was amplified using Promega PCR Master Mix and 0.5 µM of primers AZ87 and an oligo-dT anchor primer (Roche). Amplified cDNA products were purified using a ZymoClean Gel DNA Recovery Kit (Zymo), cloned into pCR2.1-TOPO (Invitrogen), and sequenced with AZ143. 2-step RT-PCR reactions: cDNA was synthesized using the Promega Reverse Transcription System except random hexamers were replaced with primer AZ87. Either 200 ng of K204 RNA or 3 µg of H37Rv RNA was used as template. 2 µL of cDNA was added to PCR reactions containing the primers (AZ179 and AZ87) or (AZ180 and AZ87) using Promega PCR Master Mix. PCR conditions: initial denaturation at 97°C for 5 min, 30 cycles of 97°C for 20 sec, 60°C for 20 sec, and 72°C for 45 sec; followed by a final elongation step at 72°C for 2 minutes. PCR products were analyzed on agarose gels.

ACKNOWLEDGEMENTS

The authors thank Richard Friedman for generously providing the anti-Eis antibody. Special thanks to Daniel Kalman, Patrick M. Reeves, & Angela Starks for critical reading of the manuscript and helpful suggestions.

References

1. World Health Organization. in

Antituberculosis Drug Resistance in the World Report No. 4 (Geneva, Switzerland, 2008).

2. CDC. Emergence of *Mycobacterium tuberculosis* with extensive resistance to second line drugs-worldwide, 2000-2004. *MMWR* 55, 301-5 (2006).

3. Gandhi, N. R., et al,. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* 368, 1575-80 (2006).

4. Johnson, R., et al. Drug resistance in *Mycobacterium tuberculosis*. Curr Issues Mol Biol 8, 97-111 (2006).

5. Magnet, S. & Blanchard, J. S. Molecular insights into aminoglycoside action and resistance. Chem Rev 105, 477-98 (2005).

6. Maus, C. E., Plikaytis, B. B. & Shinnick, T. M. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 49, 3192-7 (2005).

7. Alangaden, G. J., et al, . Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 42, 1295-7 (1998).

8. Kruuner, A., Jureen, P., Levina, K., Ghebremichael, S. & Hoffner, S. Discordant resistance to kanamycin and amikacin in drug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 47, 2971-3 (2003).

9. Suzuki, Y., et al. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. J Clin Microbiol 36, 1220-5 (1998).

10. Taniguchi, H., et al. Molecular analysis of kanamycin and viomycin resistance in *Mycobacterium smegmatis* by use of the conjugation system. J Bacteriol 179, 4795-801 (1997).

11. NCCLS. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes; approved standard M24-A. (2003).

12. Tuberculosis Coalition for Technical Assistance. in The Hague: Tuberculosis Coalition for Technical Assistance, 2006. .

13. Samuel, L. P., et al. Expression, production and release of the Eis protein by *Mycobacterium tuberculosis* during infection of macrophages and its effect on cytokine secretion. Microbiology 153, 529-40 (2007).

14. Vetting, M. W., et al. Structure and functions of the GNAT superfamily of acetyltransferases. Arch Biochem Biophys 433, 212-26 (2005).

15. Wu, S., et al. Activation of the eis gene in a W-Beijing strain of Mycobacterium tuberculosis correlates with increased SigA levels and enhanced intracellular growth. Microbiology 155, 1272-81 (2009).

16. Gomez M. and Smith I. in Molecular genetics of Mycobacteria (ed. Hatfull GF, J. W., Jr.) 111-29. (American Society for Microbiology Press, Washington, DC, 2000).

17. Roberts, E. A., Clark, A., McBeth, S. & Friedman, R. L. Molecular characterization of the *eis* promoter of *Mycobacterium tuberculosis*. J Bacteriol 186, 5410-7 (2004).

18. Jackson, M., et al. The *Mycobacterium tuberculosis* purine biosynthetic pathway: isolation and characterization of the *purC* and *purL* genes. Microbiology 142 (Pt 9), 2439-47 (1996).

19. Timm, J., et al.,. Transcription and expression analysis, using *lacZ* and *phoA* gene fusions, of *Mycobacterium fortuitum* beta-lactamase genes cloned from a natural isolate and a high-level beta-lactamase producer. Mol Microbiol 12, 491-504 (1994).

20. Dhandayuthapani, S., Mudd, M. & Deretic, V. Interactions of OxyR with the promoter region of the *oxyR* and *ahpC* genes from *Mycobacterium leprae* and *Mycobacterium tuberculosis*. *J Bacteriol* 179, 2401-9 (1997).

21. Mick, V., et al. Transcriptional analysis of and resistance level conferred by the aminoglycoside acetyltransferase gene *aac(2')-Id* from *Mycobacterium smegmatis*. J Antimicrob Chemother 61, 39-45 (2008).

22. Wei, J., et al. Identification of a *Mycobacterium tuberculosis* gene that enhances mycobacterial survival in macrophages. J Bacteriol 182, 377-84 (2000).

23. Lella, R. K. & Sharma, C. Eis (enhanced intracellular survival) protein of Mycobacterium tuberculosis disturbs the cross regulation of T-cells. J Biol Chem 282, 18671-5 (2007).

24. Dahl, J. L., Wei, J., Moulder, J. W., Laal, S. & Friedman, R. L. Subcellular localization of the Intracellular survival-enhancing Eis protein of *Mycobacterium tuberculosis*. *Infect Immun* 69, 4295-302 (2001).

25. Hamano, Y., Hoshino, Y., Nakamori, S. & Takagi, H. Overexpression and characterization of an aminoglycoside 6'-N-acetyltransferase with broad specificity from an epsilon-poly-L-lysine producer, *Streptomyces albulus* IFO14147. *J Biochem* 136, 517-24 (2004).

Figure Legends

Figure 1: Characterization of *eis* promoter and expression. (A) *eis* promoter sequence and predicted promoter elements in *M. tuberculosis*. Mutations identified in clinical isolates are denoted by arrows. The mutation identified in the K204 *eis* promoter region is noted by the asterisk. The *eis* transcription start site is denoted by a bent arrow. The -10 and -35 regions are underlined, and the start codon is boxed. The extended -10 region (TGn motif) is located directly upstream of the -10 region (B) Immunoblot analysis of cell lysates generated from strains harboring either WT (H37Rv, K214, K215) or the C-14T *eis* allele (K204, K315, K317). The lysates were probed with either anti-Eis or anti-Groel2 serum. (C) RNA from H37Rv (Lanes 1-4) and K204 (Lanes 5-8) was used in one-step RT-PCR reactions. Odd numbered lanes contain primer pair AZ198 (anneals at -20 to -1 of the *eis* start codon) and AZ87; even lanes contain primers AZ199 (anneals at +1 to +20) and AZ87. H37Rv genomic DNA (lanes 9 and 10) was used as a positive control for each primer set. The + and – signs indicate whether reverse transcriptase was added to the reaction and serve as negative controls for each primer set and RNA sample.

Figure 2: Analysis of *eis* expression and acetyltransferase activity. The ratio of *eis/sigA* transcripts (A-D) was determined by qRT-PCR and normalized to H37Rv (A) H37Rv, K204, and allelic exchange derivatives (B) H37RvΔ*eis* complemented with *eis* promoter truncation constructs. Base pairs (bp) indicates the length of sequence upstream from the *eis* start codon present on the complementing plasmid. (C) H37RvΔ*eis* complemented with *eis* promoter alleles (D) Clinical isolates. Acetyltransferase activity (E-H) was measured from crude cell lysates and is expressed in nmol/mg/min. (E) H37Rv, K204,

and allelic exchange derivatives (F) H37RvΔ*eis* complemented with *eis* promoter truncation constructs. (G) H37RvΔ*eis* complemented with different *eis* promoter alleles (H) Clinical isolates. In all panels, error bars represent three independent experiments.

Figure 3: Eis acetylates kanamycin and amikacin. (A) Acetylation rates KAN, AMK, and SM. (B) Michaelis-Menton plots for KAN and AMK. Acetyltransferase reactions were performed using either (C) KAN or (D) AMK as substrate, and the products were developed on TLC plates. Lane 1: unmodified antibiotic, Lane 2: no acetyl-CoA control, Lane 3: complete acetyltransferase reaction, Lane 4: no Eis control. R_f values under these solvent conditions: unmodified KAN, 0.31; acetyl-KAN, 0.44; unmodified AMK, 0.09; acetyl-AMK, 0.46.

Table 1: Eis mutations and aminoglycoside resistance levels

		MIC(μg/mL)	
Strain	***eis***	**KAN**	**AMK**
H37Rv	WT	2	0.5
K214	WT	2	0.5
K215	WT	2	0.5
K204	C-14T	25	3
K315	C-14T	25	3
K317	C-14T	20	3
Plasmid complementing H37RvΔ*eis*			
pAZ38	WT	2	0.5
pAZ29	C-14T	20	2
pAZ40	G-37T	20	1
pAZ39	G-10A	10	1
pAZ41	C-12T	10	1
pAZ42	A-13G	5	0.5
Clinical Isolate			
KAN1	C-14T	40	<4
KAN14	C-14T	40	<4
KAN3	G-37T	20	<4
KAN4	G-37T	20	<4
KAN9	G-37T	10	<4
KAN6	G-10A	10	<4
KAN7	G-10A	10	<4
KAN26	G-10A	80	<4
KAN20	C-12T	10	<4
KAN33	C-12T	<5	<4
KAN39	A-13G	<5	<4
KAN31	WT	<5	<4
KAN32	WT	<5	<4

Table 2: Distribution of *eis* mutations in clinical isolates

eis allele	Number of isolates	KAN MIC[a] (µg/mL)
C-14T	10 (24%)	25-40
G-10A	14 (33%)	10-20
G-37T	8 (19%)	20
C-12T	1 (2%)	10
Wild type	9 (21%)	10-40
Total	42 (100%)	
C-12T	3 (19%)	<5
A-13G	1 (6%)	<5
Wild type	12 (75%)	<5
Total	16 (100%)	

[a]Isolates with MICs > 5 µg/mL are considered resistant to KAN.

Figure 1.

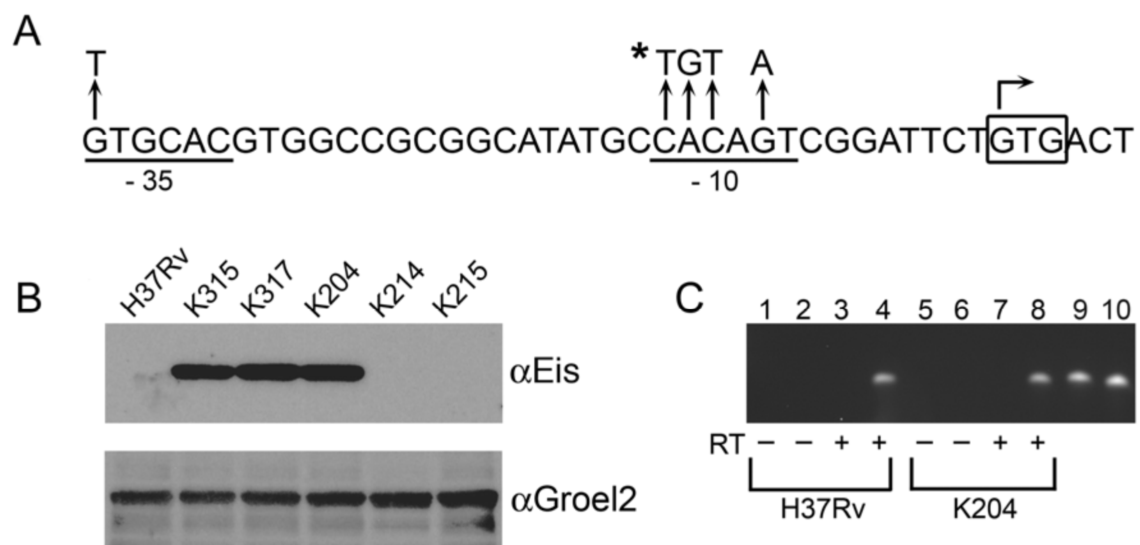


Figure 2.

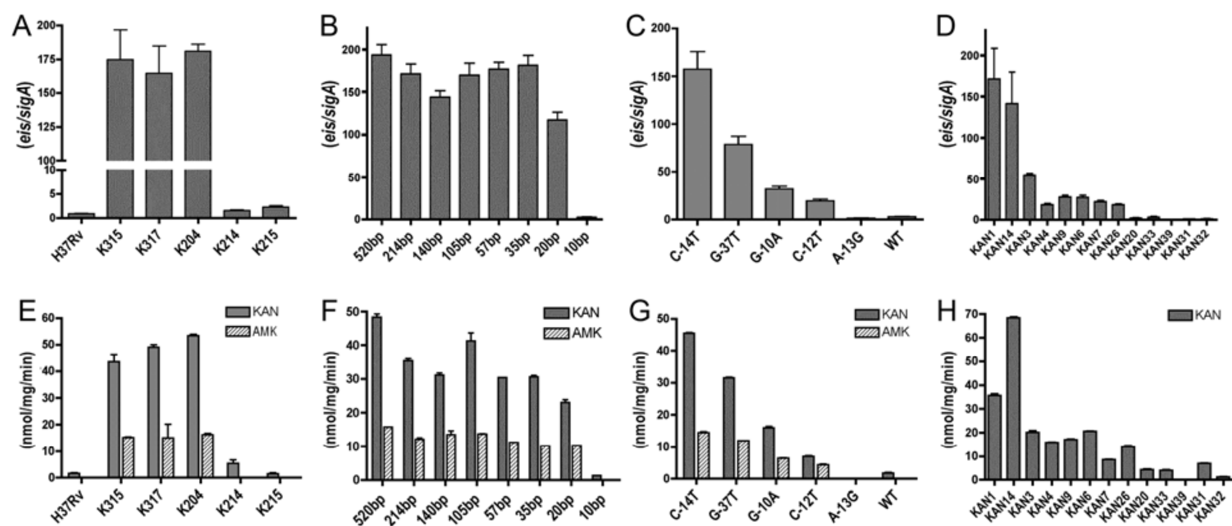
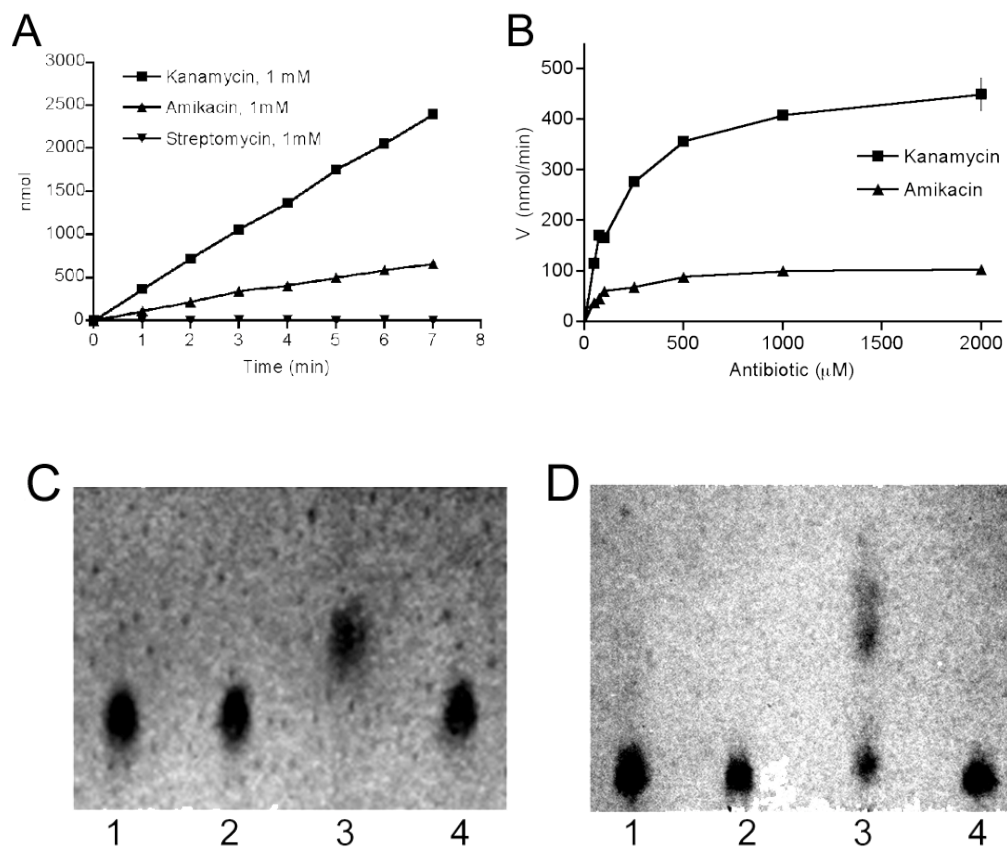


Figure 3.



Supporting Information

Supplementary methods

Bacterial strains, media, and DNA manipulations. The media were supplemented with hygromycin (HYG) (75 μg/ml), kanamycin (KAN) (5 μg/ml or 25 μg/ml), or sucrose (5%) when needed for cultivation of *M. tuberculosis* strains. For *E. coli* strains, Luria media was supplemented with HYG (200 μg/ml), KAN (50 μg/ml), or carbenicillum (100 μg/ml) when needed. Antibiotics were purchased from Sigma. Standard protocols or the manufacturer's instructions were used for all DNA manipulations (New England Biolabs (NEB)/ Invitrogen/ Finnzymes). All oligonucleotide primers (Table S3) were synthesized at the Biotechnology Core facility, National Center for Preparedness, Detection, and Control of Infectious Diseases, CDC. Clinical isolates were obtained from the culture collection at the Mycobacteriology Laboratory Branch, CDC. Human subject information linked to the clinical isolates used in this study is protected by the protocol approved by the CDC institutional review board.

Construction and screening of the K204 cosmid library. A cosmid library of Sau3AI partial digest products of *M. tuberculosis* K204 in pYUB178-hyg (a derivative of pYUB178 [136] in which the KAN^R cassette has been replaced with a HYG^R cassette) was constructed and electroporated into *M. tuberculosis* H37Rv bacteria using standard methods. Transformants were selected on 7H10 agar plates containing either HYG alone or HYG and KAN. Genomic DNA was extracted from individual KAN^R transformants and analyzed by RATE (Rapid Amplification of Transposon Ends) [137] using primers specific to the pYUB178-hyg plasmid.

Construction of recombinant cosmids pAZ10 (pYUB854::*eis*::*Rv2415c*) and pAZ19 (pYUB854::*eisC-14T*::*Rv2415c*) for allelic exchange

The *Rv2415c* gene was amplified from the H37Rv genome using primers AZ151 and AZ152 and cloned into the XhoI/SpeI sites of pYUB854 after digestion with XhoI and SpeI, forming pAZ09 (pYUB854::*Rv2415c*). The *eis* gene and 1 kb of upstream sequence were amplified from the H37Rv genome or the K204 genome using primers AZ149 and AZ150. The fragments were digested with StuI and XbaI and cloned into pAZ09 (pYUB854::*Rv2415c*) to generate pAZ10 (pYUB854::*eis*::*Rv2415c*) or pAZ19 (pYUB854::*eisC-14T::Rv2415c*). These cosmids harbor the *eis* and downstream gene, *Rv2415c* separated by a HYG^R cassette.

Construction of specialized transducing phages phAlexWT and phAlexC-14T

The specialized transducing phages phAlexWT or phAlexC-14T were generated from pAZ10 (pYUB854::*eis*::*Rv2415c*) or pAZ19 (pYUB854::*eisC-14T*::*Rv2415c*) and phAE159 as previously described [136]. *M. tuberculosis* H37Rv bacteria or K204 bacteria were infected with phAlexC-14T or phAlexWT at an MOI of 10:1 and selected on 7H10 containing HYG. Genomic DNA from individual transductants was sequenced and analyzed by MAMA-PCR using primers optimized for either the *eis*C-14T (AZ199 and AZ87) or wild type (AZ198 and AZ87) allele using Promega Master Mix Taq.

Construction of mutants and complemented strains. An unmarked deletion of *eis* (bases -32 to 1204 were removed) was constructed using pYUB854, phAE159, and pYUB870 as previously described [136]. The final unmarked deletion of *eis* was confirmed by PCR. To complement the knockout strain, the *eis* open reading frame and 520bp upstream of *eis* were amplified by PCR from genomic DNA of representative clinical

isolates using primers AZ107 and AZ108, cloned into pHIN-STOP, and electroporated into H37RvΔ*eis*. Complemented strains were confirmed by PCR using internal *eis* primers AZ80 and AZ87 and sequencing. Promoter truncation derivatives of the *eis* promoter were amplified by PCR from K204 genomic DNA using primers AZ167 to AZ174 and AZ108, cloned into pHIN-STOP, and electroporated into H37RvΔ*eis*. Integration of the truncated promoter constructs was confirmed by PCR using primers HYG9F and AZ87 and sequencing.

MIC determination. The susceptibilities to KAN and AMK were determined according to guidelines and definitions stated by the CLSI[122], using 7H10 agar containing KAN (Sigma) at concentrations of 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 50, and 80 µg/ml or AMK (Sigma) at concentrations of 0.25, 0.5, 1, 2, 3, and 4 µg/ml. The MIC was defined as the lowest concentration of drug resulting in complete inhibition of growth or in growth of ≤1% of the initial inoculum after 4 weeks of incubation at 37°C.

Supplemental Figure Legends

Figure S1 *sigA* transcript levels in H37Rv and K204. The ratio of *sigA/rrs*(16S rRNA) transcripts was determined by qRT-PCR and normalized to H37Rv. Error bars represent the standard deviation of three independent experiments.

Figure S2 Immunoblot analysis of cell filtrate proteins generated from strains harboring either WT (H37Rv, K214) or the C-14T *eis* allele (K204, K315) shows Eis is present in the culture supernatants of C-14T promoter mutants. The lysates were probed with either anti-Eis or anti-Ag85 complex serum (Colorado State University).

Figure S3 Promoter truncation constructs in the pHIN-STOP plasmid backbone (Table S2). A plasmid containing each construct was used to complement the H37RvΔ*eis* strain. Eis expression and activity for each complement was analyzed by qRT-PCR and acetyltransferase activity analysis (data in Figure 2). Length indicates the amount of sequence upstream from the *eis* start codon present on the complementing plasmid. Black and grey boxes represent the -35 and -10 promoter regions respectively.

Figure S4 Disk-diffusion assays for KAN, AMK, and SM on *M. smegmatis.* 20 µl volume of acetyltransferase assays were spotted onto Whatman filter disks on a lawn of *M. smegmatis* cells and incubated at 37°C for 3 days; Column 1: unmodified antibiotic, Column 2: no acetyl-CoA control, Column 3: no Eis control, Column 4: 20 µl of complete acetyltransferase reaction. 5: 40 µl volume of complete acetyltransferase reaction (not performed with SM). Similar results were obtained with *E. coli*.

Table S1: Strains used in this study

Strain	Genotype/Relevant Characteristics	Reference or Source
Escherichia coli		
HB101	F-Δ(gpt-proA)62, leuB6, glnV44, ara-14, galK2, lacY1, Δ(mcrC-mrr), rpsL20(Str^r), xyl-5, mtl-1, recA13, thi-1	NEB
BL21 Gold	F– ompT hsdS(rB– mB–) dcm+ Tet^r gal (DE3) endA Hte	Novagen
M smegmatis		
LR222	Easily transformable strain, used for phage preparation	Miller et al. (1994)
M. tuberculosis		
H37Rv	Pansusceptible laboratory strain	
K204	Spontaneous Kan^r mutant of H37Rv	Maus et al. (2005)
K315, K317	H37Rv with *eis*C-14T allele, Hyg^r introduced by allelic exchange	This study
K214, K215	K204 with *eis* wildtype allele, Hyg^r introduced by allelic exchange	This study
RvΔ*eis*	Unmarked *eis* deletion strain of H37Rv	This study
RvΔ*eis*+WT	RvΔ*eis* with an integrated copy of pAZ38 *eisWT::attB*	This study
RvΔ*eis*+C-14T	RvΔ*eis* with an integrated copy of pAZ29 *eisC-14T::attB*	This study
RvΔ*eis*+G-10A	RvΔ*eis* with an integrated copy of pAZ39 *eisG-10A::attB*	This study
RvΔ*eis*+G-37T	RvΔ*eis* with an integrated copy of pAZ40 *eisG-37T:attB*	This study
RvΔ*eis*+C-12T	RvΔ*eis* with an integrated copy of pAZ41 *eisC-12T::attB*	This study
RvΔ*eis*+A-13G	RvΔ*eis* with an integrated copy of pAZ42 *eisA-13G::attB*	This study
RvΔ*eis*+pHIN-STOP	RvΔ*eis* with an integrated copy of pHIN-STOP *::attB*	This study
RvΔ*eis* 520	RvΔ*eis* with an integrated copy of pAZ29 *eisC-14T::attB*	This study
RvΔ*eis* 214	RvΔ*eis* with an integrated copy of pAZ30 *eisC-14T::attB*	This study
RvΔ*eis* 140	RvΔ*eis* with an integrated copy of pAZ31*eisC-14T::attB*	This study
RvΔ*eis* 105	RvΔ*eis* with an integrated copy of pAZ32*eisC-14T::attB*	This study
RvΔ*eis* 57	RvΔ*eis* with an integrated copy of pAZ33*eisC-14T::attB*	This study
RvΔ*eis* 35	RvΔ*eis* with an integrated copy of pAZ34*eisC-14T::attB*	This study
RvΔ*eis* 20	RvΔ*eis* with an integrated copy of pAZ35*eisC-14T::attB*	This study
RvΔ*eis* 10	RvΔ*eis* with an integrated copy of pAZ36*eisC-14T::attB*	This study

Abbreviations: Hyg^r, hygromycin resistant. Amp^r, ampicillin resistant. Kan^r, kanamycin resistant

Table S2: Plasmids and Phage used in this study.

Plasmid	Relevant Characteristics	Reference or Source
pET19b	pET vector containing N-terminal His tag, Amp^r	Novagen
pET19b-eis	*eis* open reading frame cloned into pET19b, Amp^r	This study
pYUB178-hyg	Cosmid vector, Hyg^r	This study
pYUB854	Cosmid vector, with res sites flanking the Hyg^r gene	Bardarov et al. (2002)
pEIS-KO	pYUB854 + 900 bp flanking sequence of *eis*	This study
pYUB870	Helper plasmid, *tnpR, sacB*, Kan^r	Bardarov et al. (2002)
pAZ09	*Rv2415c* cloned into pYUB854 for allelic exchange, Hyg^r	This study
pAZ10	pAZ09 + *eis*WT and 1.2 kb upstream sequence	This study
pAZ19	pAZ09 + *eis*C-14T 1.2 kb upstream sequence	This study
pHIN-STOP	Integrating mycobacterial vector, Hyg^r, Transcription stop sequence, used for complementation experiments	This study
pAZ29	pHIN-STOP + *eis*C-14T and 520 bp upstream sequence	This study
pAZ30	pHIN-STOP + *eis*C-14T and 214 upstream sequence	This study
pAZ31	pHIN-STOP + *eis*C-14T and 140 upstream sequence	This study
pAZ32	pHIN-STOP + *eis*C-14T and 105 upstream sequence	This study
pAZ33	pHIN-STOP + *eis*C-14T and 57 bp upstream sequence	This study
pAZ34	pHIN-STOP + *eis*C-14T and 35 bp upstream sequence	This study
pAZ35	pHIN-STOP + *eis*C-14T and 20 bp upstream sequence	This study
pAZ36	pHIN-STOP + *eis*C-14T and 10 bp upstream sequence	This study
pAZ38	pHIN-STOP + *eis*WT and 520 bp upstream sequence	This study
pAZ39	pHIN-STOP + *eis*G-10A and 520 bp upstream sequence	This study
pAZ40	pHIN-STOP + *eis*G-37T and 520 bp upstream sequence	This study
pAZ41	pHIN-STOP + *eis*C-12T and 520 bp upstream sequence	This study
pAZ42	pHIN-STOP + *eis*A-13G and 520 bp upstream sequence	This study
Phage		
phAE159	Mycobacterial phage	Bardarov et al. (2002)
phΔ*eis*	pEIS-KO cloned into phAE159	This study
phALEX-WT	phAE159 + pAZ10, hyg^r	This study
phALEX-C-14T	phAE159 + pAZ19, hyg^r	This study

Abbreviations: Hyg^r, hygromycin resistant. Amp^r, ampicillin resistant. Kan^r, kanamycin resistant

Table S3: Primer Sequences

Primer Name	**Sequence 5'-3'**
178F	CTTTCCTGACAGTGACAGAC
178R	TACTGTTTATGTAAGCAGACAG
HYG9F	ACGACTTCGAGGTGTTCGAGGAG
AZ56	ATCCCGCGTTCTTCCCTC
AZ77	CCCCTTGTATTACTGTTTATGTAAG
AZ78	TTTGAGACACAACGTCGCTTTG
AZ80	GCGTAACGTCACGGCGAAATTC
AZ85	GCCTTCAGAACTCGAACG
AZ87	GTCAGCTCATGCAAGGTG
AZ109	CGCCATATGACTGTGACCCTGTGTAG
AZ120	ACAGGATCCACCGCGATCGAGAGTGTAC
AZ139	AGCTGGCCAAAGAGATGG A
AZ140	GGGCGTATTGCTGGATTTC
AZ141	GCGATCTACGAGCGATGG
AZ142	CTCGTCCCAGAGCACCTG
AZ143	CCGCAGATCCATGTACAG
AZ144	ACGTCACGGCGAAATTCG
AZ145	TCATGCAAGGTGGTAGCG
AZ146	GGGTTCTCTCGGATTGACG
AZ147	GGCTGCTGGCACGTAGTT
AZ149	CGAAGGCCTTGCAGAGCGAAGCGATGAGG
AZ150	CGATCTAGACACGGCCTTCAGAACTCGAAC
AZ151	CGACTCGAGGTGCTAGGCCGGCGCTAG
AZ152	CGAACTAGTTGTCTTAGGGCCTGTCGTG
AZ167	GCATCTAGATGTAGCGCGGTTGGACAATC
AZ168	ACGTCTAGAACCGGTACTTGCTCTGCACG
AZ169	GCATCTAGAACGTCACGGCGAAATTCG
AZ170	CGATCTAGAGCGTCACGCTGGCGGGGCTA
AZ171	GCATCTAGAACTGTCGTCGTAATATTCAC
AZ172	GCATCTAGAGCACGTGGCCGCGGCATATG
AZ173	AGCTCTAGAATATGCCACAGTCGGATT
AZ174	GCATCTAGAGTCGGATTCTGTGACTGT
AZ179	GTGACTGTGACCCTGTGTAG
AZ180	CATATGCCACAGTCGGATTC
AZ198	ACGTGGCCGCGGCATATGCC
AZ199	ACGTGGCCGCGGCATATGGT

Figure S1.

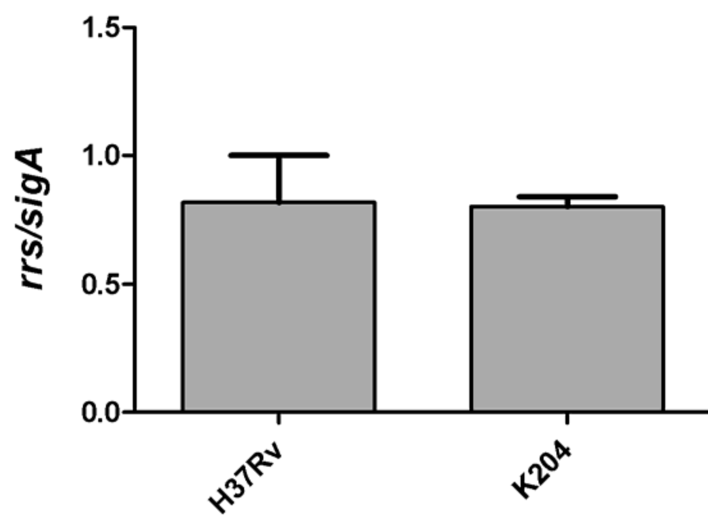


Figure S2.

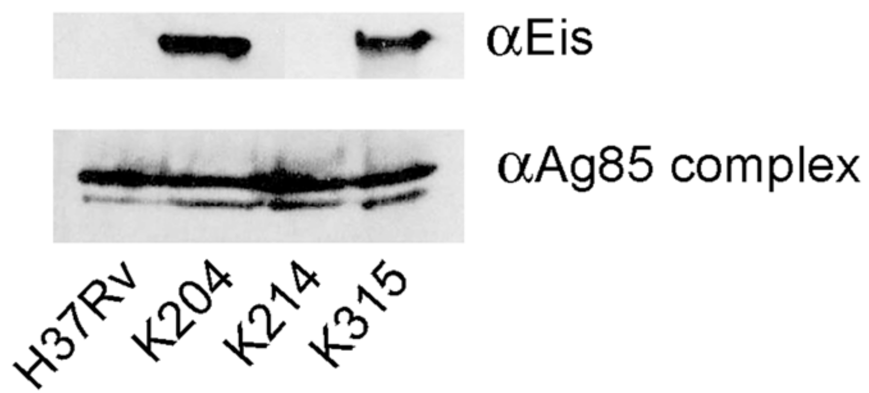


Figure S3.

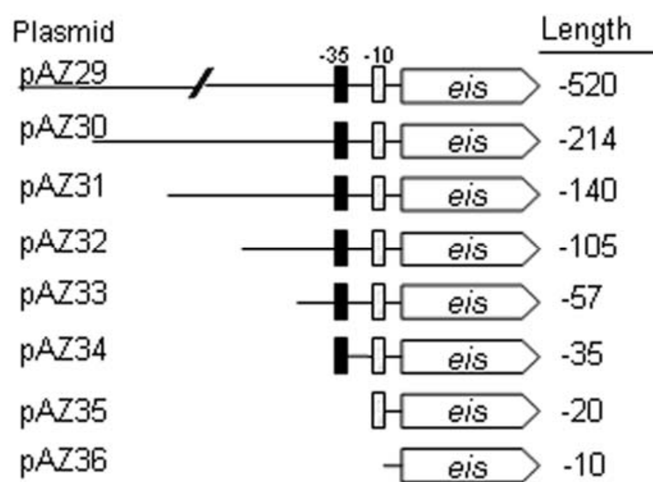
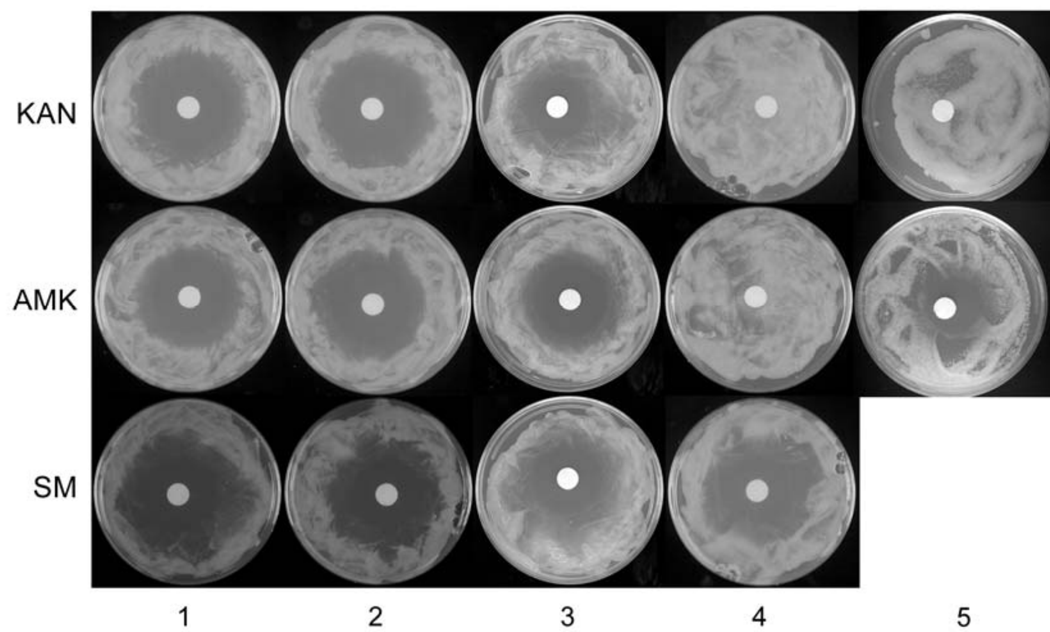


Figure S4.



Chapter 3

Mutations in the *whiB7* promoter lead to cross resistance of kanamycin and streptomycin in *Mycobacterium tuberculosis*

Submitted to Proceedings of the National Academy of Sciences
September 2010

This manuscript was written by Mary Analise Zaunbrecher, Thomas Shinnick and James Posey. Additional contributing authors include Patricia Campbell, Razvan Sultana, and Megan Murray

Mary Analise Zaunbrecher was intellectually involved in the concept, experimental design, and implementation of this project from its conception as well as the analysis and interpretation of the results.

Mutations in the *whiB7* promoter Lead to Cross Resistance of Kanamycin and Streptomycin in *Mycobacterium tuberculosis*

M. Analise Zaunbrecher[a,b], Patricia H Campbell[b], Razvan Sultana[c], Megan Murray[d], Thomas M Shinnick[b] & James E Posey[b*]

[a]Microbiology and Molecular Genetics Graduate Program, Department of Microbiology and Immunology, Emory University, Rollins Research Center, Suite 3001, 1510 Clifton Rd, Atlanta, GA, 30322 USA,

[b]Division of TB Elimination, National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention, Centers for Disease Control and Prevention, 1600 Clifton Rd, Mail Stop G35, Atlanta, GA, 30333 USA,

[c]Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA, 02115 USA

[d]Department of Epidemiology, Harvard School of Public Health, Boston, MA, USA.

ABSTRACT

Since the discovery of streptomycin's bactericidal activity against *Mycobacterium tuberculosis*, aminoglycosides have been utilized to treat tuberculosis (TB). Today, the aminoglycosides kanamycin (KAN) and amikacin (AMK) are used to treat multi-drug resistant (MDR) TB, and resistance to any of the second-line injectable antibiotics, including KAN, AMK, or capreomycin, is a defining characteristic of extensively drug-resistant (XDR) TB. Resistance to KAN and streptomycin (STR) is thought to be due to separate mechanisms. However, we identified nine mutations in the promoter region of the transcriptional activator, *whiB7*, that confer low-level resistance to both aminoglycosides. The mutations lead to a 23- to 145-fold increase in *whiB7* transcripts and subsequent increased expression of both *eis (Rv2416c)* and *tap (Rv1258c).* Increased expression of *eis* confers KAN resistance in these mutants while increased expression of the *tap* efflux pump is a previously uncharacterized mechanism of low-level STR resistance. Additionally, *whiB7* promoter mutants evolve to high-level STR resistance at a much higher frequency than wild type. These results may have important clinical implications given that a *whiB7* mutation was identified in a clinical isolate of *M. tuberculosis*. As drug resistance continues to develop and spread, understanding the mechanisms and molecular basis of antibiotic resistance is valuable for the development of rapid molecular tests to diagnose drug-resistant TB strains and ultimately for designing regimens to treat drug-resistant cases of TB.

INTRODUCTION

Drug resistant strains of *Mycobacterium tuberculosis* present a great challenge to global tuberculosis (TB) control efforts[1]. Multidrug-resistant (MDR) TB (resistance to rifampin (RIF) and isoniazid (INH) and extensively drug-resistant (XDR) TB strains of *M. tuberculosis* (MDR with additional resistance to a flouroquinolone and at least one of the injectable antibiotics; kanamycin (KAN), amikacin (AMK), and capreomycin(CAP)) are difficult to diagnose and complicated and expensive to treat[1]. The World Health Organization estimates that approximately half a million cases of MDR TB occurred during 2008[1]. As the number of drug-resistant cases increase each year, the need to supplement the current time consuming, media-based resistance detection methods with more rapid molecular drug susceptibility tests becomes more pressing. However, a better understanding of the molecular mechanisms that confer drug resistance is required for the development of these methods. The combination of new technologies, such as next-generation sequencing, with improved molecular genetic systems in *M. tuberculosis* creates a robust method to identify and confirm the role of particular mutations with drug resistance and the ability to differentiate between mutations that confer drug resistance and those that are not involved. These strategies are especially important for understanding resistance mechanisms to the less commonly studied second-line antibiotics that are needed to treat patients with MDR TB.

The aminoglycoside KAN is central in treating MDR TB, and resistance to KAN is one of the defining characteristics of XDR TB strains[2, 3]. Yet our understanding of KAN resistance in *M. tuberculosis* remains incomplete. KAN inhibits protein synthesis by binding to the 16S ribosomal RNA (rRNA) in the 30S ribosomal subunit and

resistance is observed at two phenotypic levels: high and low[4, 5]. High-level resistance (MIC ≥ 80 μg/ml) occurs as a result of mutations in the 1400 bp region of the 16S rRNA (*rrs*) where KAN binds to the ribosome[6, 7]. These *rrs* mutations (such as the A1401G or G1484T alleles) are also associated with cross resistance to other ribosome-binding antibiotics including the structurally similar aminoglycoside, AMK, and the cyclic peptide CAP[8]. In contrast, low-level KAN resistant (5 μg/ml ≥ MIC > 80 μg/ml) strains generally exhibit resistance to KAN only[5, 7, 9, 10]. In a recent study, we found that up to 80% of strains displaying low-level KAN resistance harbored mutations in the promoter region of the aminoglycoside acetyltransferase, *eis (Rv2416c)*[5]. However, the mechanism of KAN resistance in the remaining isolates was unexplained. In this study, we used whole genome sequencing and molecular genetics to identify mutations in the promoter region of *whiB7 (Rv3197A)*, a transcriptional activator of *eis*[11], that confer low level resistance in *M. tuberculosis* by enhancing *eis* expression. Further analysis revealed that *whiB7* promoter mutants confer cross resistance to streptomycin (STR) due to overexpression of the *tap* (*Rv1258c)* efflux pump.

RESULTS

Spontaneous KAN resistant mutants harbor *whiB7* promoter mutations

In a previous study, we isolated 20 spontaneous kanamycin resistant (KAN^R) mutants derived from the pansusceptible *M. tuberculosis* strains H37Rv and CDC1551[8]. A low concentration of KAN (5 μg/ml) was used to provide selection and mutants with both high- and low-level KAN resistance phenotypes were recovered. Five of the 20 mutants (25%) harbored *rrs* mutations (A1401G or G1484T) and 2 of 20 (10%) had *eis*C-14T promoter mutations. The remaining 13 of 20 (65%) spontaneous mutants were resistant to KAN at a low level (MICs of 10-30 μg/ml) and susceptible to AMK (MIC ≤ 4 μg/ml), but did not harbor any mutations at either locus (Table 1). To identify possible mutations responsible for conferring KAN resistance, the spontaneous KAN^R mutant K301 was analyzed by whole genome sequencing. Sequence comparison between K301 and the pansusceptible parental strain, CDC1551, revealed a C deletion in the promoter region of the gene encoding the putative transcriptional activator, WhiB7 (Fig 1). Subsequently, nine distinct *whiB7* promoter mutations were identified in each of the 13 spontaneous mutants with unexplained low level KAN resistance (Fig 1 and Table 1). Using Rapid Amplification of cDNA Ends (RACE), we mapped the transcription start site of *whiB7* in the mutant, K301, to an A residue 90 bp upstream of the start codon (Fig 1). This nucleotide was chosen as the reference for numbering the location of the *whiB7* mutations (Table 1). The most commonly identified mutation occurred in a string of six cytosine residues 125 bp upstream of the transcriptional start site and included either a deletion (6 mutants) or an insertion (1 mutant) of a cytosine (Fig 1 and Table 1). Six

other mutants harbored either cytosine or guanine deletions or transversions scattered throughout the promoter region (Fig 1 and Table 1).

whiB7 promoter mutations cause enhanced _whiB7_ expression and low-level KAN resistance

Although the *whiB7* promoter mutations did not map to any known regulatory region in *whiB7*, we hypothesized that these mutations could affect *whiB7* expression. Using quantitative RT-PCR, we compared *whiB7* expression levels in the spontaneous mutants to the parental strains CDC1551 and H37Rv (Fig 2a). When these results were normalized to the housekeeping sigma factor *sigA* mRNA in each strain, the relative expression levels of *whiB7* varied between a 23–145 fold increase in the spontaneous mutants (Fig 2a).

To investigate whether *whiB7* promoter mutations are sufficient to confer *whiB7* overexpression and low-level KAN resistance in *M. tuberculosis*, several mutants were reverted back to a wild-type sequence through allelic exchange using the specialized transducing phage, phAZ70 (Table S2). Three spontaneous mutants were chosen that represented each type of mutation identified; K301 harboring the ΔC-125 deletion, K205 harboring the +C-125 insertion, and K209 harboring the A-26G transversion. The allelic exchange revertants displayed wild-type levels of *whiB7* mRNA expression when compared to CDC1551 and H37Rv, confirming that the presence of any of these point mutations leads to highly increased *whiB7* expression (Fig 2b). Additionally, the allelic exchange derivatives were susceptible to KAN, confirming the role of *whiB7* mutations in conferring low-level KAN resistance (Table 1 and Fig 3).

KAN resistance in _whiB7_ mutants is due to _eis_ overexpression

We recently showed that overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* (*Rv2416c*) confers low-level KAN resistance in *M. tuberculosis* through acetylation and inactivation of the drug[5]. Additionally, previously described microarray analysis indicated that *eis* is a member of the *whiB7* regulon and that increased expression of *whiB7* leads to enhanced levels of *eis* mRNA[11]. We measured *eis* mRNA levels in *whiB7* mutants by quantitative RT-PCR assays and found that expression was increased from 36–150 fold (Fig 2a). The amount of *eis* transcripts in the *whiB7* mutants is comparable to *eis* promoter mutants that are known to be resistant to KAN such as K204, which harbors an *eis* C-14T allele (Table 1 and Fig 2a). Additionally, all of the *whiB7* promoter mutant lysates exhibited acetyltransferase activity against both KAN and AMK while activity in the parental strains, CDC1551 and H37Rv, was either very low or undetectable (Fig 2c). The amount of activity detected when KAN was used as a substrate was three times higher than AMK, which is characteristic of Eis protein activity (Fig 2c)[5]. In the *whiB7* allelic exchange derivatives, *eis* mRNA expression and acetyltransferase activity reverted to wild-type expression levels indicating that *whiB7* point mutations are sufficient to increase expression of *eis* (Fig 2b and 2d). To confirm the acetyltransferase activity was due to Eis, we performed immunoblots on the cell lysates of each *whiB7* mutant and allelic exchange derivative with an anti-Eis antibody. As expected, Eis was only detected in lysates generated from strains harboring *whiB7* mutations, suggesting that the mechanism of KAN resistance in *whiB7* mutants is due to inactivation of the drug by Eis (Fig 2e and 2f).

We reasoned that if enhanced expression of *whiB7* could confer KAN resistance through Eis, then disrupting *whiB7* may have the opposite effect and increase KAN

susceptibility. When *whiB7* was deleted from the parental strain CDC1551 (CDC1551Δ*whiB7*), *eis* transcripts were reduced by 7.5-fold and the Eis protein was no longer detectable by immunoblot demonstrating that *whiB7* is necessary for basal level expression of *eis* (Fig 4a and 4b). However, the reduction in Eis did not cause increased susceptibility to KAN suggesting that neither *whiB7* nor the basal level Eis protein is a limiting factor in the intrinsic resistance of *M. tuberculosis* to KAN (Table 1). Intriguingly, disruption of *whiB7* in an *eis* promoter mutant such as K204 (which harbors a C-14T mutation) has no effect on *eis* expression, indicating that expression from the *eis* mutant promoter is independent of WhiB7 (Fig S1).

***whiB7* promoter mutants are resistant to streptomycin**

WhiB7 is thought to regulate expression of several genes involved in intrinsic antibiotic resistance of mycobacteria[11]. We reasoned that additional genes in the *whiB7* regulon may be overexpressed in the *whiB7* mutants and that this modulation in gene expression could contribute to resistance to additional antibiotics besides KAN. A survey of drug MICs was performed comparing wild type and *whiB7* mutant strains on several clinically used anti-TB drugs; streptomycin (STR), capreomycin (CAP), rifampin (RIF), isoniazid (INH), and ofloxacin (OFX). While there was no difference in MICs against CAP, RIF, INH, or OFX, the MIC to STR increased 16-fold to 8 μg/ml for strains harboring *whiB7* mutations (Table 1). Sequence analysis of the *rrs, rpsL,* and *gidB* loci in each mutant confirmed that no second site mutations were present that would account for the STR resistance[12, 13]. The Clinical Laboratory Standards Institute document currently recommends testing STR susceptibility at two levels (2 μg/ml and 10 μg/ml) to distinguish between high- and low-level STR resistant isolates[14]. Because the MIC is

below 10 μg/ml, *whiB7* mutants are considered low-level STR resistant[14]. The exception was K205 which harbors a +C-125 *whiB7* allele and a slightly higher STR MIC of 10 μg/ml (Table 1). The *whiB7* allelic exchange derivatives were susceptible to STR (MICs of 0.5 μg/ml, Table 1 and Fig 3) confirming that a single *whiB7* promoter point mutation confers cross resistance to both KAN and STR and also suggested that a component of the *whiB7* regulon is responsible for the STR resistance. However, Eis was ruled out as a contributing factor to resistance because STR is not a substrate of Eis[5], and *eis* promoter mutants such as K204 that overexpress the *eis* gene are susceptible to STR (Table 1).

Streptomycin resistance in *whiB7* mutants is due to overexpression of the *tap* efflux pump

Examination of the previously characterized *whiB7* regulon revealed the *tap* (*Rv1258c*) gene encoding an efflux pump as a strong candidate that could potentially contribute to STR resistance[11, 15]. Numerous studies have indicated a role for efflux pumps in antibiotic resistance in *M. tuberculosis* and the Tap homolog from the related mycobacteria *M. fortuitum* is involved in efflux of macrolides and aminoglycosides, including STR[15-17]. Therefore, we hypothesized that STR could also be a substrate of the *M. tuberculosis* Tap efflux pump. Initially, we confirmed that *tap* mRNA levels were highly elevated in *whiB7* mutants by 30–175 fold (Fig 2a). Also, *whiB7* promoter mutations confer increased *tap* expression given that reversion back to wild type sequence in the allelic exchange derivatives yields *tap* expression levels similar to those of parental strains CDC1551 and H37Rv (Fig 2b).

To determine whether the Tap efflux pump was responsible for conferring STR resistance, we constructed *tap* deletion strains in both a wild type and *whiB7* mutant

background. However, *tap* is the first gene in a two-gene operon that includes the cotranscribed gene, *Rv1257c*, which is annotated as a putative oxidoreductase with no known role in antibiotic resistance in *M. tuberculosis*[11]. To circumvent the possibility that disruption of *tap* would introduce a polar downstream deletion of the *Rv1257c* gene, we constructed deletions of each gene individually and then assessed the STR and KAN resistance phenotypes. Deletion of the *Rv1257c* gene in either CDC1551 (WT) or K301 (ΔC-125) had no effect on the STR or KAN MIC and disrupting the *tap* gene did not significantly affect KAN resistance (Table 1 and Fig 3). However, deleting *tap* from either background caused the mutants to become hyper-susceptible to STR, decreasing the MIC approximately 5-fold as compared to wild type strains (MIC of 0.1 μg/ml compared to 0.5 μg/ml, Table 1 and Fig 3). These data confirm that the STR resistance in *whiB7* mutants is due to enhanced expression of *tap*, which probably results in increased efflux of STR. Additionally, this data provides the first direct genetic evidence that an efflux pump in *M. tuberculosis* confers resistance to a clinically used anti-TB drug.

In agreement with a previous report[11], our results found that *whiB7* is required for the intrinsic STR resistance in *M. tuberculosis* (MIC of 0.5 μg/ml) since the CDC1551Δ*whiB7* deletion strain is 2-fold more susceptible to STR than wild type (Table 1). The loss of *whiB7* leads to a 15-fold reduction in basal level *tap* mRNA which likely contributes to the reduced MIC of STR by decreasing efflux of STR from the cell (Fig 4a). Overall, this data suggests that the cross resistance between KAN and STR in *whiB7* mutants is due to two distinct mechanisms: inactivation of KAN by the Eis protein and efflux of STR out of the bacterial cell by the Tap efflux pump.

***whiB7* promoter mutations lead to a high frequency of emergence of high-level STR resistant mutants**

The use of STR as a chemotherapeutic for TB patients has been hindered by the high frequency of emergence of STR resistant strains[18]. Until now, STR resistance in *M. tuberculosis* has been thought to be associated with the binding site of STR at the ribosome[19]. Point mutations in either *rrs* or *rpsL* have been shown to confer high-level STR resistance with MICs of 10 μg/ml or greater[20, 21]. Additionally, mutations throughout the open reading frame of the rRNA methyltransferase *gidB* confer low-level STR resistance (2 μg/ml ≤ MIC < 10 μg/ml) presumably by inhibiting efficient STR binding to the ribosome[12, 22]. Intriguingly, *gidB* mutants are also 2000 times more likely to acquire high-level STR resistance than a strain with a wild type *gidB* locus[12]. This observation led us to hypothesize that low-level STR resistant mutants with *whiB7* mutations may demonstrate a similar phenomenon. By plating CDC1551 (wild type) and K301 (ΔC-125 *whiB7* mutant) on plates containing a high concentration of STR (10 μg/ml), we compared the frequency of the emergence of high-level STR resistant isolates in the different backgrounds. Similar to the *gidB* study, high-level STR resistant mutants arose at a frequency 850-fold higher than wild type cells (Table 2). However, in contrast to the previous study that found numerous second site mutations at the *rpsL* locus[12], greater than 90% of the high-level STR resistant mutants derived from K301 harbored mutations in *gidB* and not *rpsL* nor *rrs*. The high mutation frequency of the *whiB7* mutant was specific to STR since the emergence of high-level KAN resistant mutants remained the same in either strain background indicating that the overall mutation rate of *whiB7* mutants is not affected (Table 2).

A whiB7* mutation was identified in a clinical isolate of *M. tuberculosis

We sequenced the *whiB7* promoter region in 16 clinical isolates with unexplained low- level KAN resistance but only identified one clinical isolate (CL-27) that harbored a *whiB7* mutation and demonstrated acetyltransferase activity (Table 1 and Fig S2). Notably, CL-27 was one of only three isolates (33%) that also demonstrated low-level STR resistance. A single cytosine insertion at position -125 was mapped to the *whiB7* locus in isolate CL-27 (Table 1). This isolate was cross resistant to both KAN (MIC 20 μg/ml) and STR (MIC 10 μg/ml) with a drug resistance profile that matched the spontaneous mutant K205, which also harbors a +C-125 *whiB7* mutation (Table 1). The remaining unexplained KAN resistant isolates contained either very low or undetectable acetyltransferase activity (Fig S2) insinuating that another mechanism of KAN resistance is acting in these clinical isolates that is distinct from *eis* or *rrs* mutations.

DISCUSSION

WhiB7 coordinates expression of a regulon of genes including target and drug modifying enzymes (*erm* and *eis*) and efflux pumps (*tap* and *Rv1473*) that contribute to intrinsic antibiotic resistance in *M. tuberculosis*[11]. In the absence of *whiB7,* isolates are more susceptible to several structurally distinct antibiotics including chloramphenicol, several macrolides, and STR[11]. In contrast, we show that increased expression of *whiB7* due to naturally occurring promoter mutations confers cross resistance between KAN and STR. The resistance to KAN and STR is conferred through two distinct mechanisms involving different components of the *whiB7* regulon.

We have recently shown that Eis is an aminoglycoside acetyltransferase which acetylates and inactivates KAN and that enhanced *eis* expression confers low-level KAN resistance in *M. tuberculosis*[5]. In *whiB7* promoter mutants, the induction of high levels of *eis* accounts for the low-level KAN resistance phenotype, although the mechanism of induction is uncertain. The transcriptional regulator WhiB3, is closely related to WhiB7 and regulates genes by interacting with the principal sigma factor SigA and directly binding to promoter regions[23]. We hypothesize that WhiB7 may act similarly and directly regulate *eis* expression by binding to the *eis* promoter region, leading to induction. Alternatively, it is possible that another factor regulated by WhiB7 could act at the *eis* promoter, thereby exerting an indirect affect on *eis* expression.

The Tap protein is homologous to members of the Major Facilitator Superfamily (MFS) of efflux pumps, which generally have relaxed substrate specificity and can accommodate a breadth of antimicrobials[15]. The Tap homolog from *M. fortuitum* extrudes tetracycline as well as various structurally distinct aminoglycoside compounds,

including STR, from the cell[15, 24]. The increased expression of *tap* in *whiB7* promoter mutants presumably leads to efficient efflux of STR from the *M. tuberculosis* cell by the Tap efflux pump, leading to STR resistance. Tap was proposed to be involved in the efflux of INH, RIF, and OFX [25-27]. However, we found no evidence of increased resistance to any of these compounds or CAP when *tap* was overexpressed, suggesting that none of these drugs are Tap substrates.

The observation that the majority of the *whiB7* mutants have a similar MIC to STR (8 μg/ml) regardless of having variable levels of *tap* transcripts suggests that some form of post-transcriptional or post-translational control may limit how much active Tap can be present in the cell at one time. It is possible that the membrane becomes saturated with the Tap protein at the amount capable of extruding enough STR to confer an MIC of 8 μg/ml. However, without an antibody against the Tap protein, we were unable to determine Tap protein levels in the *whiB7* mutants.

A recent study investigating STR resistant isolates from Brazil found 22% of clinical isolates examined had no mutations in *rpsL, rrs*, or *gidB*[13]. These isolates showed increased susceptibility to STR in the presence of the proton motive force inhibitors CCCP (carbonyl cyanide m-chlorophenylhydrazone) and verapamil, suggesting an efflux pump contributed to the resistance[13]. Our data suggest that the STR resistance in these clinical isolates could be mediated by the Tap efflux pump. Another report identified an MDR clinical isolate of *M. tuberculosis* with increased *tap* expression, which might be explained by *whiB7* overexpression[26]. It has also been documented that *tap* expression is induced in clinical isolates in the presence of subinhibitory concentrations of INH and RIF, although it is unclear if this upregulation is mediated by

WhiB7[25]. These data also suggest that mutations in the *tap* promoter that enhance *tap* expression could represent another uncharacterized mechanism of STR resistance. Based on these studies, Tap-mediated STR resistance likely contributes to clinically relevant STR resistance in clinical isolates.

An intriguing observation from this work is that low-level STR resistant *whiB*7 mutants evolved to high-level resistant strains at a frequency much higher than their wild-type counterparts. This phenomenon has been previously described in strains that are resistant to low-level STR and harbor *gidB* mutations, which also develop high-level STR resistance at a very high frequency (2000 times higher than a wild-type strain)[12]. In both instances, the emergence of high-level resistance is specific to STR and not other antibiotics, but the mechanism for this drug specificity is not clear. In the case of the *whiB7* promoter mutants, high-level STR resistance emerged at a frequency ($7.2x10^{-5}$) similar to the mutation frequency reported for single-step *gidB* mutations ($2.8x\ 10^{-6}$)[12]. Indeed, greater than 90% of the high-level STR resistant mutants harbored *gidB* mutations, suggesting that the frequency of emerging high-level STR resistant strains is largely a reflection of the spontaneous mutation rate at the *gidB* locus. Conversely, single-step point mutations that result in high-level STR resistance (such as mutations in *rrs* or *rpsL*) occur at a much lower frequency (10^{-8})[28] and this is consistent with the frequency of high-level STR resistant mutants emerging from the wild type CDC1551 strain ($8.7x10^{-7}$). The presence of both a *whiB7* and *gidB* mutation likely creates a synergistic effect on STR resistance through both enhanced efflux of the drug via the Tap efflux pump and decreased binding affinity of STR to the ribosome. This data shows that a mutation at either the *whiB7* or *gidB* locus leads to the acquisition of high-level STR

resistance at an elevated frequency, which may partly explain why STR resistance can evolve so quickly in the host.

A major question that remains is the mechanism by which several types of base pair alterations (insertions, deletions, or transversions) throughout the promoter region of *whiB7* confer a similar phenotype of greatly enhanced *whiB7* expression and subsequent upregulation of downstream genes in the *whiB7* regulon. One hypothesis is that alterations in the DNA sequence could affect binding of one or more regulatory proteins that interact at the *whiB7* intergenic region. However, given the variety of mutation types and the lack of clustering to a single location in the promoter (spacing between mutations is up to 200 bp apart); it seems unlikely that the mutations affect the binding of a single regulatory protein. At least seven proteins are predicted to interact at the promoter region of *whiB7* including the extracytoplasmic sigma factor SigK, the two component system response regulator, RegX3, and WhiB7 itself[11, 29]. Some regulatory proteins have been shown to bind to the *whiB7* promoter in a bacterial one-hybrid system, but the residues responsible for these interactions are not known[29]. An IdeR (Iron-dependant regulator) consensus binding site is predicted in the *whiB7*promoter, but no mutations map to this region[30]. Also, the WhiB family of proteins are predicted to respond to changes in the redox state of the cell through an iron-sulfur cluster in the structure, but the correlation between redox state and induction of antibiotic resistance genes remains to be elucidated[23].

A very complex and sensitive regulatory system controls *whiB7* expression and activity. Modulations in *whiB7* expression are observed following exposure to subinhibitory concentrations of several structurally and functionally diverse classes of

antibiotics, fatty acids, and lung surfactant, and *whiB7* is induced in response to heat shock, low iron, and upon entry into macrophages[11, 31-33]. However, antibiotics that target the 16S rRNA including KAN, STR, and tetracycline are potent inducers of *whiB7*, suggesting that *whiB7* responds to ribosomal stress signals in the cell[11, 31]. An intriguing aspect of our data is that the WhiB7-mediated response to ribosomal stress upregulates at least two genes that specifically alleviate aminoglycoside-induced ribosomal stress due to drug inactivation or extrusion. The mechanism by which WhiB7 senses ribosomal stress is still unclear, but is more likely receiving a general stress signal from the cell rather than directly sensing numerous structurally different antibiotics. These observations raise the question of whether exposure to stressful conditions such as antibiotic treatment can induce phenotypic antibiotic resistance on *M. tuberculosis*. If so, this may have implications on designing treatment for tuberculosis.

A caveat of the approach used in this study is that mutations identified through *in vitro* methods may not correlate with the selective pressures experienced during drug treatment of tuberculosis *in vivo* and may not reflect the requirements for bacterial growth *in vivo*. Investigation of low-level KAN^R clinical isolates from our limited strain collection only revealed three isolates with a phenotype of low-level cross resistance to KAN and STR, one of which (33%) harbored a *whiB7* mutation (CL-27, Table 1). Therefore, the abundance of *whiB7* mutations in circulating clinical strains of *M. tuberculosis* is not clear at this time and warrants further investigation. Alternatively, it is possible that *whiB7* mutations impart a fitness cost on *M. tuberculosis* that abrogates the growth advantage afforded by antibiotic resistance. This possibility remains to be examined experimentally. To our knowledge, this is the first account of single point

mutations conferring antibiotic resistance due to two distinct mechanisms. Additionally because the majority of KAN resistant clinical isolates analyzed in this study harbored no mutations known to confer KAN resistance, this suggests that another as-yet-unidentified mechanism of KAN resistance remains. Continuing to examine the molecular mechanisms responsible for conferring resistance and cross resistance to second-line drugs is important for the future development of accurate and rapid molecular tests to diagnose drug-resistant strains.

METHODS

Generating spontaneous KAN^R mutants

Spontaneous KAN^R mutants were generated from the pansusceptible strains, H37Rv and CDC1551 as described. Each strain was grown to a high cell density (~$1x10^8$ cfu/ml) and portions of the cell suspension were plated on 7H10 plates supplemented with 5 μg/ml of KAN. Serial 10-fold dilutions were plated on 7H10 agar containing no antibiotics to determine the number of viable cells in the suspension. Isolated colonies were inoculated into 7H9 broth containing 5 μg/ml of KAN. Genomic DNA was harvested from each isolate and analyzed by PCR and sequencing. Mutation frequency was determined by dividing the number of colonies on selective media by the number of viable cells after 28 days at 37ºC.

Immunoblot analysis. Immunoblots were performed as described[36]. Briefly, 10 μg of whole cell lysate were separated on 12% Bis-Tris SDS PAGE gels (Invitrogen) and transferred to PVDF membranes by electrophoresis at 30V for 1 hour. Membranes were incubated with primary antibody specific to Eis (1:2500) or GroES (1:20) for 1 hour in 1mM Phosphate Buffered Saline with Tween20 (PBS-Tw20) containing 0.25% skim milk, washed three times with PBS-Tw20 and then probed for 1 hour with anti-IgG secondary antibody (1:10,000) conjugated to horseradish peroxidase (HRP). The reactive bands were visualized using ECL detection reagents (Amersham). αGroES antibodies were obtained from Colorado State University TB Vaccine Testing and Research Materials Contract.

Cell lysate collection and acetyltransferase assays. *M. tuberculosis* strains were grown to mid-log phase, washed, harvested, resuspended in 50 mM Tris HCl pH 8.0, transferred

to tubes containing glass beads (Biospec Products, Inc), and lysed using a MiniBead Beater (BioCold Scientific). Acetyltransferase assays were carried out as described[5]. Reaction mixtures (1mL) contained 0.1 mM acetyl-CoA (Sigma), 1 mM KAN or AMK, 1 mM DTNB (5,5'-dithio-bis(2-nitrobenzoate)) (Sigma), 50 mM Tris-HCl pH 8.0, and 50 μg cell lysate. To quantify acetylation, the degree of colorimetric change was subtracted from that of a control mixture that lacked antibiotic substrate. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol TNB (2-nitro-5-benzoate) (14,151 $M^{-1}cm^{-1}$) per min at room temperature at A_{412} .

Quantitative RT-PCR. *M. tuberculosis* strains were grown to mid-log phase (OD_{600} 0.4-0.8) in Middlebrook 7H9 medium, bacteria were harvested and lysed using a FastPrep 120 (Bio 101 Savant) and RNA purified using an RNeasy kit (Qiagen). 1 μg of DNAse-treated RNA from each *M. tuberculosis* strain was used to generate cDNA using the Promega Reverse Transcriptase System. Quantification of transcripts from 2 μL cDNA was performed by real-time PCR using Probes Master Mix (Roche) in a LightCycler480 detection system. Reactions to detect specific transcripts used 0.4 μM of each primer and 0.1 μM of the probe (Roche) using the probe and primer pairs listed in Table S3. The relative amounts of each PCR product was calculated from standard curves obtained from PCR with the same primers and probes and serially diluted K204 or K301 cDNA.

REFERENCES

1. World Health Organization. Global tuberculosis control - epidemiology, strategy, financing. World Report 2009. WHO/HTM/TB/2009.411 (2009).
2. Notice to readers: Revised definition of extensively drug-resistant tuberculosis. MMWR 55, 1176 (2006).
3. World Health Organization. Anti-Tuberculosis drug resistance in the world, Report No. 4. WHO/HTM/TB/2008.394 (2008).
4. Magnet, S. & Blanchard, J. S. Molecular insights into aminoglycoside action and resistance. Chem Rev 105, 477-98 (2005).
5. Zaunbrecher, M. A., Sikes, R. D., Jr., Metchock, B., Shinnick, T. M. & Posey, J. E. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in Mycobacterium *tuberculosis*. Proc Natl Acad Sci U S A 106, 20004-9 (2009).
6. Taniguchi, H., et al. Molecular analysis of kanamycin and viomycin resistance in *Mycobacterium smegmatis* by use of the conjugation system. J Bacteriol 179, 4795-801 (1997).
7. Suzuki, Y., et al. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. J Clin Microbiol 36, 1220-5 (1998).
8. Maus, C. E., Plikaytis, B. B. & Shinnick, T. M. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 49, 3192-7 (2005).

9. Alangaden, G. J., et al, . Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 42, 1295-7 (1998).

10. Kruuner, A. e. a. Discordant resistance to kanamycin and amikacin in drug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 47, 2971-3 (2003).

11. Morris, R. P. et al. Ancestral antibiotic resistance in *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 102, 12200-5 (2005).

12. Okamoto, S. et al. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. Mol Microbiol 63, 1096-106 (2007).

13. Spies, F. S., da Silva, P. E., Ribeiro, M. O., Rossetti, M. L. & Zaha, A. Identification of mutations related to streptomycin resistance in clinical isolates of *Mycobacterium tuberculosis* and possible involvement of efflux mechanism. Antimicrob Agents Chemother 52, 2947-9 (2008).

14. NCCLS. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes; approved standard M24-A. (2003).

15. Ainsa, J. A. et al. Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*. J Bacteriol 180, 5836-43 (1998).

16. Louw, G. E. et al. A balancing act: efflux/influx in mycobacterial drug resistance. Antimicrob Agents Chemother 53, 3181-9 (2009).

17. Gupta, A. K. et al. *jefA* (*Rv2459*), a drug efflux gene in *Mycobacterium tuberculosis* confers resistance to isoniazid & ethambutol. Indian J Med Res 132, 176-88.

18. STREPTOMYCIN treatment of pulmonary tuberculosis. Br Med J 2, 769-82 (1948).

19. Johnson, R. et al. Drug resistance in *Mycobacterium tuberculosis*. Curr Issues Mol Biol 8, 97-111 (2006).

20. Sreevatsan, S. et al. Characterization of *rpsL* and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. Antimicrob Agents Chemother 40, 1024-6 (1996).

21. Meier, A., Sander, P., Schaper, K. J., Scholz, M. & Bottger, E. C. Correlation of molecular resistance mechanisms and phenotypic resistance levels in streptomycin-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 40, 2452-4 (1996).

22. Via, L. E. et al. Polymorphisms associated with resistance and cross-resistance to aminoglycosides and capreomycin in *Mycobacterium tuberculosis* isolates from South Korean Patients with drug-resistant tuberculosis. J Clin Microbiol 48, 402-11.

23. Singh, A. et al. *Mycobacterium tuberculosis* WhiB3 maintains redox homeostasis by regulating virulence lipid anabolism to modulate macrophage response. PLoS Pathog 5, e1000545 (2009).

24. Ramon-Garcia, S., Martin, C., Ainsa, J. A. & De Rossi, E. Characterization of tetracycline resistance mediated by the efflux pump Tap from *Mycobacterium fortuitum*. J Antimicrob Chemother 57, 252-9 (2006).

25. Jiang, X. et al. Assessment of efflux pump gene expression in a clinical isolate *Mycobacterium tuberculosis* by real-time reverse transcription PCR. Microb Drug Resist 14, 7-11 (2008).

26. Siddiqi, N. et al. *Mycobacterium tuberculosis* isolate with a distinct genomic identity overexpresses a tap-like efflux pump. Infection 32, 109-11 (2004).

27. De Rossi, E. et al. The multidrug transporters belonging to major facilitator superfamily in *Mycobacterium tuberculosis*. Mol Med 8, 714-24 (2002).

28. Springer, B. et al. Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. Antimicrob Agents Chemother 45, 2877-84 (2001).

29. Guo, M. et al. Dissecting transcription regulatory pathways through a new bacterial one-hybrid reporter system. Genome Res 19, 1301-8 (2009).

30. Gold, B., Rodriguez, G. M., Marras, S. A., Pentecost, M. & Smith, I. The *Mycobacterium tuberculosis* IdeR is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages. Mol Microbiol 42, 851-65 (2001).

31. Geiman, D. E., Raghunand, T. R., Agarwal, N. & Bishai, W. R. Differential gene expression in response to exposure to antimycobacterial agents and other stress conditions among seven *Mycobacterium tuberculosis* whiB-like genes. Antimicrob Agents Chemother 50, 2836-41 (2006).

32. Schwab, U. et al. Transcriptional responses of *Mycobacterium tuberculosis* to lung surfactant. Microb Pathog 46, 185-93 (2009).

33. Rohde, K. H., Abramovitch, R. B. & Russell, D. G. *Mycobacterium tuberculosis* invasion of macrophages: linking bacterial gene expression to environmental cues. Cell Host Microbe 2, 352-64 (2007).

Figure Legends

Figure 1. *whiB7* promoter and mutations. The location of mutations identified in spontaneous mutants are denoted by arrows; deletions (▼), insertions (▽), and transversions (▼). The *whiB7* transcription start site is denoted by a bent arrow and the start codon is boxed. A predicted IdeR binding region is underlined.

Figure 2. *whiB7* mutations confer increased expression of *whiB7*, *eis*, and *tap*. The ratio of gene transcripts to *sigA* (A-B) was determined by qRT-PCR and normalized to H37Rv. Error bars represent the SEM from at least 3 experiments. (A) *whiB7* spontaneous mutants and parental strains. K300 strains are derivatives of CDC1551 and K200 strains are derivatives of H37Rv. K204 harbors an *eis*C-14T promoter mutation and is wild type at the *whiB7* locus. (B) Gene expression from one representative strain of each type of *whiB7* promoter mutation and the corresponding allelic exchange derivative reverted to the wild type sequence; deletion, K301 (ΔC-125), insertion, K205 (+C-125), and transversion, K209 (A-26G). Parental strains CDC1551 and H37Rv are included for comparison. Acetyltransferase activity (C-D) due to Eis was measured from crude cell lysates and is expressed in nmole/mg/min. (C) *whiB7* spontaneous mutants and parental strains. (D) *whiB7* mutants and representative allelic exchange derivatives. Error bars represent the SEM from at least 3 experiments. Immunoblot analysis of cell lysates from (E) spontaneous *whiB7* mutants and parental strains and (F) representative allelic exchange derivatives. Lysates were probed with either anti-Eis or anti-GroES serum.

Figure 3. *M. tuberculosis* strains were streaked on 7H10 agar containing either (A) 5 μg/ml KAN or (B) 2 μg/ml STR and incubated for 28 days at 37ºC.

Figure 4. *eis* and *tap* expression in a *whiB7* knockout. (A) The relative expression of gene transcripts was determined by qRT-PCR and normalized to *sigA* in CDC1551. Error bars represent the SEM from at least 3 experiments. (B) Immunoblot analysis of cell lysates. 20 μg of cell lysate was probed with anti-Eis antibodies to ensure that basal level Eis expression could be detected.

Table 1

Table 1: Spontaneous mutant survey derivatives

Spontaneous Mutants*†	***whiB7***	**MIC µg/ml**	
		KAN	**STR**
CDC1551	WT	2	0.5
H37Rv	WT	2	0.5
K301, K203, K302, K304, K305, K307	ΔC-125	20	8
K303	ΔC-177	20	8
K306	ΔG-83	20	8
K308	ΔG-133	20	8
K309	ΔC-138	20	8
K205	+C -125	20	10
K206	G35A	10	8
K209	A-26G	10	8
CL-27‡	+C -125	20	10
K204	WT, *eis*C-14T	25	0.5
Allelic exchange derivatives			
K301-WT	WT	<5	0.5
K205-WT	WT	<5	2
K209-WT	WT	<5	0.5
Knockout strains			
CDC1551Δ*whiB7*	n/a	2	0.25
CDC1551Δ*tap*	WT	<5	0.1
CDC1551Δ*Rv1257c*	WT	<5	0.5
K301Δ*tap*	ΔC-125	10	0.1
K301Δ*Rv1257c*	ΔC-125	10	8

*All strains had AMK MICs <4 µg/ml
†K200 strains are derivatives of H37Rv, and K300 strains are derivatives of CDC1551
‡Clinical Isolate

Table 2

Effect of *whiB7* ΔC-125 mutation on
the emergence of high level SM-resistant mutants

	Mutant Frequency		
Strain	SM 2 μg/ml	SM 10 μg/ml	KAN 40 μg/ml
CDC1551	$1.8x10^{-6}$	$8.7x10^{-9}$	$9.8x10^{-7}$
K301	n/a	$7.2x10^{-6}$	$7.5x10^{-7}$

Figure 1

```
-180   caacaacgca tttggtgtcg gcgcggctgc cgcggtgcca accgcgggaa
-130   cccccatag caagaaccgt gtcgccgccg cgactgacga gtccgtggag
 -80   cggggctcta cgtaagcgct acgtaatcag ccccgtttcg ccagatggcc
 -30   acggacccga agtcacagga tccgtggcca aactttcggg acatctgccc
 +21   accgcagcat ccctggtcct ...50bp... GTGtcggtac tgacagtccc
```

Figure 2

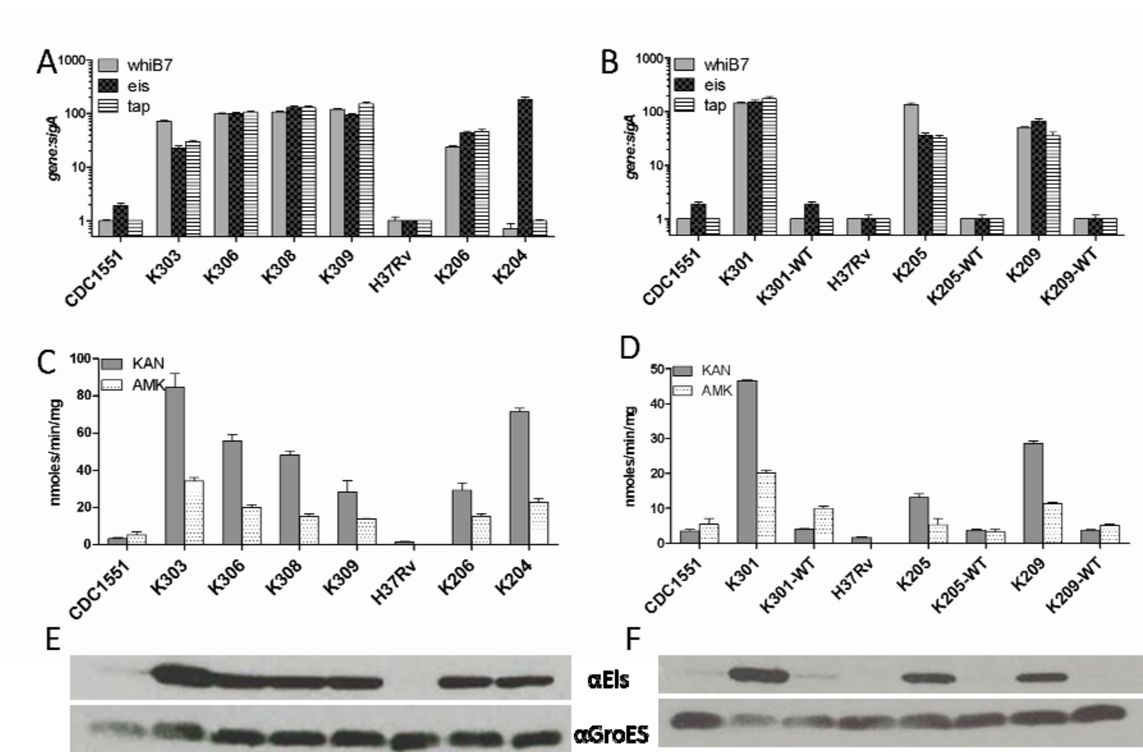
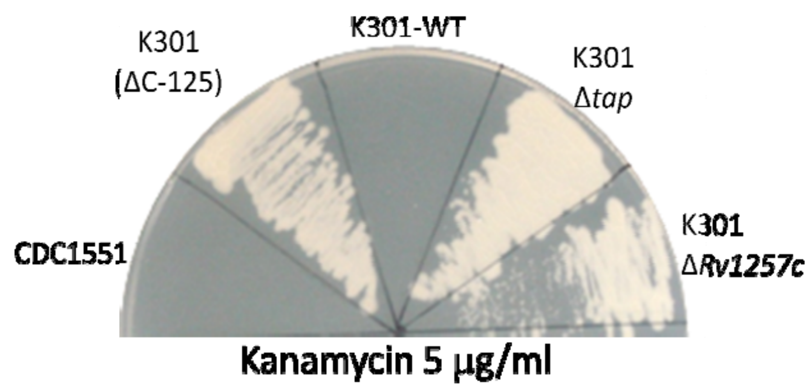


Figure 3



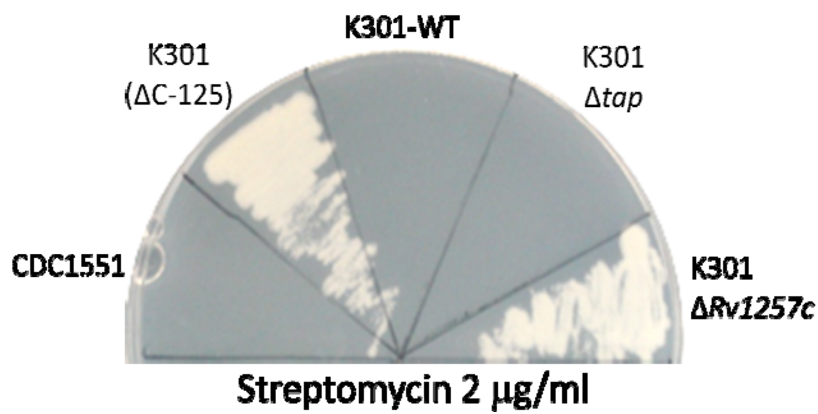
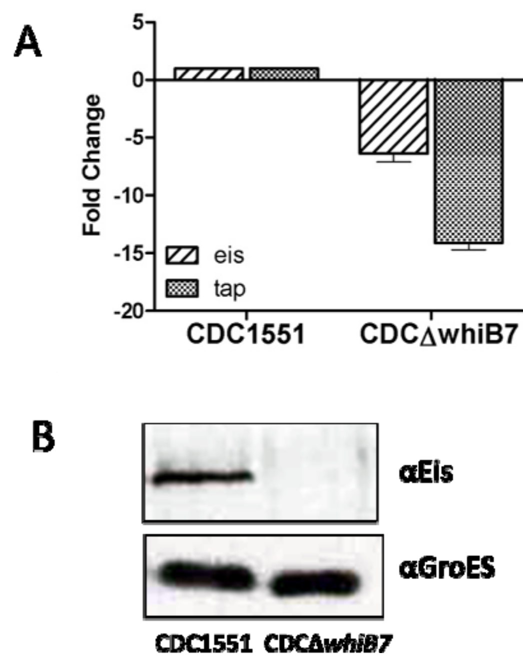


Figure 4



Supplemental Methods

Bacterial strains, media, and DNA manipulation. The strains and plasmids used in this study are listed in Table S1 and were cultivated as previously described[1]. The media were supplemented with hygromycin (HYG) (75 µg/ml), kanamycin (KAN) (5 µg/ml or 25 µg/ml), or streptomycin (SM) (2 µg/ml) when needed for cultivation of *M. tuberculosis*. For *E. coli* strains, Luria media was supplemented with HYG (200 µg/ml) or KAN (50 µg/ml) when needed. Antibiotics were purchased from Sigma. Standard protocols or the manufacturer's instructions were used for all DNA manipulation (New England Biolabs (NEB), Invitrogen, Finnzymes). All oligonucleotide primers (Table S3) were synthesized at the Biotechnology Core facility, National Center for Preparedness, Detection, and Control of Infectious Diseases, CDC. Clinical isolates were obtained from the culture collection at the Mycobacteriology Laboratory Branch, CDC. Human subject information linked to the clinical isolates used in this study is protected by the protocol approved by the CDC institutional review board.

Minimal Inhibitory Concentration (MIC) determination. The susceptibilities to KAN and STR were determined according to guidelines and definitions stated by the CLSI[2], using 7H10 agar containing KAN (Sigma) at concentrations of 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 50, and 80 µg/ml or STR at 0.25, 0.5, 1, 2, 4, 8, 10, and 16 µg/ml. The MIC was defined as the lowest concentration of drug resulting in complete inhibition of growth of ≤1% of the initial inoculum after 4 weeks of incubation at 37°C.

Construction of *whiB7* revertants and knockout strains. The specialized transducing phage, phAZ70, was constructed using methods previously described[3] and harbors the wild type *whiB7* gene and promoter and downstream gene, *Rv3197* separated by a HYG^R

cassette (Table S2). *M. tuberculosis* K301, K205, and K209 bacteria were infected with phAZ70 at an MOI of 10:1 (phage to bacteria) and selected on 7H10 containing HYG. The *whiB7* promoter region was PCR amplified and sequenced in each HYG^R transductant to confirm the reversion of the *whiB7* promoter point mutations to wild type sequence. *whiB7* knockout strains (bases -40 to 266 were removed) were constructed as previously described[1,2]. *M. tuberculosis* strains H37Rv, K204, and CDC1551 were transduced with phΔ*whiB7* at an MOI of 10:1 (phage to bacteria) and selected on 7H10 containing HYG. The marked deletion of *whiB7* was confirmed by PCR using the primers listed in Table S3 which amplify a 1.8 kb product when phΔ*whiB7* integrates into the *whiB7* locus. The *tap* (*Rv1258c*) knockout (bases 90 to 1230 removed) and *Rv1257c* knockout (bases 134 to 1188 removed) strains were similarly constructed and confirmed. All plasmids and phage used in this study are listed in Table S2.

Rapid Amplification of cDNA Ends (RACE). RACE was performed using a 5'/3' RACE Kit, 2nd Generation (Roche). For K301, 300 ng of RNA was used for cDNA synthesis respectively using primer RACE1 (12.5 μM) and 80 U transcriptor reverse transcriptase (Roche). cDNA was purified using a High Pure PCR Product Purification Kit (Roche). A homopolymeric A-tail was added to the 3'end of the purified cDNA by incubation of the cDNA with recombinant terminal transferase (80 U) and dATP (200 nM) for 20 min at 37°C. 5 μL of poly-adenylated cDNA was amplified using Promega PCR Master Mix and 0.5 μM of primers RACE2 and an oligo-dT anchor primer (Roche). Amplified cDNA products were purified using a ZymoClean Gel DNA Recovery Kit (Zymo), cloned into pCR2.1-TOPO (Invitrogen), and sequenced with RACE3.

Supplemental References

1. Zaunbrecher, M. A., Sikes, R. D., Jr., Metchock, B., Shinnick, T. M. & Posey, J. E. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 106, 20004-9 (2009).
2. NCCLS. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes; approved standard M24-A. (2003).
3. Bardarov, S., et al,. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis, M. bovis* BCG and *M. smegmatis*. *Microbiology* 148, 3007-17 (2002).

Supplemental Figure Legends

Figure S1. The ratio of *eis* transcripts to *sigA* was determined by qRT-PCR and normalized to H37Rv. Error bars represent the SEM from at least 3 experiments.

Figure S2. 16 clinical isolates with unexplained KAN resistance were analyzed by sequencing the *eis*, *rrs*, and *whiB7* loci. With the exception of isolate CL-27 (which harbors a +C-125 *whiB7* mutation), all the loci were wild type. Acetyltransferase activity due to Eis was measured from crude cell lysates from 12 of the isolates and is expressed in nmole/mg/min. However, the remaining four clinical isolates grew very poorly in 7H9 media and did not reach an OD_{600} sufficient to perform the acetyltransferase assays. Isolates CL-27, MLB100 and MLB126 displayed low-level cross resistance to KAN and STR. H37Rv is a wild type laboratory strain and K204 harbors an *eis* C-14T mutation.

Table S1: Strains used in this study

Strain	Genotype/Relevant Characteristics	Reference or Source
Escherichia coli		
HB101	F-Δ(gpt-proA)62, leuB6, glnV44, ara-14, galK2, lacY1, Δ(mcrC-mrr), rpsL20(Str^r), xyl-5, mtl-1, recA13, thi-1	NEB
M. smegmatis		
LR222	Easily transformable strain, used for phage preparation	Miller et al. (1994)
M. tuberculosis		
H37Rv	Pansusceptible laboratory strain	
K204	Spontaneous KAN^R mutant of H37Rv, C-14T *eis* mutation	Maus et al. (2005)
CDC1551	Pansusceptible isolate	This study
K301	Spontaneous Kan^R, SM^R mutant of CDC1551, ΔC-125 *whiB7* mutation	This study
K301-WT	K301 revertant, WT *whiB7* allele, HYG^R, introduced by allelic exchange	This study
K205	Spontaneous KAN^R,SM^R mutant of H37Rv, +C-125 *whiB7* mutation	This study
K205-WT	K205 revertant, WT *whiB7* allele, HYG^R, introduced by allelic exchange	This study
K209	Spontaneous KAN^R, SM^R mutant of H37Rv, A-26G *whiB7* mutation	This study
K209-WT	K209 revertant, WT *whiB7* allele, HYG^R, introduced by allelic exchange	This study
Knockouts		
CDC1551Δ*whib7*	*whiB7* knockout in CDC1551, *whiB7*::HYG^R	This study
H37RvΔ*whib7*	*whiB7* knockout in H37Rv, *whiB7*::HYG^R	This study
K204Δ*whib7*	*whiB7* knockout in K204, *whiB7*::HYG^R	This study
CDC1551Δ*tap*	*tap (Rv1258c)* knockout in CDC1551, *tap*::HYG^R	This study
K301Δ*tap*	*tap (Rv1258c)* knockout in K301, *tap*::HYG^R	This study
CDC1551Δ*Rv1257c*	*Rv1257c* knockout in CDC1551, *Rv1257c*::HYG^R	This study
K301Δ*Rv1257c*	*Rv1257c* knockout in K301, *Rv1257c*::HYG^R	This study

Abbreviations: HYG^R, hygromycin resistance cassette, KAN^R, kanamycin resistant, SM^R, streptomycin resistant, ORF, open reading frame

Table S2: Plasmids and Phage used in this study.

Plasmid	**Relevant Characteristics**	**Reference or Source**
pYUB854	Cosmid vector, with res sites flanking the HYG^R gene	Bardarov et al. (2002)
pJC01	pYUB854 + 900 bp flanking sequence of *whiB7*	This study
pJC02	pYUB854 + 900 bp flanking sequence of *tap(Rv1258c)*	This study
pJC03	pYUB854 + 900 bp flanking sequence of *Rv1257c*	This study
pAZ70	pYUB854 + *whiB7* WT promoter and ORF and *Rv3197* separated by HYG^R cassette, for *whiB7* revertant construction	This study
pAZ69	pYUB854 + *whiB7* +C-125 promoter and ORF and *Rv3197* separated by HYG^R cassette, for *whiB7* revertant construction	
pHIN-STOP	Integrating mycobacterial vector, HYG^R, Transcription stop sequence upstream of MCS	Zaunbrecher et. al (2009)
pAZ91	pHIN-STOP + C-terminal FLAG tag fusion of WhiB7	This study
Phage		
phAE159	Mycobacterial phage	Bardarov et al. (2002)
phΔ*whiB7*	phAE159 + pJC01, HYG^R	This study
phΔ*tap*	phAE159 + pJC02, HYG^R	This study
phΔ*Rv1257c*	phAE159 + pJC03, HYG^R	This study
phAZ70	phAE159 + pAZ70, HYG^R	This study
phAZ69	phAE159 + pAZ69, HYG^R	This study

Abbreviations: HYG^R, hygromycin resistant, Kan^r, kanamycin resistant, WT, wild type, ORF, open reading frame, MCS, multi-cloning site

Table S3, primer sequences

Gene/Loci	Forward primer (5'-3')	Reverse primer (5'-3')	UPL#
whiB7 upstream for allelic exchange	ACA**GGTACC**TACGTCGGAATCACACGG	TCG**TCTAGA**ATCGCGGGCTATGCAACAG	
whiB7 downstream for allelic exchange	CAC**AAGCTT**GATGCTGTTGCATAGCCC	GAC**ACTAGT**CTCAAACAGCTCTCACCC	
Δ *whiB7* upstream region	ACA**GGTACC**GTTCCTCAACGGCATTGC	ACC**TCTAGA**GAAAGTGATCCGGACCAG	
Δ *whiB7* downstream region	CAC**AAGCTT**GATGCTGTTGCATAGCCC	GAC**ACTAGT**CTCAAACAGCTCTCACCC	
Δ *whiB7* confirmation PCR	CTGTTGCCGACGATGGAGAAG	ACGACTTCGAGGTGTTCGAGGAG	
Δ *tap* upstream region	AGC**GGTACC**ATTCTGCTGGCTCGGGTGGTAG	CAC**TCTAGA**AAACGCGACTATCGAGAC	
Δ *tap* downstream region	ACC**AAGCTT**ATCGATCGGCCCGTAGGATC	ACC**ACTAGT**GCCACGTTCATCAGAACC	
Δ *tap* confirmation PCR	CGAACACGGCGGCCATCACGG	ACGACTTCGAGGTGTTCGAGGAG	
Δ *Rv1257c* upstream region	CAA **GGTACC** TGA TGA TCG CGA CGG TTG	CAA**TCTAGA**TGATTGCCAGCGGTTTGC	
Δ *Rv1257c* downstream region	ACC**AAGCTT**TACGGCGAAATCATGGAC	ACC**ACTAGT**TGAAGTAGGCCATCATCG	
Δ *Rv1257c* confirmation PCR	ACGCCAGTATGGACAGTGTG	ACGACTTCGAGGTGTTCGAGGAG	
sigA qRT-PCR	AGCTGGCCAAAGAGATGGA	GGGCGTATTGCTGGATTTC	133
eis qRT-PCR	GCGATCTACGAGCGATGG	CTCGTCCCAGAGCACCTG	62
whiB7 qRT-PCR	CGGATCACTTTCGAAGAACC	CAGTACCGACACGTCCTGTTT	38
tap qRT-PCR	CAACCTGGCCTTTATTGTCG	CGTCGCGGTAATCCACAT	9
erm qRT-PCR	GAAATTGGATTGATCGCAGTG	GGACACGACGTACCGTCAC	105
whiB7 RACE1	GACTCACGATCGAGCCTTGG		
whiB7 RACE2	CCAAGCACTGCCGCCTGATC		
whiB7 RACE3	GACACTGTGTGTGAGCTG		

Figure S1

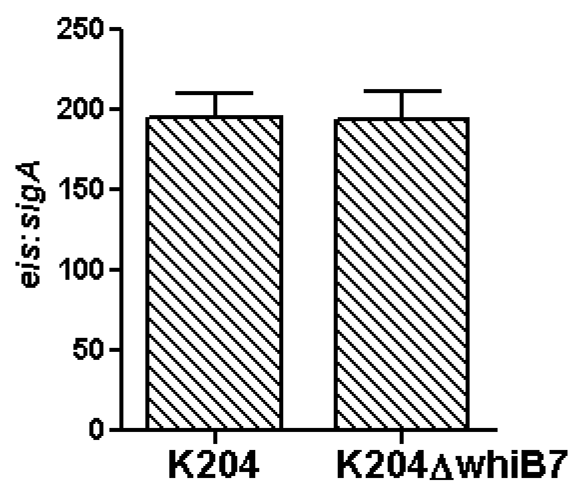
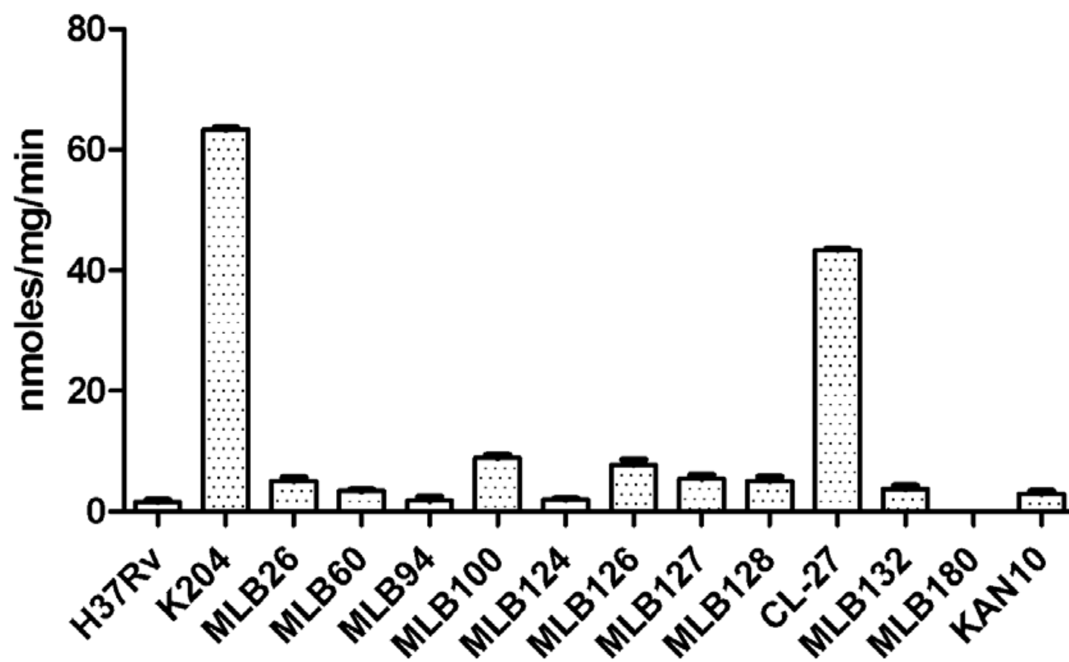


Figure S2



Chapter 4

Unpublished Research

Kanamycin resistant *Mycobacterium tuberculosis* harboring mutations in the *eis* promoter display enhanced growth in macrophages

This manuscript was written by Mary Analise Zaunbrecher
Additional contributing authors include Patricia Campbell, Thomas Shinnick, and James Posey

Mary Analise Zaunbrecher was intellectually involved in the concept, experimental design, and implementation of this project from its conception as well as the analysis and interpretation of the results.

Kanamycin resistant *Mycobacterium tuberculosis* harboring mutations in the *eis* promoter display enhanced growth in macrophages

M. Analise Zaunbrecher[1,2], Patricia H Campbell[2], Thomas M Shinnick[2] & James E Posey[2]

[1]Microbiology and Molecular Genetics Graduate Program, Department of Microbiology and Immunology, Emory University, Rollins Research Center, Suite 3001, 1510 Clifton Rd, Atlanta, GA, 30322 USA;

[2]Division of TB Elimination, National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention, Centers for Disease Control and Prevention, 1600 Clifton Rd, Mail Stop G35, Atlanta, GA, 30333 USA

INTRODUCTION

The ability to survive and replicate within macrophages is a central component of MTB pathogenesis. Several mechanisms contribute to the intracellular survival of mycobacteria including the inhibition of phago-lysosomal fusion and resistance to acid and reactive nitrogen and oxygen intermediates[1, 2]. However, our understanding of the genes and gene products responsible for these processes is lacking. One of the many factors proposed to be involved in macrophage survival of MTB is the Eis (*Rv2416c*) protein[3]. *eis* was originally identified in a screen that selected for genes that enhanced the intracellular survival of the nonpathogenic mycobacteria, *M. smegmatis*, in a human macrophage cell line[3]. Although Eis shares amino acid homology with the GCN5-related family of N-acetyltransferases (GNAT superfamily) and appears to be released into macrophage cytoplasm during an infection, little is known about the true function[4]. Several studies document an involvement of Eis in immune modulation[4, 5]. When recombinant Eis protein is used to stimulate monocytes or T cells, an anti-inflammatory cytokine response is elicited[4, 5]. This modulation of cytokine expression may produce a zone of localized immunosuppression that facilitates MTB survival in the host. Additionally, Eis may play a role as an immunogen since Eis was detected in the serum of 40% of purified protein derivative (PPD) positive patients analyzed with anti-Eis antibodies[6]. We have previously confirmed a function of Eis in aminoglycoside resistance in MTB[7]. Eis is able to acetylate and inactivate the aminoglycosides kanamycin (KAN) and amikacin (AMK) and *eis* promoter mutations, such as the C-14T mutation, that cause increased *eis* expression confer low level mono-KAN resistance (5 μg/ml $\geq$ MIC $\geq$ 40 μg/ml)[7]. These two drugs are the only known substrates of Eis,

however, the relatively low affinity of Eis to these drugs when compared with other characterized aminoglycoside acetyltransferases (AACs) suggests that neither of these compounds are the true substrate of Eis[7]. The true substrate(s) remains unknown.

Studies investigating the role of Eis in intracellular survival are somewhat conflicting. One study comparing the laboratory strain H37Rv with an H37Rv *eis* knockout and complemented strain found no difference in survival in either U-937 human-derived macrophages or in the lungs or spleens of aerosol-infected mice[4]. However, another study found that deleting *eis* from a W-Beijing derivative of MTB correlated with a diminished growth phenotype in the human monocyte cell line, MonoMac6[9]. Given that different monocyte cell lines were used in these studies, it is difficult to directly compare these results. Notably, the W-Beijing strain naturally expresses *eis* 10-times higher than H37Rv[9], suggesting that genetic background and *eis* expression level may influence *eis*-mediated macrophage survival. The lack of consistency in these studies exemplifies the need for a better understanding of the role of *eis* in macrophage survival. In this study, we demonstrate that *eis* C-14T promoter mutants derived from either the H37Rv or Beijing background confer an enhanced growth phenotype in murine J774A.1 macrophages. This phenotype is not observed in strains that over-express Eis due to *whiB7* mutations. Importantly, because the same *eis* mutation that confers enhanced intracellular survival can be selected for by KAN treatment in clinical isolates[7], this data may have important clinical implications for multi-drug resistant (MDR) TB treatment. Additionally, we present data that suggests AMK may not be a suitable alternative drug choice for treating Eis mutants given that *eis* C-14T mutants grow in the presence of AMK in intracellular susceptibility assays.

RESULTS

eis C-14T promoter mutants demonstrate enhanced intracellular survival in murine macrophages.

The original report that linked *eis* to the intracellular survival of MTB in macrophages employed a system that expressed Eis on a multicopy plasmid[3]. However, no survival phenotype was observed in a subsequent study with an *eis* deletion strain derived from H37Rv[4]. Given that the original experimental design artificially over-expressed *eis* in *M. smegmatis*, we reasoned that the enhanced survival phenotype may be a consequence of increased *eis* expression. *eis* over-expression occurs naturally in strains harboring *eis* promoter mutations[7]. For example, *M. tuberculosis* strain K204 is a derivative of the laboratory strain H37Rv and harbors an *eis* C-14T promoter mutation that confers a substantial increase in *eis* transcripts, protein levels, and KAN resistance[7]. In a survey of low-level KAN resistant clinical isolates, *eis* promoter mutations were commonly found in isolates of the Beijing family origin[7]. These isolates originated primarily in former soviet countries (data not shown) where KAN is commonly used in MDR-TB treatment, suggesting that treatment with KAN selects for *eis* mutations in this population. As Beijing strains are widely circulated throughout the world, we sought to investigate the effects of *eis* over-expression in this clinically relevant MTB background. A Beijing *eis* C-14T mutant strain was constructed by allelic exchange in a clean genetic background (parental strain Beijing F2) using the specialized transducing phage, phALEX-C-14T (Table 1). The phALEX-C-14T phage also introduces a hygromycin (HYG) cassette downstream of the *eis* open reading frame. To allow for direct comparison of isogenic strains, the HYG cassette was also inserted into the same area of the genome in the wild type background using phage phALEX-WT (Beijing F2::*hyg,* Table 1). Eis expression in

each strain was measured by qRT-PCR and compared to the parental strain. Introduction of the *eis* C-14T allele into Beijing F2 resulted in a 157-fold increase in *eis* expression whereas insertion of just the HYG cassette did not alter the amount of *eis* transcript when compared to the parental Beijing F2 strain (Fig 1).

To investigate whether increased *eis* expression enhances growth in macrophages, the murine macrophage cell line J774A.1 was infected with either wild type or *eis* C-14T mutants derived from either the H37Rv or Beijing background at a multiplicity of infection (MOI) of 1:1. After 5 days, the *eis* mutants exhibited a marked increase in intracellular growth when compared to wild type strains. Bacteria were enumerated by colony forming units per ml (CFU/ml). By this measurement, K204 outgrew H37Rv by 8.2-fold while Beijing *eis* C-14T outgrew Beijing F2::*hyg* by 6.1 fold (Figure 2a and 2b). These data indicate that the *eis* C-14T mutation confers enhanced growth in macrophages, and this phenotype is likely due to enhanced Eis expression.

whiB7 promoter mutants also exhibit increased *eis* expression (Zaunbrecher et al., 2010, unpublished data). Strain K301 is a spontaneous mutant derivative of the MTB background CDC1551 that harbors a *whiB7* ΔC-125 mutation. The *whiB7* ΔC-125 mutation confers enhanced *whiB7* expression that leads to an upregulation of the genes in the *whiB7* regulon, including an increase in *eis* expression that is comparable to K204 and Beijing *eis* C-14T (Fig 1). However, there was no significant difference in intracellular growth when K301 was compared to CDC1551 (p value = 0.0931, Mann-Whitney test) (Fig 2c) suggesting that modulated expression of other genes in the *whiB7* regulon besides *eis* may affect the growth advantage conferred by overexpression of *eis* alone.

All of the strains described above have similar *in vitro* growth curves in 7H9 broth suggesting the intracellular growth phenotype of the *eis* mutants is due to interactions with the macrophage (Fig 3).

Enhanced *eis* expression confers increased intracellular resistance to AMK

Eis mutants are resistant to KAN (MIC ≥ 5 μg/ml), but are classified as susceptible to AMK (MIC < 4 μg/ml) under the current guidelines set by Clinical Laboratory Standards Institute (CLSI)[7, 10]. This observation has led to the suggestion that AMK may be a suitable treatment alternative for patients infected with strains harboring an *eis* mutation. It would be very difficult to experimentally test this assumption in humans without conducting clinical trials. Therefore, we examined the intracellular susceptibility of *eis* mutants to AMK while growing inside of macrophages in a model system believed to mimic physiologic circumstances inside the host. This approach takes into consideration that an antibiotic must penetrate the eukaryotic cell wall before exerting an effect on the internalized bacteria and also takes into account that gene expression profiles may be altered by bacteria inside a macrophage in a manner that may contribute to drug resistance.

To investigate whether *eis* mutants are susceptible to AMK in a macrophage infection model, we followed intracellular growth and survival of wild type H37Rv and *eis* mutant K204 bacteria in J774.1 macrophages exposed to increasing concentrations of AMK for 5 days. Notably, although K204 is classified as susceptible to AMK, the AMK MIC (3 μg/ml) is 6 times greater than the AMK MIC of H37Rv (0.5 μg/ml)[7]. In the absence of AMK, K204 grows 8.2 fold better than H37Rv after 5 days in macrophages (Fig 2a). When a low concentration of AMK (5 μg/ml) is added to H37Rv infected

macrophages, a 4 fold reduction in CFU/ml is observed after 5 days (Fig 4a). When infected macrophages were exposed to 10 μg/ml or 20 μg/ml of AMK, we observed an 8.3 fold reduction and 25.6 fold reduction respectively (Fig 4a). These data suggest that AMK is able to penetrate the macrophage cell wall in sufficient concentrations to kill H37Rv cells (AMK is present in the cell at a concentration greater than 0.5 μg/ml). Additionally, since the number of CFU/ml is decreasing over time in the 10 μg/ml and 20 μg/ml samples, this suggests that AMK is having a bactericidal effect on the internalized H37Rv bacteria. Conversely, K204 bacteria grew practically uninhibited in AMK treated macrophages (Fig 4b). Even in the presence of 20 μg/ml AMK, only a 2.4 fold reduction in CFU/ml was observed for K204 (Fig 4b) and the bacterial numbers were 10 fold higher than those for H37Rv at the same AMK concentration. Only when the AMK concentration was increased to 40 μg/ml (well above clinically used doses) was a 6.6 fold reduction in CFU/ml observed (Fig 4b). This data indicates that *eis* mutants residing inside of macrophages are sufficiently resistant to AMK to continue growing. This observation may have implications on using AMK to treat *eis* mutants.

DISCUSSION

We demonstrate here that an *eis* mutation confers enhanced growth in macrophages. These data may have several clinical implications for patients infected with *eis* mutants. Enhanced macrophage growth is associated with increased transmission and spread of a strain through a population[11, 12]. Studies investigating the spread of Beijing isolates found that the strains responsible for large clusters of TB cases also demonstrated consistently better growth in macrophages when compared to isolates that caused single TB infections or a small cluster of cases[11]. This suggests that *eis* mutants may possess an increased fitness that could allow for more efficient disease transmission. In fact, this idea has already been demonstrated. The Beijing lineage strain 210 (and derivatives) is known to overexpress *eis* and has an enhanced capacity to grow in murine macrophages, THP-1 cells, and mice[9, 11, 12]. Additionally, this strain harbors an increased capacity to spread through a population since it is documented in at least 5 states and is responsible for outbreaks throughout the south central and south western United States[9]. At this point, it is not clear whether *eis* mutants contribute to TB outbreaks, but retrospective studies on the epidemiology of Beijing lineage outbreaks may represent a population of interest for this type of study, particularly in a region where KAN is used for treatment.

Treatment with KAN can select for mutations in *eis*, indicating that exposing patients to KAN may simultaneously select for mutants that are more adapted to grow in macrophages[7]. When designing treatment regimens for MDR-TB patients, this data may need to be considered when deciding whether or not KAN should be included. In

addition to detecting KAN resistance, the *eis* promoter region may serve as a molecular marker for enhanced fitness or enhanced capacity to spread within a population.

This data raises the question of the mechanism for *eis*-mediated enhanced macrophage survival. Since Eis is secreted into the macrophage cytoplasm, it is possible that host factors are affected. *Salmonella typhimurium* uses a host-directed acetyltransferase to inhibit innate immune responces[13]. The AvrA acetyltransferase penetrates the nucleus of intestinal epithelial cells and acetylates the transcription factor p53 leading to cell cycle arrest and inhibition of apoptosis and inflammation[13]. Eis shares homology to histone acetyltransferases[4], so an intriguing possibility is that Eis could directly affect host gene expression by modulating histone acetylation patterns. To approach this hypothesis, *in vitro* acetyltransferase assays with purified histones or synthetic amino-terminal histone tails as substrate for recombinant Eis could be performed along with careful localization studies of Eis during an infection. The *aac(2')-Ia* acetyltransferase in *Providenica stuartii* is involved in peptidoglycan acetylation modifications that are critical for proper cellular morphology[14]. Thus, it is also possible that Eis modulates the bacterial cell wall or lipids in a way that improves resistance to macrophage stresses. This idea could be tested by performing HPLC analysis on lipid extractions from a wild type and mutant strain and examining for any obvious differences in lipid composition or polarity. Alternatively, *in vitro* susceptibility assays comparing the wild type and *whiB7* mutant strain could be performed under conditions mimicking macrophage stresses, such as acid or oxidative stress. We also cannot rule out the possibility that Eis may contribute to metabolism. Infection studies in mice examining

organ burden, histology, and lung pathology would also be useful to help understand whether *eis* mutants are more virulent than a wild type infection.

The enhanced macrophage growth phenotype was ablated in the *whiB7* mutant, K301. Microarray analysis of K301 predicts that 800 genes have modulated expression when *whiB7* is overexpressed, including a dramatic upregulation of many genes involved in the stress response to antibiotics (microarray data is unpublished). Constitutive expression of stress response genes may result in a loss of fitness of the *whiB7* mutant in a macrophage that cancels out the advantage afforded by *eis* overexpression. This hypothesis is supported by the observation that only a single clinical isolate harboring a *whiB7* promoter mutation has been identified (Zaunbrecher et al., 2010 unpublished). Additionally, the *dosR* regulon, which is responsive to oxidative stresses such as those experienced within a macrophage[15], is down regulated in K301, possibility increasing susceptibility to macrophage defenses. Determining whether *whiB7* mutants have decreased fitness *in vivo* will require mouse infection studies where an intact immune system is in place. Alternatively, it appears that metabolism may be altered in a *whiB7* mutant since genes involved in histidine and arginine biosynthesis are upregulated and this may affect growth in a macrophage (unpublished data).

The second major finding of this study is that growth of *eis* mutants in macrophages is not inhibited when exposed to physiological concentrations of AMK. When using AMK according to the recommended treatment guidelines, the blood serum level of amikacin peaks at approximately 46 µg/mL and has a median half life of 2.5 hours[16-18]. Therefore, the AMK concentration in the serum throughout the duration of treatment is typically much lower than 40 µg/ml, a concentration that still permits growth

of the *eis* C-14T mutant. These data suggest that AMK may not be a suitable treatment alternative for *eis* mutants. Additionally, this suggests the CLSI may need to consider decreasing the recommended concentration of AMK used to diagnose AMK resistance to include *eis* mutant strains. The *eis* promoter region may need to be reconsidered as a molecular marker for low-level cross resistance between KAN and AMK.

MATERIALS & METHODS

Bacterial Strains and media *M. tuberculosis* strains H37Rv, CDC1551, Beijing F2 and derivatives were propagated in 7H9 media containing 10% ADC, 0.4% glycerol and 0.5% Tween 20. The media were supplemented with hygromycin (HYG) (75 µg/ml) when necessary. Antibiotics were purchased from Sigma. Standard protocols or the manufacturer's instructions were used for all DNA manipulations (New England Biolabs (NEB)/ Invitrogen/ Finnzymes).

Construction of Beijing *eis*C-14T mutant (FAX) The specialized transducing phage phAlexC-14T was generated from pAZ19 (pYUB854::*eisC-14T*::*Rv2415c*) and phAE159 as previously described[7, 19]. *M. tuberculosis* Beijing F2 bacteria were infected with phAlexC-14T or phAlex-WT at an MOI of 10:1 and selected on 7H10 containing HYG. Genomic DNA from individual transductants was sequenced to confirm the presence of the *eis*C-14T allele.

Quantitative RT-PCR. *M. tuberculosis* strains were grown to mid-log phase (OD_{600} 0.4-0.6) in Middlebrook 7H9 medium; bacteria were harvested and lysed using a FastPrep 120 (Bio 101 Savant); and RNA purified using an RNeasy kit (Qiagen). 1µg of DNAse-treated RNA from each *M. tuberculosis* strain was used to generate cDNA using the Promega Reverse Transcriptase System. Quantification of transcripts from 2 µL cDNA was performed by real-time PCR using Probes Master Mix (Roche) in a LightCycler480 detection system. Reactions to detect specific transcripts used 0.4 µM of each primer and 0.1 µM of the probe (Roche) using probe and primer pairs. To detect the *sigA* transcript we used primers AZ139 and AZ140 and probe UPL#133 (Roche). Primers AZ141 and AZ142 and UPL#62 probe (Roche) were used to detect the *eis*

transcript. The relative amounts of each PCR product was calculated from standard curves obtained from PCR with the same primers and probes and serially diluted K204 cDNA.

Macrophage Survival Assays The murine macrophage cell line J774A.1 (ATCC) was cultured in DMEM medium (Gibco) containing 10% fetal bovine serum (FBS) (DMEM+FBS)(Sigma) and maintained at 37°C with 5% CO_2. *M. tuberculosis* bacteria were resuspended in DMEM+FBS and sonicated 2 x 5 sec before infecting J774 cells at an MOI of 1:1 for 2 hrs. Monolayers were washed 5x with DMEM+FBS, exposed to DMEM+FBS medium containing 200 µg/ml amikacin for 2 hrs to kill any extracellular bacteria, and then washed 2x with DMEM+FBS. At different time points after the infection, the supernatant was aspirated and macrophages were lysed with 0.1% Triton X-100 for 10 min at room temp. The bacterial suspensions in cell lysates were ultrasonically dispersed, serially diluted in phosphate buffered saline + 0.05% Tween 20 (PBS-Tw20), and plated in triplicate on 7H10-OADC agar plates. CFUs were enumerated after 3-4 weeks at 37°C and served as a measure of intracellular survival. Amikacin (AMK) susceptibility assays were performed using same method except that AMK was maintained in the media throughout the infection at concentrations of 5, 10, 20, or 40 µg/ml.

Acknowledgements

Thank you to Ruth Napier for critical reading of this chapter and helpful conversations.

REFERENCES

1. Nguyen, L. & Pieters, J. The Trojan horse: survival tactics of pathogenic mycobacteria in macrophages. Trends Cell Biol 15, 269-76 (2005).
2. Pieters, J. Mycobacterium tuberculosis and the macrophage: maintaining a balance. Cell Host Microbe 3, 399-407 (2008).
3. Wei, J., et al. Identification of a *Mycobacterium tuberculosis* gene that enhances mycobacterial survival in macrophages. J Bacteriol 182, 377-84 (2000).
4. Samuel, L. P., et al. Expression, production and release of the Eis protein by *Mycobacterium tuberculosis* during infection of macrophages and its effect on cytokine secretion. Microbiology 153, 529-40 (2007).
5. Lella, R. K. & Sharma, C. Eis (enhanced intracellular survival) protein of Mycobacterium tuberculosis disturbs the cross regulation of T-cells. J Biol Chem 282, 18671-5 (2007).
6. Dahl, J. L., Wei, J., Moulder, J. W., Laal, S. & Friedman, R. L. Subcellular localization of the Intracellular survival-enhancing Eis protein of *Mycobacterium tuberculosis*. *Infect Immun* 69, 4295-302 (2001).
7. Zaunbrecher, M. A., Sikes, R. D., Jr., Metchock, B., Shinnick, T. M. & Posey, J. E. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 106, 20004-9 (2009).
8. Via, L. E. et al. Polymorphisms associated with resistance and cross-resistance to aminoglycosides and capreomycin in Mycobacterium tuberculosis isolates from

South Korean Patients with drug-resistant tuberculosis. J Clin Microbiol 48, 402-11.

9. Wu, S., et al. Activation of the eis gene in a W-Beijing strain of Mycobacterium tuberculosis correlates with increased SigA levels and enhanced intracellular growth. Microbiology 155, 1272-81 (2009).

10. National Committee for Clinical laboratory Standards. Susceptibility Testing of *Mycobacteria, Norcardiae,* and other aerobic actinomycetes. Approved Standard M24-A.

(Wayne, Pa, 2003).

11. Zhang, M., et al. Enhanced capacity of a widespread strain of Mycobacterium tuberculosis to grow in human macrophages. J Infect Dis 179, 1213-7 (1999).

12. Theus, S., Eisenach, K., Fomukong, N., Silver, R. F. & Cave, M. D. Beijing family Mycobacterium tuberculosis strains differ in their intracellular growth in THP-1 macrophages. Int J Tuberc Lung Dis 11, 1087-93 (2007).

13. Wu, S. et al. Salmonella typhimurium infection increases p53 acetylation in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 298, G784-94.

14. Payie, K. G., Rather, P. N. & Clarke, A. J. Contribution of gentamicin 2'-N-acetyltransferase to the O acetylation of peptidoglycan in *Providencia stuartii*. J Bacteriol 177, 4303-10 (1995).

15. Park, H. D. et al. Rv3133c/dosR is a transcription factor that mediates the hypoxic response of Mycobacterium tuberculosis. Mol Microbiol 48, 833-43 (2003).

16. American Thoracic Society. Centers for Disease Control and Prevention. and Infectious Diseases Society of America. in American Journal of Respiratory and Critical Care Medicine 603-62 (2003).

17. American Thoracic Society Centers for Disease Control and Prevention. Diagnostic standards and classification of tuberculosis in adults and children. *Am J Respir Crit Care Med* 161, 1376-1395 (2000).

18. Peloquin, C. A., et al. Aminoglycoside toxicity: daily versus thrice-weekly dosing for treatment of mycobacterial diseases. Clin Infect Dis 38, 1538-44 (2004).

19. Bardarov, S., et al,. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis, M. bovis* BCG and *M. smegmatis*. *Microbiology* 148, 3007-17 (2002).

Figure Legends

Figure 1. Analysis of *eis* expression. The ratio of *eis/sigA* transcripts in each strain was determined by qRT-PCR and normalized to H37Rv.

Figure 2. Comparison of intracellular growth. (A) H37Rv and K204, (B) Beijing F2::*hyg* and Beijing F2::*eis* C-14T, (C) CDC1551 and K301. J447A.1 cells were infected at an MOI of 1:1 and CFU/ml were enumerated at 4 hrs, 2 days, and 5 days after infection. Data points represent the mean ± standard deviation from a representative experiment performed in triplicate.

Figure 3. *In vitro* growth curves in 7H9 broth comparing wild type strains to point-mutant derivatives. (A) H37Rv vs K204 (*eis* C-14T). (B) Beijing F2::*hyg* vs Beijing *eis* C-14T. (C) CDC1551 vs K301 (*whiB7* ΔC-125).

Figure 4. Amikacin intracellular survival assays. J774A.1 monolayers were infected with *M. tuberculosis* cultures at an MOI of 1:1 and CFU/ml were enumerated at 4 hrs, 2 days, and 5 days after infection. AMK was included in the extracellular media as indicted throughout the duration of the experiment. (A) H37Rv, wild type (B) K204, *eis* C-14T mutant. Data points represent the mean ± standard deviation from a representative experiment performed in triplicate.

Table 1: Strains and Phage used in this study

Strain	**Genotype/Relevant Characteristics**	**Reference or Source**
M. tuberculosis		
H37Rv	Pansusceptible laboratory strain	
K204	Spontaneous KAN^R mutant of H37Rv, C-14T *eis* mutation	Zaunbrecher (2009)
CDC1551	Pansusceptible isolate	This study
K301	Spontaneous Kan^R, STR^R mutant of CDC1551, ΔC-125 *whiB7* mutation	This study
Beijing F2	Pansusceptible Beijing isolate	This study
Beijing F2::*hyg*	Beijing F2 with HYG^R cassette introduced by allelic exchange at the *eis* locus	This study
Beijing F2::*eis* C-14T	Beijing F2 with HYG^R cassette and *eis* C-14T mutation introduced by allelic exchange at the *eis* locus	This study
Phage		
phAE159	Mycobacteriophage used for allelic exchange	Bardarov (2002)
phALEX-C-14T	phAE159+*eis* C-14T promoter and ORF and HYG^R cassette	Zaunbrecher (2009)
phALEX-WT	phAE159+wild type *eis* promoter and ORF and HYG^R cassette	Zaunbrecher (2009)

Abbreviations: HYG^R, hygromycin resistance cassette, KAN^R, kanamycin resistant, STR^R, streptomycin resistant, ORF, open reading frame

Figure 1

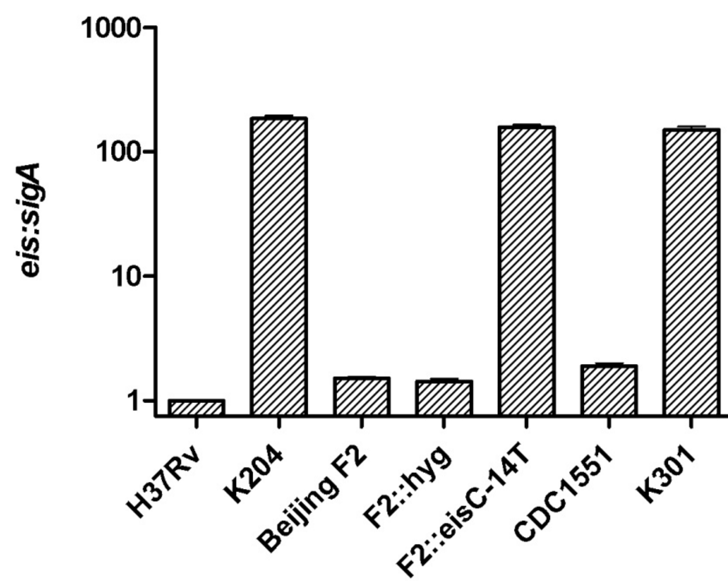


Figure 2

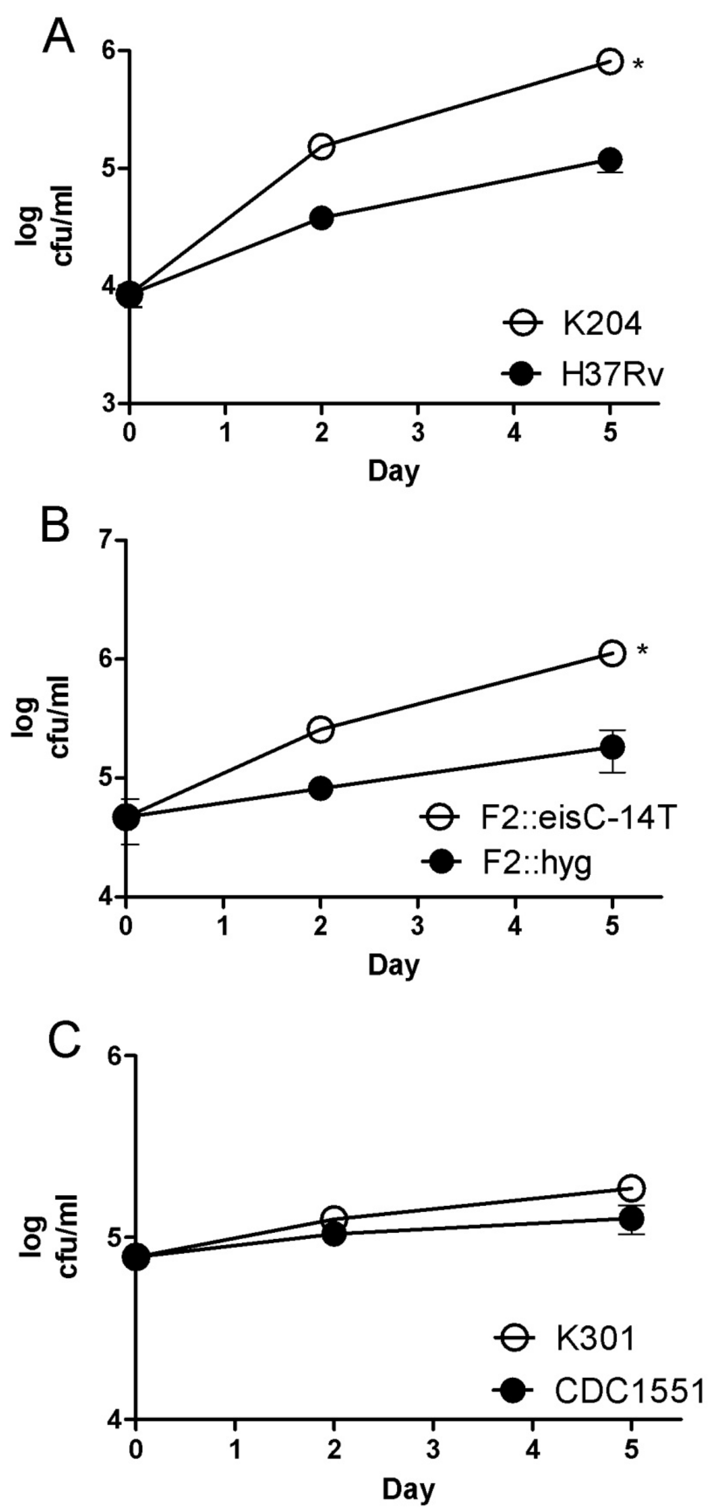


Figure 3

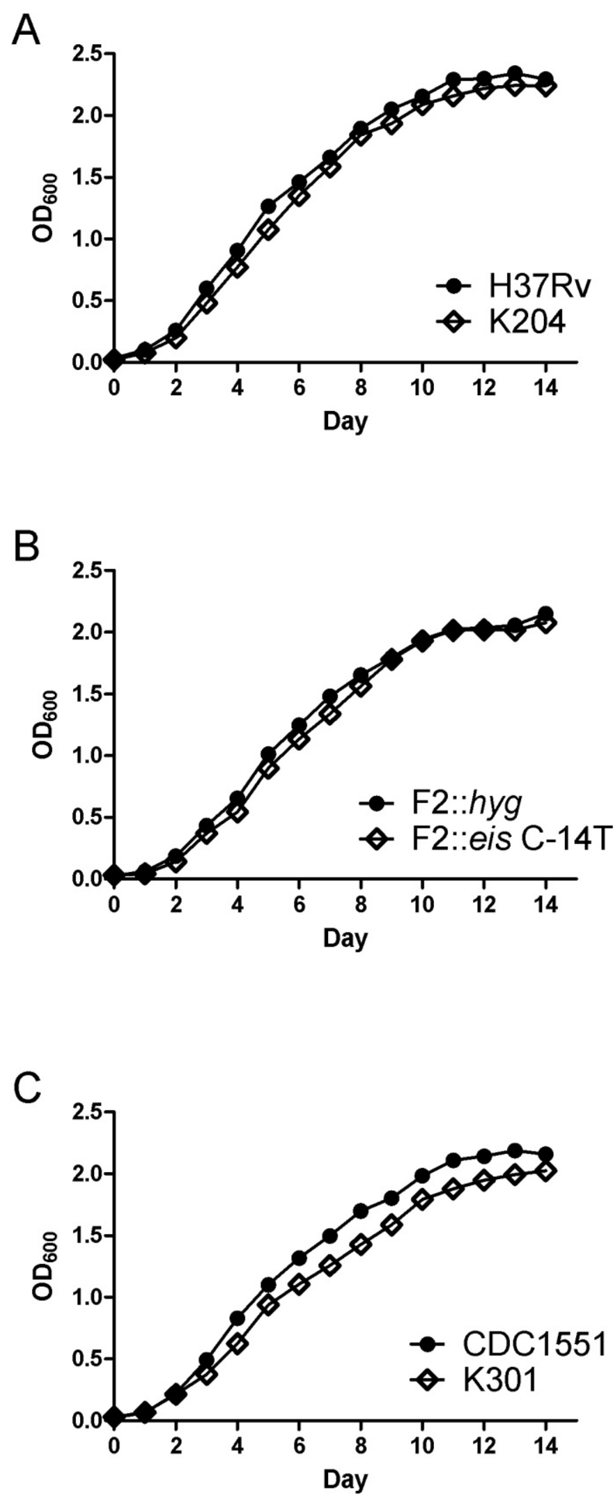
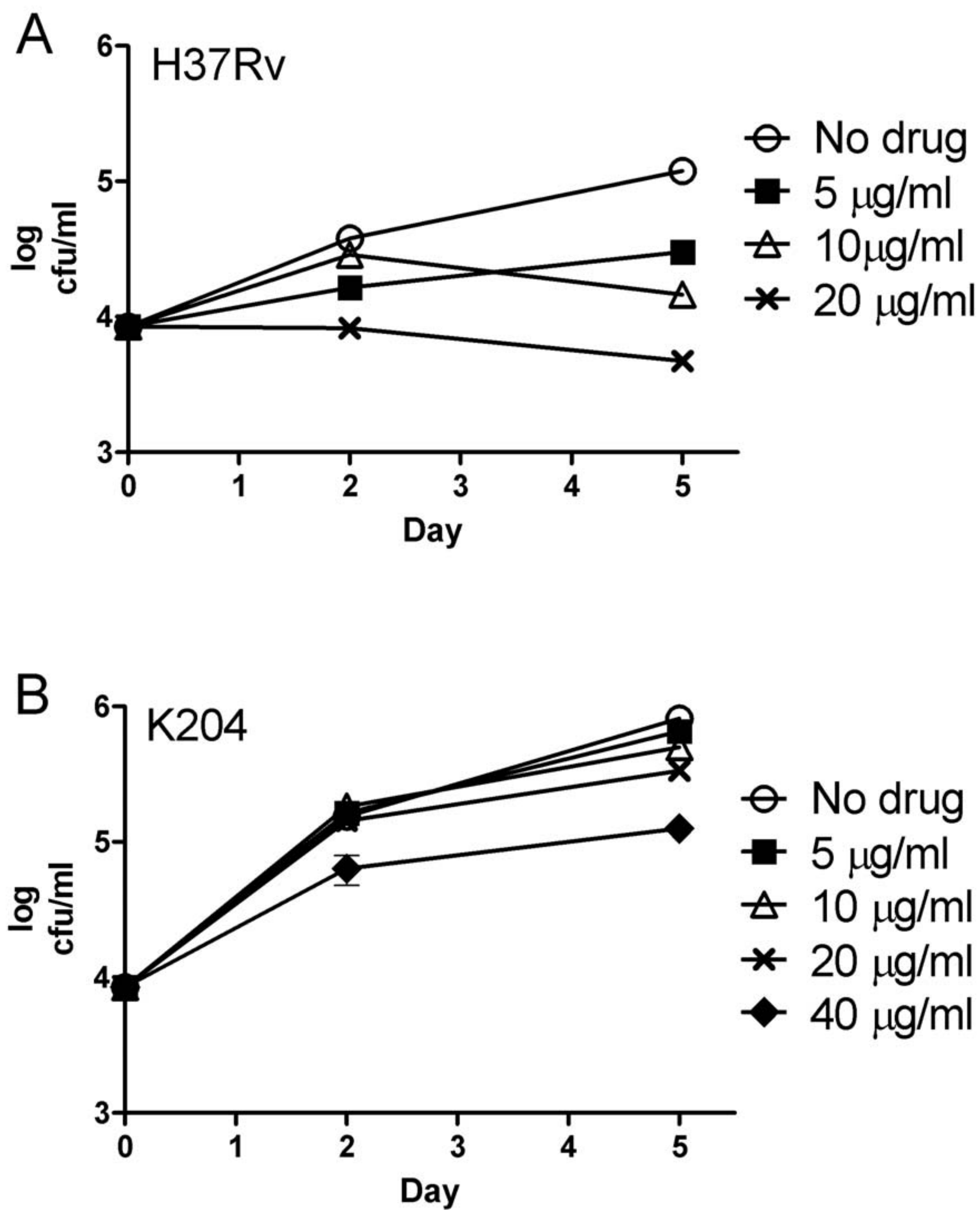


Figure 4



Chapter 5

CONCLUSION

Mycobacterium tuberculosis (MTB) infects one third of the world's population and is considered the world's most successful pathogen[1]. The emergence of multi-drug resistant (MDR) variants of MTB has fueled the spread of the disease and complicated control efforts[1]. Second line antibiotics, such as the aminoglycosides kanamycin (KAN) and amikacin (AMK) are recommended to treat MDR-TB strains[3, 4]. However, as resistance arises to second line drugs, an even more dangerous and nearly untreatable form of MTB develops; extensively drug-resistant (XDR) TB[4, 5]. KAN resistance is a defining characteristic of XDR TB[4], yet prior to this study, little was known about the molecular mechanisms of KAN resistance in *M. tuberculosis.* Earlier investigations established that high-level KAN resistant isolates often harbor mutations in the 16S ribosomal RNA (*rrs*), and display cross resistance to AMK and capreomycin (CAP)[6]. However, the existence of low-level mono-KAN resistant strains was acknowledged in several reports and suggested that a mechanism of KAN resistance existed that was independent of the *rrs* mutations[7-9]. Given that low-level KAN resistance is reported in up to 80% of KAN resistant isolates, understanding this mechanism of resistance is an important question[9]. The purpose of this study was to determine the mechanism(s) of low-level KAN resistance in *M. tuberculosis*.

Chapter 2 of this study describes the identification of *eis*, an aminoglycoside acetyltransferase that acetylates and inactivates KAN. Various molecular studies confirmed that *eis* promoter mutations cause enhanced *eis* expression and confer low-level KAN resistance in spontaneous mutants and 80% of clinical isolates surveyed. An important observation was that *eis* mutants remain susceptible to AMK under current Clinical Laboratory Standards Institute (CLSI) recommendations (MIC $\geq$ 4 μg/ml). This

finding challenges the common assumption that cross-resistance between KAN and AMK is complete[5].

The difference between mono-KAN resistance and cross-resistance in clinical isolates is beginning to be acknowledged and addressed. The Clinical Laboratory Standards Institute (CLSI) standards previously encouraged the use of KAN as the class representative for drug susceptibility testing for both KAN and AMK resistance in *M. tuberculosis*[10]. This year, the CLSI is updating their recommendations for aminoglycoside susceptibility to suggest testing each drug individually. Individual drug resistance results should distinguish between strains that are completely cross resistant to KAN and AMK (such as *rrs* mutants) and those that are mono-KAN resistant (such as *eis* mutants), hopefully leading to more catered drug regimens for patients. However, it should be noted that the data presented in chapter 4 suggests that AMK may not be a suitable treatment alternative for *eis* mutants. Instead, this study suggests that the current CLSI recommendation for determining AMK resistance at a concentration of 4 μg/ml may be too high. Decreasing the concentration of AMK used to determine resistance may be necessary in the future to incorporate *eis* mutants.

Chapter 2 also proposed using the *eis* promoter region as a molecular marker of KAN resistance. Since this study, the Mycobacteriology Laboratory Branch (MLB) of the Centers for Disease Control has incorporated the *eis* locus into a Molecular Detection of Drug Resistance (MDDR) service that is offered to Public Health laboratories across the United States. This service uses PCR and sequencing to determine whether any mutations associated with drug resistance are present in clinical isolates. The MDDR service examines eight different loci including *rpoB, katG,* and *inhA* which detect MDR-

TB isolates. To date, 136 isolates have been submitted to the service and 2 *eis* mutations have been identified; one G-10A mutation (from Belgium) that was phenotypically KAN resistant and a novel C-26T mutation (isolated in New Mexico) that was KAN susceptible (personal communication). Additionally, as a precursor to launching the MDDR service, the MLB recently completed a large-scale survey of drug resistant clinical isolates examining which mutations correlate with drug resistance. The *eis* promoter region was included in this survey. Of 314 clinical isolates examined, 111 were resistant to KAN, and 38 (34%) had *eis* mutations. Additionally, several other laboratories across the world (for example, in Latvia) have now documented *eis* mutations in clinical isolates, including at least two novel mutations that occur at the -8 and -15 position in the promoter (personal communication). Whether or not these mutations contribute to KAN resistance needs to be determined experimentally.

As a follow-up of the initial study, chapter 3 describes another previously undescribed mechanism of KAN resistance in *M. tuberculosis* based on the regulation of *eis* expression. Mutations in the promoter region of the *whiB7* transcriptional activator lead to enhanced expression of genes in the *whiB7* regulon, including *eis*. The increased *eis* expression confers KAN resistance in a manner similar to *eis* promoter mutants, but with an unexpected additional consequence: low-level cross resistance to the aminoglycoside, streptomycin (STR)[12]. This study identified both *whiB7* and the *tap* efflux pump as novel loci associated with STR resistance. Overexpression of *tap* in *whiB7* mutants likely causes enhanced efflux of STR from the cell which leads to resistance. This is the first confirmed case of an efflux pump contributing to clinically relevant antibiotic resistance in a naturally occurring MTB strain and supports the long-

standing hypothesis that efflux pumps are involved in anti-TB drug resistance. At this time, the clinical implications of *whiB7* promoter mutations are not clear and may only play a minor role in aminoglycoside cross resistance in MTB.

Another observation from this work is that treatment with certain antibiotics can cause unintended additional consequences that may need to be considered when TB treatment regimens are designed. Low-level STR resistant isolates that harbor either a *whiB7* or *gidB* mutation quickly progress to high-level STR resistance. Also, KAN resistant *eis* mutants demonstrate enhanced survival in macrophages and possibly increased transmissibility. These inadvertent consequences may greatly affect the outcome of an infection. In the case of KAN, the studies here certainly question the utility of a drug that selects for mutants with two phenotypes: drug resistance and increased fitness. Additionally, the *whiB7* system responds to ribosomal stress by upregulating genes that specifically alleviate this stress through drug extrusion or inactivation. Similarly, the *iniBAC* system of MTB is believed to induce antibiotic tolerance to isoniazid and ethambutol[13]. These data show links between stress response systems and antibiotic resistance and introduces the possibility that treatment with drugs can lead to inducible antibiotic resistance. Whether these stress response systems contribute to drug resistance development *in vivo* remains an unanswered, but important question.

A mutual interest in the role of *whiB7* in antibiotic resistance, stress response, and MTB pathogenesis has initiated a collaboration with Kyle Rohde, PhD in the Russell laboratory at Cornell University. Possible future collaboration projects include microarray analysis of *whiB7* knockouts and over-expressing strains in the presence of

antibiotics in either broth or in macrophages and more detailed examination of growth kinetics of these strains in macrophages and the mouse model.

Overall, this study identifies two genes involved in kanamycin resistance and a novel mechanism of streptomycin resistance. The data from these studies may influence treatment strategies for patients with MDR-TB. Indeed, the *eis* promoter region has already begun to be utilized as a molecular marker of kanamycin resistance. Finally, this study has also revealed that a single protein involved in drug resistance can also affect MTB stress response and macrophage growth, illustrating that antibiotic resistance is intimately linked to bacterial physiology and pathogenesis.

REFERENCES

1. Hingley-Wilson, S. M., Sambandamurthy, V. K. & Jacobs, W. R., Jr. Survival perspectives from the world's most successful pathogen, Mycobacterium tuberculosis. Nat Immunol 4, 949-55 (2003).
2. Via, L. E. et al. Polymorphisms associated with resistance and cross-resistance to aminoglycosides and capreomycin in Mycobacterium tuberculosis isolates from South Korean Patients with drug-resistant tuberculosis. J Clin Microbiol 48, 402-11.
3. American Thoracic Society. Centers for Disease Control and Prevention. and Infectious Diseases Society of America. in American Journal of Respiratory and Critical Care Medicine 603-62 (2003).
4. World Health Organization. Anti-Tuberculosis drug resistance in the world, Report No. 4. WHO/HTM/TB/2008.394 (2008).
5. Matteelli, A., et al. Multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis:* epidemiology and control. Expert Rev Anti Infect Ther 5, 857-71 (2007).
6. Maus, C. E., Plikaytis, B. B. & Shinnick, T. M. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 49, 3192-7 (2005).
7. Suzuki, Y., et al. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. J Clin Microbiol 36, 1220-5 (1998).

8. Taniguchi, H., et al. Molecular analysis of kanamycin and viomycin resistance in *Mycobacterium smegmatis* by use of the conjugation system. J Bacteriol 179, 4795-801 (1997).

9. Kruuner, A. e. a. Discordant resistance to kanamycin and amikacin in drug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 47, 2971-3 (2003).

10. National Committee for Clinical laboratory Standards. Susceptibilty Testing of *Mycobacteria, Norcardiae,* and other aerobic actinomycetes. Approved Standard M24-A.

(Wayne, Pa, 2003).

11. Maus, C. E., Plikaytis, B. B. & Shinnick, T. M. Mutation of *tlyA* confers capreomycin resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 49, 571-7 (2005).

12. Johnson, R. et al. Drug resistance in Mycobacterium tuberculosis. Curr Issues Mol Biol 8, 97-111 (2006).

13. Colangeli, R. et al. Transcriptional regulation of multi-drug tolerance and antibiotic-induced responses by the histone-like protein Lsr2 in *M. tuberculosis*. PLoS Pathog 3, e87 (2007).