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ROLE OF SigE IN *M.TUBERCULOSIS* DRUG SUSCEPTIBILITY AND PERSISTENCE

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Summary

M.tuberculosis (*Mtb*) is one of the most important pathogens that infect humans and it is the causative agent of tuberculosis (TB), a disease that dates back to ancient times.

The World Health Organisation (WHO) estimates that more than one third of the total whole population is infected with *Mtb* and each year about 1,5 millions people die from tuberculosis.

Two of the main problems in *M. tuberculosis* control are the lengthy treatment and the enormous reservoir of latent carriers.

A common explanation for the need of lengthy treatment is that all or part of the population enters a nonreplicating persistent state characterized by some degree of dormancy. These are called “dormant” or “persistent” cells. This phenomenon, whose physiology is not well known, is very important for the treatment of infections, given the fact that dormant cells are resistant to many drugs.

The aim of this study was to find new potential molecular targets that can be used to enhance the effectiveness of traditional antibacterial drugs, in order to develop new therapies effective against this population of non-replicating cells.

Sigma factors (σ) regulate gene expression by binding to the RNA polymerase core enzyme and they could be important targets of new therapeutic approaches in the future, as some evidences recently showed they may play an essential role during *Mtb* persistence.

In this work, we focused on one of these sigma factors, SigE, that was previously described to have a role in response to the different types of stresses bacteria encounter during infection, such as heat shock, surface and oxidative stresses (Manganelli et al., 2001).

Using a *sigE null* mutant and through a series of different molecular and phenotypic investigations, we demonstrated how SigE plays a crucial role in response to stresses caused by different classes of antimicrobials. Furthermore, we observed a strong decrease in persister cells production when the *sigE* mutant was exposed to high concentrations of antibiotics. Finally, we tested other sigma mutants, to investigate if such a feature was shared across sigma factors. Although we demonstrated *sigB*

(which expression is controlled by *sigE* under stress conditions) involvment in persisters production, the vast majority of the features we described in this work were unique of the SigE sigma factor.

To conclude, with this work we were able to demonstrate how SigE may be a valid molecular target to develop new effective therapies against *M.tuberculosis* infections.

Sommario

M.tuberculosis (*Mtb*) è uno dei patogeni più importanti che infettano gli essere umani ed è l'agente eziologico della tubercolosi (TB), una malattia che risale a tempi antichi.

L'Organizzazione Mondiale della Sanità (OMS) stima che più di un terzo dell'intera popolazione mondiale sia stata infettata da *Mtb* e che ogni anno circa 1,5 milioni di persone muoiano di tubercolosi.

Due dei principali problemi nel controllo di questa malattia sono l'enorme serbatoio di portatori latenti e la lunghezza del trattamento terapeutico.

Una spiegazione condivisa relativamente alla necessità di un trattamento terapeutico prolungato è che la totalità o una parte della popolazione batterica, nel corso di un'infezione, entri in uno stato non replicativo, caratterizzato da un certo grado di "dormienza". Queste cellule quiescenti sono chiamate "persisters".

Questo fenomeno, la cui fisiologia non è ben nota, è molto importante per il trattamento delle infezione, in considerazione del fatto che le cellule dormienti sono tolleranti ad alte concentrazioni di molti farmaci e perciò non vengono da essi uccise.

Lo scopo di questo studio è stato pertanto quello di trovare nuovi potenziali bersagli molecolari che possano essere utilizzati per migliorare l'efficacia dei trattamenti terapeutici tradizionali, al fine di sviluppare nuove terapie efficaci contro questa popolazione di cellule persistenti.

I fattori sigma (σ) regolano l'espressione genica legandosi all'apoproteina polimerasica e, in futuro, potrebbero essere importanti bersagli molecolari di nuovi approcci terapeutici, in quanto alcuni recenti studi hanno dimostrato come essi potrebbero avere un ruolo chiave nel fenomeno della persistenza di *M.tuberculosis*.

In questo lavoro ci siamo concentrati su uno di questi fattori sigma in particolare, SigE, che era stato descritto in precedenza nel giocare un ruolo di primaria importanza nella risposta ai diversi tipi di

stress che *Mtb* incontra durante un'infezione, come shock termico, stress di superficie e ossidativo. (Manganelli et al., 2001).

Utilizzando un mutante di delezione per *sigE* e, attraverso una serie di indagini molecolari e fenotipiche, siamo stati in grado di dimostrare come SigE rivesta un ruolo cruciale nella risposta ai diversi tipi di stress causati dall'esposizione a differenti classi di farmaci antibatterici. Inoltre, abbiamo osservato una forte riduzione nella produzione di cellule "persister" quando il mutante *sigE* è stato esposto ad elevate concentrazioni di farmaci antimicrobici.

Infine, per verificare se queste caratteristiche fossero condivise tra i fattori sigma, abbiamo testato altri mutanti per differenti fattori sigma. Nonostante siamo stati in grado di dimostrare che anche *sigB* (la cui espressione è controllata da *sigE* in condizioni di stress) sia coinvolto nella produzione di cellule persistenti, tuttavia la maggior parte delle caratteristiche molecolari e fenotipiche che abbiamo descritto in questo lavoro sono attribuibili al solo fattore SigE.

Per concludere, con questo lavoro siamo stati in grado di dimostrare come SigE possa essere un valido bersaglio molecolare per lo sviluppo di nuove terapie efficaci contro le infezioni causate da *M.tuberculosis*.

INTRODUCTION

Mycobacterium tuberculosis: an overview

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), that continues to reign as one of the world's deadliest human diseases. According to the data provided by WHO (WHO, 2014), in 2013, 9 milions of new TB cases have been estimated (5,7 milions of newly diagnosed TB cases notified) and about 1,5 milions of people died from TB. Furthermore, while TB incidence, mortality and prevalence have been falling globally over the last years, it is estimated that one-third of the world's population has been infected with TB.

TB is caused by members of the *Mycobacterium tuberculosis* complex (MTBC), which includes: *Mtb*, the etiologic agent of TB in humans; *M. africanum*, that causes TB in humans only in certain regions of Africa; *M. bovis*, *M. caprae* and *M. pinnipedii*, causing TB in wild and domesticated mammals; *M. microti*, that causes TB in voles. The MTBC members are genetically extremely closely related and the genome of *M. tuberculosis* shows <0.05% difference with that of *M. bovis.*

Tuberculosis is one of the most prevalent human infections and remains a formidable public health challenge that shows little sign of abating. The current TB epidemic is being sustained by two important factors: the human immunodeficiency virus (HIV) infection and the selection of *Mtb* strains resistant to the most effective (first-line) anti-TB drugs. (WHO, 2009). Other contributing factors include population expansion, poor case detection and cure rates in impoverished countries, active transmission in overcrowded hospitals, prisons and other public places, migration of individuals from high-incidence countries due to wars or famine, drug abuse, social decay and homelessness. (Suhail A., 2011)

Generally, TB therapy is based on the combination of four drugs, rifampicin, isoniazid, ethambutol (EMB) and pyrazinamide (PZA) for 2 months, followed by rifampicin and isoniazid for 4 months. (WHO, 2013) Unfortunately, poor patient compliance favored the selection of Multi-Drug-

Resistant (MDR)-TB strains that require at least 20 months of treatment with second line drugs (fluoroquinolones, amikacin, kanamycin and capreomycin), more toxic and less efficient. (Lienhardt C. et al., 2012)

MDR-TB are strains resistant to the most potent first-line drugs, rifampicin and isoniazid. In 2012, 450,000 people developed MDR-TB around the world. It is estimated that about 9.6% of these cases were extensively-drug-resistant (XDR-TB), showing additional resistance to at least one fluoroquinolone and one injectable drug (amikacin, kanamycin or capreomycin) (WHO 2013; Green K.D. et al., 2013). For patients affected by XDR-TB, the therapeutic efficacy is quite limited.

Recently, a few reports have claimed about the emergence of "totally drug-resistant TB" with no chance of cure. (Sotgiu G. et al., 2013; Elayati A. et al., 2009)

Taking into account all these factors, one of the most important challenges today is to find new antimicrobial therapies useful to both shorten the treatment regimen and to be active against the MDR and XDR-TB strains.

Hystory of the disease

Tuberculosis is an infection disease whose origins date back to ancient times. First traces of tuberculosis were found in some skeletons of the Neolithic age (400 BC) as well as in Egyptian mummies (3000 BC) and South America (5000 BC). This disease, well known in the Greco-Roman world, where it was called as "phthisis" or "consumption". (Grmek M.D., 1983) At that time, this condition was considered incurable and hopeless, especially if the illness was contracted at a young age.

During the Middle Ages and the Renaissance, the high prevalence of other infectious diseases (for example, leprosy and bubonic plague) reduced the interest of physician towards tuberculosis that seemed to disapper from the European continent (Daniel T.M., 2006).

Between 1820 and 1830s, the dramatic rise of TB in the Western countries led European physicians and scientists to seek new remedies to fight this infectious disease. New chemical compounds and old herbal preparations were often used simultaneously in the nineteenth-century pharmacopoeia, such as cod liver oil, chest massages with acetic acid and cantharidin tincture, pills of copper sulfate, morphine. (Riva M.A., 2014) Physicians also recommended the inhalation of vapors of tar, iodine, chlorine, hemlock, turpentine, prussic acid and hydroiodate of potassium and other stimulant or sedative substances. (Tognotti E., 2012) These remedies were useful to relieve the symptoms of the disease, but a better nutrion and “fresh air” seemed to be the only efficacy methods to improve the lives of patients affected by tuberculosis.

It was only in the last part of the nineteenth century, with the development of clinical practice, medicine and laboratory research that etiology and pathogenesis of this disease have begun to be clarified.

Introduction of the term “tuberculosis” by Johann L Schonlein (1793–1864), diffusion of Laennec’s stethoscope and institution of the first hospitals entirely devoted to consumptives, all contributed to better clinical practices needed to cure patients affected by TB.

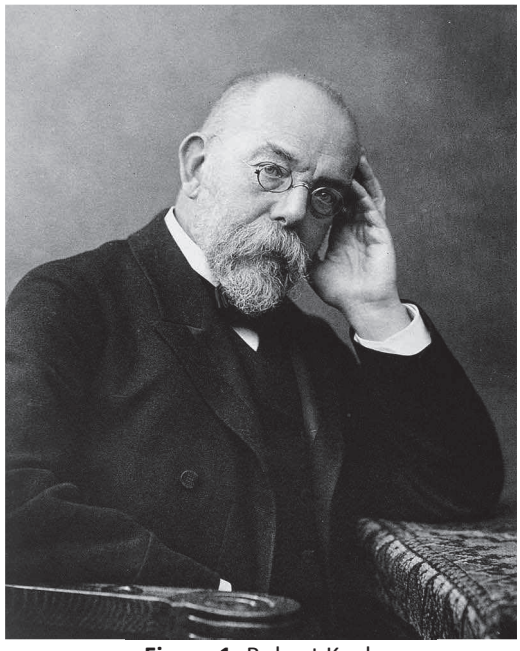

Figure 1: Robert Kock

In 1865, a French military surgeon, Jean-Antoine Villemin (1827–1892) was the first to prove that tuberculosis is an infectious disease by inoculating a rabbit with purulent material from an infected individual. But it was in 1882 when Robert Koch (1843–1910) conducted a series of experiments leading him to shed light on the etiology of the disease and formulating the "Koch's postulates" that the history of tuberculosis and its treatment changed dramatically. (Cambau E. et al., 2014) He was able to isolate

Mtb, demonstrating in the guinea pig that the same mycobacterium is the causative agent of tuberculosis in humans. (Koch R., 1882)

In 1890, during the Tenth International Medical Congress in Berlin, Kock announced to have found a "substance" able to inhibit the growth of tubercle bacillus in laboratory cultures and guinea pigs. It was a glycerin concentrate extract of tubercle bacilli, called by the German bacteriologist 'tuberculin' and renamed by contemporary mass media as "Koch's lymph". (Friedman M. et al., 1998) Unfortunately, as injection of this substance came into vogue for treatment of tuberculosis, it was shown to be ineffective. (Sbarbaro J.A. et al., 1997)

Even today, tuberculin is an important diagnostic tool utilized as a screening test for the presence of latent tuberculosis, thanks to the contributions of Clemens von Pirquet (1874–1929) and Charles Mantoux (1877–1947) (Yang H. et al., 2012).

Following the failures of science and clinical practices in the fight against the disease, only a suggestion appeared still effective: individuals with tuberculosis needed high-quality assistance, bed rest and better nutrition. This led to the development of "sanatoria" that contributed in the control of the disease. Establishment of these structures was primarily linked to new theories on climatic benefits against tuberculosis. According to these theories "fresh air" and mountain climate would be preferred for the people affected with TB. With the apparent therapeutic success of these structures located in mountain resorts, "aerotherapy" became the standard of care for the treatment of tuberculosis. In these hospitals, the most common treatments were better nutrition and sunbathing (heliotherapy) that probably contributed to restore vitamin D deficiency, essential for macrophage function. Moreover, bed rest and moderate physical exercise would improve perfusion to areas of the lung most affected by cavitary tuberculosis. (Diacon A.H. et al., 2012)

However, despite some successes of the sanatorium approach, tuberculosis continued to be a problem for the society in mid-1900s, especially because of the deprivation and poor hygienic conditions due to

the two global wars. For this reason, alternative immuno-therapies and new preventive measures were experimented between 1920 and 1940.

In 1921, the BCG vaccine was first used in humans, being developed by the French bacteriologist Albert Calmette (1863–1933) and his colleague Camille Guérin (1872–1961), by sub-culturing virulent strains of *Mycobacterium bovis* for more than 10 years to obtain a mutant attenuated enough to be considered safe for use as a vaccine. (Hawgood B.J., 2007) Mass vaccination did not start until after World War II, but because efficacy against pulmonary tuberculosis appeared to be variable it was of limited utility as a preventive measure.

However, with industrialization of pharmacotherapy things began to change. In 1944 the first two drugs with clear effects against *Mycobacterium tuberculosis*, streptomycin and para-aminosalicylic acid (PAS), were discovered. Indeed, a famous clinical trial conducted in 1948 demonstrated a lower rate of streptomycin resistance in the streptomycin-PAS treated patients. (Medical Research Council, 1948; Fox W. et al., 1956) These results provided the first evidence of the importance of combination therapies to prevent drug resistance.

The next step was the discovery, in 1951, of the antitubercular activity of Isoniazid. After some initial skepticism, various clinical trials proved Isoniazid to be the most effective drug yet discovered. Addition of Isoniazid to Streptomycin and PAS (“triple therapy”) further reduced the rates of drug resistance and improved efficacy of treatment, although a 24 months period of therapy was still requested. (Medical Research Council, 1955)

In 1960s, reduction of treatment to 18 months was possible thanks to the substitution of PAS with Ethambutol, a much better tolerated drug, discovered by Lederle laboratories in 1961. (Doster B. et al., 1973)

In that decade research into antitubercular drugs continued leading to the discovery of a large number

of compunds today used as a second line therapy: viomycin, cycloserine and terizidone, kanamycin and amikacin, capreomycin and the thioamides ethionamide and prothionamide.

It was with Rifampicin, discovered in 1965 in Milan, by the research group headed by Pietro Sensi (1920 – 2013) and Maria Teresa Timbal (1925-1969), that the duration of the treatment was reduced from 18 to 9 months.

Finally, with pyrazinamide, synthetized by Dalmer and Walter in 1936 and rediscovered in 1972, the duration of treatment was reduced to 6 months when used in combination with Rifampicin and Isoniazid (short-course chemoterapy).

M. tuberculosis: a global human pathogen

In the last two decades, deciphering the TB genome allowed the reconstruction of *Mtb* as a global human pathogen (Cole S.T. et al., 1998). Historically, *Mtb* emerged as a human pathogen in Africa around 70.000 years ago and then spread out of the continent following human migrations (Gutierrez M.C. et al., 2005; Hershberg R. et al., 2006). It is hypothesized that ancient *M. tuberculosis* strains originated from enviromental mycobacteria. (Supply P. et al., 2013)

These ancient strains evolved to be able to persist in low density populations, causing disease following a long period of latent infection. (Blaser M.J. et al., 2007)

The introduction of agriculture, civilization and the increase in human population density in urban areas led to the selection of *Mtb* strains with enhanced virulence and transmissibility which are named “modern *Mtb* strains” (Wirth T. et al., 2008; Comas I. et al., 2013). These modern *Mtb* strains spread throughout the world causing the TB epidemics as we know it today.

Biology & Pathogenesis

Cell wall structure

The most important feature of mycobacteria is the distinguishing cell wall structure that represents a strong barrier against drugs and toxic compunds. The mycobacterial cell wall is composed of three distinct macromolecules: peptidoglycan, arabinogalactan and mycolic acids which are surrounded by a non-covalently linked outer capsule of proteins and polysaccharides. (**Figure 2**) The peptidoglycan layer surrounds the plasma membrane and comprises long polymers of the repeating disaccharide *N*-acetyl glucosamine– *N*-acetyl muramic acid (NAG–NAM) that are linked via peptide bridges. (Kieser K.J. & Rubin E.J., 2014) The high density of lipids in the cell wall prevents Gram staining and mycobacteria are known as acid-fast because they can be stained by acid-fast dyes such as the Ziehl-Neelsen stain. (Hett E.C. & Rubin E.J., 2008)

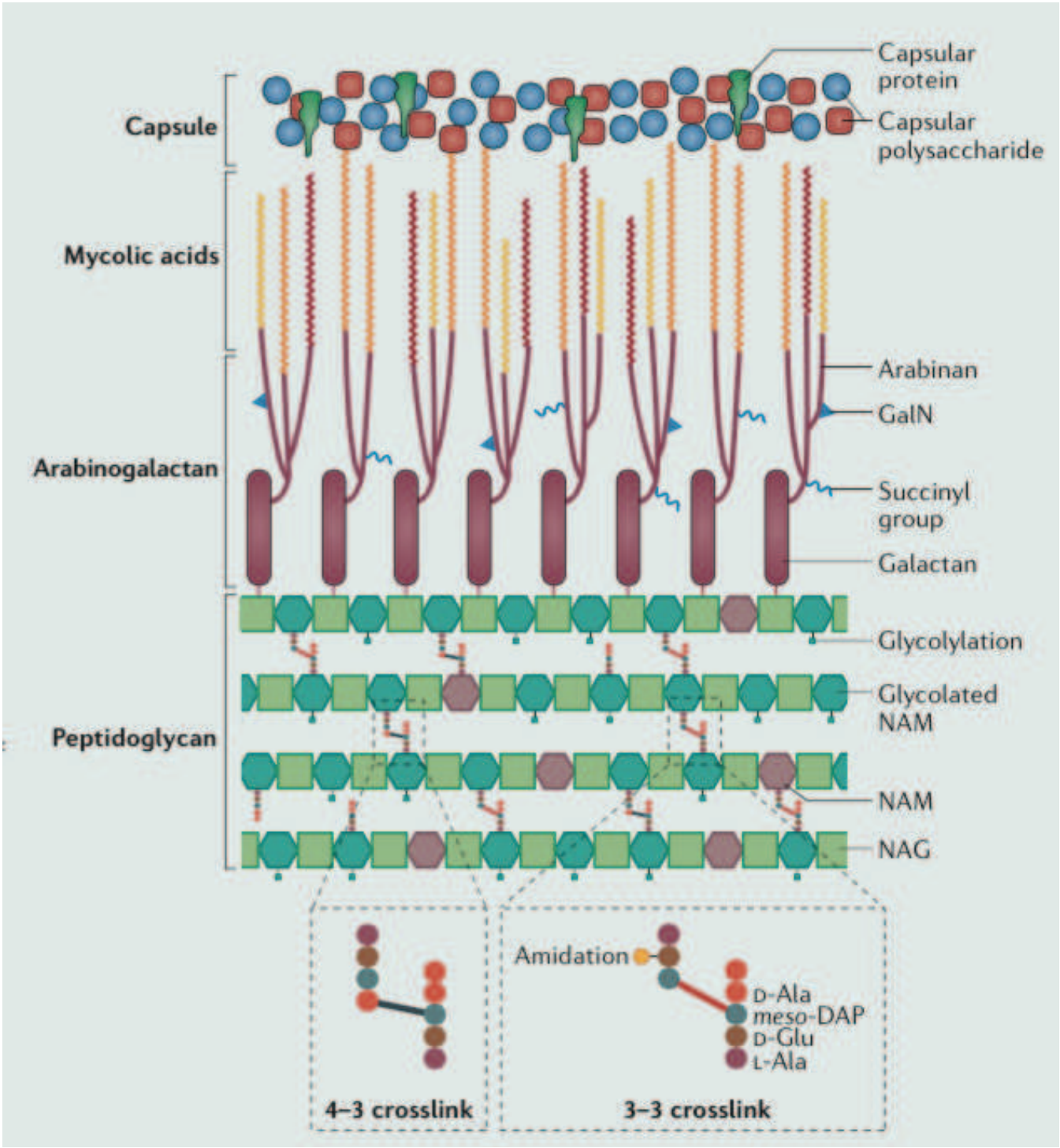

Figure 2: Representation of the cell wall structure of Mtb. It is formed by three most important macromolecules: peptydoglycan, which is surrounded by a layer of arabinogalactan and mycolic acids, which are surrounded by a capsule of proteins and polysaccharides. The peptidoglycan layer surrounds the plasma membrane and comprises long polimers of the disaccharide N-acetyl glucosamine – N-acetyl muramic acid (NAG–NAM) that are linked via peptide bridges. Compared with other bacteria, mycobacterial peptidoglycan is heavily crosslinked and most of it contains non-traditional 3-3 peptide crosslinks instead of 4-3 crosslinks. Arabinian is ligated with mycolic acids to form the characteristic waxy lipid coat that is a major contributor to impermeability of cell wall and virulence.

Mtb infection and macrophages uptake

M.tuberculosis is a slow growing aerobic-obliged bacteria with a replication time of 12-24h under optimal conditions at a temperature of 37°C. This last feature contributes to the chronic nature of the disease, requiring long times of treatment and representing one of the major obstacles to the laboratory research against this pathogen.

As a transferable disease, in tuberculosis the major source of infection are patients with pulmonary TB. Infection starts through inhalation of droplets nuclei, which are particle of 1-5 μm of diameter that contains *M.tuberculosis*, released in the air by patients affected by pulmonary TB, typically when the patient coughs.

Primary route of infection involves the lung. Because of the small size, droplets nuclei avoid the bronchus's defenses and penetrate in the alveoli where they are caught by paghocytics cells such as macrophages and dendritic cells.

Infection with *M. tuberculosis* starts with phagocytosis of the bacilli by phagocytic antigen-presenting cells in the lung including alveolar macrophages and dendritic cells. Recognition of *M.tuberculosis* and *M.tuberculosis* components occurs via different host receptors, called pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain- (NOD-) like receptors (NLRs), and C-type lectins (CD207, DC-SIGN and Dectin-1) (Harding C.V. & Boom W.H., 2010) that interact with specific pathogen-associated molecular patterns (PAMP) with different outcomes for the bacterium.

Macrophage infection

In early phases of infection, *Mtb* replicates inside the phagocytic cells.

The capacity to replicate inside macrophages is one of the most interesting features of *M. tuberculosis.* In fact*, Mtb* is able to change its behavior to adapt to the various environmental stimuli encountered

during macrophages infection. In a recent work, Rohde and colleagues (Rohde et al., 2012) used transcriptional profiling to characterize the behavior of *M. tuberculosis* during the infection process. Particularly, they showed that first days of infection were characterized by the induction of several regulons involved in stress response, among which are dormancy regulator DosR, the ECF sigma factor SigE, the heat shock regulator HspR, and genes belonging to the WhiB family as WhiB3 and WhiB7. Beyond those included in these regulons, other induced genes were involved in anaerobic respiration, Fe-S cluster assembly, propionate metabolism, regulation of acetate, glyoxylate cycle metabolism and starvation response. (Rohde et al., 2012)

After the first 5 days of infection, when the number of intracellular bacteria started to increase, a metabolic shift in *Mtb* took place and the induced genes in this phase included those encoding enzymes involved in fatty acid and cholesterol metabolism as well as genes encoding secreted antigens.

Inhibition of phagosome maturation

The alveolar macrophages, after entry of *M.tuberculosis*, produce cytokines and chemokines as a signal of infection. Monocytes, neutrophils and lymphocytes migrate to the site of infection, starting the formation of the granulome.

The strategy used by *M. tuberculosis* to survive and replicate inside macrophages is based on its ability to arrest phagosome maturation at the stage of early endosome, blocking vesicle fusion (Chen M. et al., 2006). This allows the bacterium to limit phagosome acidification, gain access to nutrients present in the endosomal compartment and to avoid the consequences of delivery to the lysosome. Many virulence factors were showed to be involved in this process and among others are worth mentioning (i) the protein tyrosine phosphatase (PtpA), a secreted protein that is able to inhibit both phagosome-lysosome fusion and the machinery responsible of phagosome acidification; (Wong D. et al., 2011) (ii) PE_PGRS62, whose expression in *Mycobacterium smegmatis* is able to block phagosome maturation;

(iii) Lipoamide dehydrogenase (LpdC), that induces phagosome maturation arrest (Deghmane A.E. et al., 2007); (iv) Nucleotide diphosphate kinase (Ndk), that causes a strong inhibition of phagosome maturation (Sun J. et al., 2010) ; (v) SecA2, an accessory secretion system required for intracellular growth in macrophages and for the arrest of phagosome maturation; (vi) Protein kinase G (PknG), secreted within the macrophage is able to inhibit phagosome-lysosome maturation; (vii) SigE, involved in stress response; (vii) Lipoarabinomannan (LAM), a glycoconjugate, able to inhibit T cell proliferation, to eliminate cytotoxic oxygen-free radicals, to inhibit protein kinase C and the activation of Interferon-γ at transcriptional level.

Following the macrophages invasion and the arrest of phagosome maturation, three other important mechanisms play a role in *M.tuberculosis* virulence: evasion from the phagosome, inhibition of autophagy and inhibition of apoptosis.

Evasion from the phagosome

Until recently, the paradigm of *M. tuberculosis* macrophage infection was that bacilli would reside inside the phagosome for the entire course of infection. However, recent findings showed that *M. tuberculosis* can escape from the phagosome gaining access to the cytoplasm of the infected cell with a process involving the type VII secretion system ESX-1. (Houben D. et al., 2012).

M. tuberculosis genome encodes four types of secretion systems (van der Woude et al., 2012): the conserved essential Sec system, the Twin-arginine translocase (Tat) export system, and two specialized secretion systems: the accessory Sec A2 pathway and the recently discovered ESX pathway (also called Type VII Secretion System, T7SS), which is only found in mycobacteria and some Gram-positive bacteria (Serafini A. et al., 2013)

M. tuberculosis has five type-7 secretion system (ESX-1 to ESX-5). ESX-1 is the most extensively characterized and it is required for the full virulence of *Mtb*. The major secretion targets of this secretion system are EsxA (ESAT-6) and EsxB (CFP-10), two small proteins forming an heterodimer. De Jonge and coll. (De Jonge M.I. et al., 2007) showed that at low pH these two proteins dissociate, allowing EsxA to associate and destabilize liposomes and so hypothesizing a direct role in phagosome rupture and *M. tuberculosis* escape in the cytosol.

Inhibition of autophagy

Autophagy is a higly conserved process, part of the innate immunity, that involves sequestration of cytosol regions with double-membrane vesicles which are delivered to lysosomes for degradation. It is an important process as it allows the immune system to eliminate intracellular pathogens such as *M.tuberculosis*.

Nevertheless, several mycobacterial components have been shown to interefere with this process and those include the glycolipid lipoarabinomannan (Shui W. et al., 2011), the type VII secretion system ESX-1 (Romagnoli A. et al., 2012) and the protein Eis (Shin D.M. et al., 2010).

Inhibition of apoptosis

While *M. tuberculosis* is able to induce apoptosis with an ESX-1- dependent mechanism, leading to the formation of *M. tuberculosis*-containing apoptotic bodies that can be engulfed by other macrophages facilitating the cell-to-cell spread (Aguilo J.I. et al., 2013), apoptosis can also boost the induction of *M. tuberculosis*-specific immune response, so that excessive apoptosis would be detrimental for the bacterium. (Shin D.M. et al., 2010) Consequently, *M. tuberculosis* has evolved at least two anti-apoptotic mechanisms enabling it to modulate this cellular process. The first one is based on NuoG, a subunit of the type I NADH dehydrogenase complex which is able to neutralize the reactive oxygen

intermediates (ROI) produced by NOX-2 resulting in the inhibition of TNF-α-mediated host cell apoptosis.

The second one is based on SecA2-dependent secretion of the superoxide dismutase SodA. Also in this case the mechanism is based on the efficient neutralization of ROI able to induce apoptosis.

Dissemination

M. tuberculosis can migrate from the primary site of infection to other sites trough the lymphatic system or the bloodstream. Apart from migration due to internalization in antigen-presenting cells, *M. tuberculosis* dissemination is also based on other mechanisms such as (Krishnan N. et al., 2010): (i) invasion of alveolar epithelial cells; (ii) translocation through the alveolar epithelium; (iii) translocation through endothelial cells; (iv) dissemination through professional phagocytes (pathogenic mycobacteria actively recruit macrophages at the site of infection and this recruitment is essential for the dissemination of the pathogen through the migration of infected macrophages to distant secondary sites of infection (Davis J.M. et al., 2009).

Formation of granuloma and latency

Once activated, the specific immune response produces primed T cells which migrate back to the focus of infection. Here, accumulation of macrophages, T cells, and other host cells (dendritic cells, fibroblasts, endothelial cells, and stromal cells) leads to the formation of granuloma. (Gonzalez-Juarrero M. et al., 2001)

Fibrotic components cover the granuloma that becomes calcified such that bacilli remain encapsulated inside and protected by the host immune response. At this point, the CD4+ cells producing IFN-*γ* recognize infected macrophages and kill them. Infection is halted.

In the vast majority of patients an effective cell-mediated immune response develops after 2-8 weeks of infection and it is enough to stop the bacilli replication.

However, in some individuals the pathogen, that, as previously described, has evolved different strategies to evade the immune response, is not completely eradicated and it is able to survive and persist in a non-replicating state in the host (LTBI) (Tufariello J.M. et al., 2003).

Some recent studies showed some differences in the immunological response from different individuals. These differences lead to the formation of physiologically different granulomatous lesions. Some of these suppress while others promote the persistence of viable *Mtb* cells in the granuloma (Rustad T.R. et al., 2009).

The microenviroment of the granuloma increases expression of several *M.tuberculosis* genes involved in dormancy induction. So, the dormant bacilli stay inside the granuloma during the lifetime of the host, but they can reactivate in case of immuno-depression.

A major corollary to this hypothesis was that reactivation of TB originated from this primary site of infection. However, in 2000, Hernandez-Pando et al. (Hernandez-Pando R. et al., 2000), demonstrated that in latent TB individuals *Mtb* bacilli can persists in cells and tissue not associated with the granuloma. Furthermore, other studies suggested that during LTBI *Mtb* can reside in different cells and organs (such as adipocytes), not associated to the site of primary infection and lacking any sign of typical granulomatous lesions.

Furthermore, recently it has been proposed that during latent infection most of the bacilli persist in a dormant state, while only some are found in an active replicating state. These replicating bacilli, called “scouts” are processed and killed by the host immuno-system and are responsible for the induction of the large number of effector/memory T cells against *Mtb* antigens found in the peripheral blood. (Chao M.C. & Rubin E.J., 2010) During latent TB dormant bacteria constantly replenish the bulk of actively replicating bacilli killed by the host. When the host immuno response, for whatever reason, fails to control these scouts the disease manifests again in the active form.

M. tuberculosis dormancy and latency

A key problem in facing *M. tuberculosis* control is the required lengthy treatment with multiple drugs for a period of at least 6 months, which makes adherence of patients to therapy really tough. This would in turn lead to an increased risk of emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains. In addition, *M. tuberculosis* has the almost unique ability to cause latent infection.

The prolonged treatment that is required to cure TB is thought to be due to the presence of "persister" bacteria refractory to antibiotic treatment.

While commonly "latency" refers to the clinical observation of infection without apparent disease, most of the research has been focused on a "dormancy" bacterial phenotype.

Dormancy has been defined as a state of nonreplication that is characterized by long-term viability despite metabolic downregulation. (Chao M.C. & Rubin E.J., 2010) Dormancy may contribute to two observations during human disease: a subpopulation of dormant cells may be responsible for 1) latency, 2) the slow response and high relapse rates seen upon antibiotic treatment of active tuberculosis.

Persisters

Persisters are a subpopulation of bacteria that resist to the action of antibiotics. They are genetically identical to susceptible bacteria and appear to be slowly growing or nonreplicating. Persisters are defined as phenotypic variants of wild-type (WT) strains. In fact, they have a non-heritable phenotypic resistance/tolerance to antibiotics, but their progeny remains fully susceptible to them upon regrowth (McDermott W., 1958).

Persisters were first described by Hobby, Meyer, and Chaffee in 1942 (Hobby G.L. et al., 1942), using a streptococcal culture, where they found that the highly bactericidal antibiotic penicillin only killed 99% of the bacteria leaving 1% of the population still alive.

Persister formation is considered a bacterial adaptation to stress conditions that the bacilli have to face both *in vivo* and *in vitro*, resulting in a quiescent state that allows this sub-population to not be killed by stress.

Persister formation take place through epigenetic factors in a stochastic manner (Veening J.W. et al., 2008) or in a deterministic way (Dorr T. et al., 2009).

They represent an heterogeneous and diverse population defined by specific conditions. (Li Y. & Zhang Y., 2007) In fact, persisters comprise different subpopulations and generally form a small and predictable portion of a culture. This proportion depends on several factors such as the age of bacterial culture, the type of antibiotics, antibiotic concentrations, length of antibiotic exposure, medium composition and aeration during antibiotic exposure, that can affect the level of persisters.

Persisters can be divided into those that can be resuscitated and propagated under standard culture conditions and those that cannot (VNBC).

Since the current and classical persister definition is based on growth in fresh medium often quantified via colony-forming unit assays (CFU assays), in which the number of bacteria growing on agar media or sometimes in liquid media is monitored, this definition excludes viable but not culturable bacteria (VNBC). For this reason a new definition to describe persister cells has been proposed: “persisters refer to genetically drug susceptible quiescent (non-growing or slow growing) organisms that survive exposure to a given cidal antibiotic or drug and have the capacity to revive (regrow or resuscitate and grow) under highly specific conditions” (Zhang Y. et al., 2014).

It is also important to distinguish “persister” phenomena from “persistence” in experimental infections. The first always relate to antimicrobial treatment while the latter may not. For example,

studies on the isocitrate lyases essential to the glyoxylate shunt and encoded by *icl1* and *icl2* demonstrate the importance of *icl1* for persistence in murine infection, and an increase in enzyme activity occurs on entry into nonreplicating persistence induced by hypoxia, but their expression has not been causally related to antibiotic tolerance. (Zhang Y. et al., 2012)

During the disease process, the tubercle bacilli reside in different enviromental conditions that include: different oxygen concentrations, nutrient starvation, oxydative stress, acidic Ph and more, all of which affect the metabolic status of the bacteria. This variety of conditions is the basis for the production of an heterogeneous bacterial population, including persisters and active bacteria with different capacities for persister formation and various sensibilities to antitubercular drugs.

A Ying-Yang model (**Figure 3**) has been proposed to describe this dynamic and heterogeneous bacterial population consisting of growing (Yang. In red) and non-growing (Yin, in black) cells that are in varying growth and metabolic states in a continuum.

Although persisters may be very rare in an actively growing log phase culture, when the growing population (Yang) reaches a certain age and density a small population of non-growing or slow-growing persister (Yin) can emerge and increase in numbers as the culture ages.

This population is heterogeneous and composed of various subpopulations with varying metabolic states *in continuum*. Persisters not killed by antibiotics could revert to replicating forms (revertants), which under appropriate conditions may have various degrees of recovery or reversion and cause relapse or prolonged infections (Zhang Y. et al., 2014).

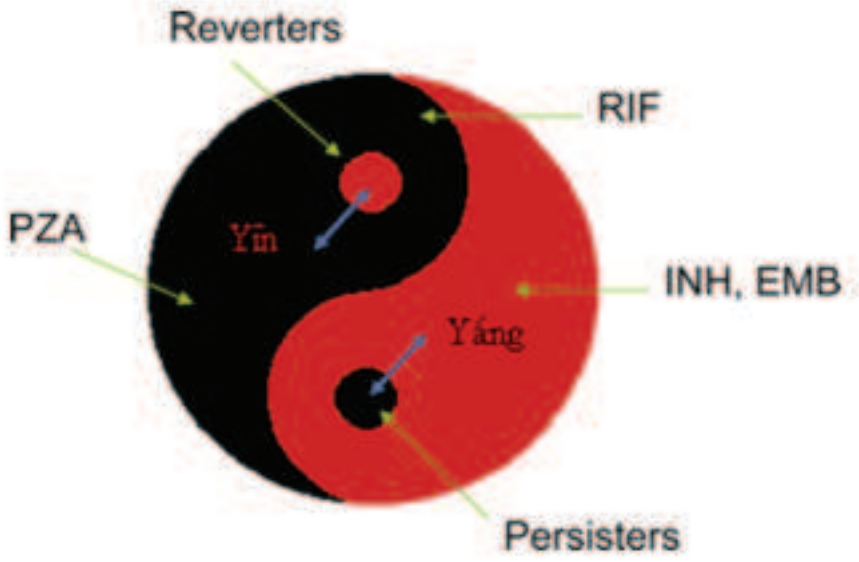

Figure 3: The yin-yang model depicts a dynamic bacterial population consisting of growing and nongrowing subpopulations in various metabolic states in a continuum. In the growing bacterial subpopulation (yang), there is a small proportion of nongrowing or slowly growing persisters (yin). As the bacteria enter stationary phase, more persisters form, with a small number of growing bacteria. The persister subpopulation is heterogeneous and consists of a continuum of various subpopulations as a result of stochastic or induced expression of persister genes. The yin-yang model is used to explain why, after a 2-month intensive-phase treatment with isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB), the remaining persister tubercle bacilli can revert to growing form which can still be killed by INH and RIF in the subsequent 4-month continuation phase of treatment. This model also explains why INH can be used for prophylactic treatment of latent TB infection, where tubercle bacilli revert from nongrowing form to growing form and become susceptible to INH. (Zhang Y. et al., 2012)

Persister phenomenon is present in virtually all bacterial species but the degree of persistence may vary among species and within species.

Furthermore, persisters pose significant challenges to the control of bacterial infections as they appear to be responsible of chronic and recurrent infection, biofilm infection and lengthy treatment.

While it is clear that *Mtb* is able to persist during chemoterapy, where *M.tuberculosis* cells reside in patients is still unclear. True persisters have been found in lesions, and candidate persisters have been demonstrated in adipose tissue (Neyrolles O. et al., 2006) and sputum (Garton N.J. et al., 2008). It is possible that persisters can reside in different locations intracellularly and extracellularly and in different cell types besides macrophages.

Mtb latency and dormancy models

Numerous *in vitro* models have been used to mimic latency and to investigate the adaptive processes that lead to TB dormancy. This has usually been obtained by limiting at least one of the conditions required for *Mtb* growth.

The most used models include: (a) starvation of essential nutrients, such as carbon, nitrogen, or phosphorus and (b) depletion of oxygen, which prevents aerobic respiration by the obligate aerobe. (For a full list of the latency/dormancy models see **Table 1**).

Adaptation to oxygen depletion is the most studied of these conditions. It is know that low oxygen levels limit *M.tuberculosis* replication and promote long-term survival, which is marked by bacteriostasis in addition to metabolic, chromosomal and structural changes of the bacilli. (Wayne L.G. & Hayes L.G. 1996)

Latency/Dormancy model	Advantages		Disadvantages	
Wayne model (*in vitro*, culture)	(a) (b) (c)	Can simulate different *Mtb* bacteria physiological dormant states Inexpensive and easy to carry out Easy for expressed genes profiling	(a) (b) (c)	Uses a single stress factor (hypoxia) Does not reflect what truly happens *in vivo* Slow simulation of dormancy
Rapid anaerobic model (*in vitro*, culture)	(a) (b) (c)	Inexpensive and easy to carry out Rapid simulation of *Mtb* dormant state Easy for expressed genes profiling	(a) (b)	Uses a single stress factor (hypoxia) Difficult to correlate with *in vivo* latency
Multi-stress model (*in vitro*, culture)	(a)	Easily achieve the stationary and non-replicating phases of *Mtb*	(a)	Difficult to correlate with *in vivo* latency

	(b) (c)	Not expensive and easy to carry out Easy for expressed genes profiling		
Cornell model (*in vivo*, mouse model)	(a) (b) (c) (d)	Inexpensive and easy to handle Availability of genetic variant strains Large number of immunological tools and reagents Can simulate latency/dormancy	(a)	Latency development does not compare well to human LTBI
Guinea pig/rabbit model (*in vivo*)	(a) (b)	Easy to handle Show necrosis and granuloma structure similar to humans	(a) (b)	Limited availability of reagents Lack of true latency which resembles human LTBI
Non-human primate model (*in vivo*)	(a) (b)	Similar immunological and infection pathology with humans Develop LTBI similar to humans	(a) (b) (c)	Expensive Require trained personnel Ethical issues

Table 1: Latency/Dormancy in vitro and in vivo models.

The Wayne Model:

Wayne and Hayes (1996) established an *in vitro* model where TB cultures were subjected to oxygen depletion to mimic the bacilli *in vivo*. In this model, a sealed standing colture of TB is left to incubate for days until the bacteria deplete the available oxygen. At this point, the colture becomes increasingly more hypoxic with a shift in the bacterial phisiology.

In this model two distinct states of non-replicating bacteria (NRP) are described: the first state, called NRP1, occurs when the declining in oxygen levels reaches the 1% of saturation. This state is characterized by a steady ATP generation and increased production of glycine dehydrogenase.

The second state, NRP2, occurs when the oxygen saturation reaches 0,06% and at this state a sharp decline of glycine dehydrogenase and sensibility to metronidazole is observed.

In the Wayne model, the dormant cells generated remain sensitive to heat shock and can be culturable when transferred to fresh aerated media. (Shleeva M. et al., 2010)

However, it is thought that the dormant state obtained with the Wayne model represents just the early state of a dormant cell and reflects bacterial adaptation to low-oxygen level rather than to the dormant state.

Other models to mimic the dormant, non-replicating state of the bacilli *in vivo* are available (see **Table 1** for a full list), each one with its one advantage and disadvantages, but the Wayne model because of its simplicity and affordability was the most used.

Persisters assay:

The current persisters assays consist of exposing bacterial cultures to bactericidal antibiotics concentrations for a different periods of time and then checking the number of surviving bacteria by CFU assays.

In some studies antibiotics are added directly to stationary phase cultures, while in other studies antibiotics are added after refreshing the culture or in fresh medium (Luidalepp H. et al., 2011).

There is a tendency in the field towards using short antibiotic exposure times in persister assays. It is important to highlight that while a short exposure time to antibiotics is sufficient to demonstrate persister's presence, it may not be sufficient to demonstrate persisters production defects in some

mutants, which become visible only after prolonged exposure times (Ma C. et al., 2010). In fact, the original studies by Hobby and Bigger (Bigger J.W. et al., 1944) used penicillin exposure times of 24-48h and even up to 3-11 days.

It is important to remember that *in vitro* persisters are not the same as *in vivo* persisters due to differences in the environments and the presence or absence of antibiotic exposure. Thus, a drug that can kill all *in vitro* persisters is not guaranteed to do so *in vivo*.

Molecular mechanisms involved in persisters production in TB

As described before, numerous *in vitro* models of *M.tuberculosis* survival in the face of lethal stresses have been developed.

Microarray analysis have identified numerous genes upregulated in nongrowing persister bacteria in the various models. Particularly, a recent study (Keren I. et al., 2011) on the trascriptome analysis of *M.tuberculosis* persisters derived from cycloserine treatment *in vitro,* identified shared downregulated genes and pathways, including energy- metabolism pathways and ribosomal proteins as already found in starvation and Wayne models. Particularly, this study revealed 56 upregulated persisters genes, including many toxin-antitoxin modules (TA), universal stress protein (UpA), SigF, anti-sigma factor RsbW, and DnaE2, with 5 genes belonging to the different persisters models illustrated before. These genes are: *acr2*, encoding the heat shock protein alpha-crystallin, *gntR*, encoding a transcriptional regulator, *pdhA*, encoding a pyruvate dehydrogenase component, Rv3290c (Lat) encoding an *l*-lysine-epsilon-amino- transferase, and Rv2517c encoding a hypothetical protein.

An *in vivo* gene expression study of persister bacilli in chronic TB in mice suggested that the persisters grow slowly and share some similarity to *in vitro* persisters. (Shi L. et al., 2005) It is of interest to note that genes required for persister formation *in vitro* may be different from those required *in vivo*, as demonstrated in the case of *relE*, overexpression of which contributes to persister formation *in vitro* but not *in vivo* (Singh R. et al., 2010).

Among all the studies, several mechanisms or pathways have been identified that may be involved in *M. tuberculosis* persisters formation or maintenance.

The functional areas involved include the following genes or pathways: (a) Energy-related pathways; (b) The stringent response regulator RelA (ppGpp synthase) (Dahl J.L. et al., 2003), which is important for persistence in *M. tuberculosis*, since its mutation caused significant defects in long-term survival *in vitro* and in mice; (c) The PhoU homolog PhoY2, a phosphate and cellular metabolism regulator, is involved in persistence, since its inactivation caused a defect in the persister phenotype as shown by reduced persister amounts upon rifampin (RIF) and pyrazinamide (PZA) exposure *in vitro*, as well as a defect in persistence in mice (Shi W. et al., 2010); (d) The target of the critical persister drug PZA has recently been shown to be RpsA (S1 protein), involved in *trans*- translation (Shi W. et al., 2011), which is required for persister survival under stress conditions (Thibonnier M. et al., 2008); (e) The protein degradation pathway mediated by proteasome PrcBA (Gandotra S. et al., 2007) has been identified as a persistence mechanism; (f) Elevated transporter activity, such as Mce4, is involved in cholesterol uptake which enriches persister (Pandey A.K. et al., 2008); (g) Mutation of the cell wall mycolic acid cyclopropane synthase PcaA led to decreased cording, persistence, and virulence in mice (Glickman M. et al., 2000); (h) Toxin-antitoxin (TA) modules have effects on bacterial antibiotic tolerance (Singh R. et al., 2010). TA modules are the most-studied persister-related mechanism in other bacteria, such as *Escherichia coli.* (Zhang Y. et al., 2012)

Toxin-antitoxin modules

Toxin/antitoxin (TA) modules are widely spread in bacteria. They consists of a stable toxin and of a labile antitoxin. It has been demonstrated that ectopic overexpression of the toxin RelE (an mRNA endonuclease) produces drug-tolerant persisters in *E.coli* (Keren I. et al., 2004). Similarly, ectopic expression of three *M. tuberculosis* RelE homologs also produces drug-tolerant cells (Singh R. et al.,

2010). TA modules are highly redundant: there are more than 20 of them in *E. coli* (Neeley W.L. et al., 2006; Rotman E. et al., 2007; Ward J.F. et al., 1987) and more than 65 in *M. tuberculosis* (TADB; http: //bioinfo-mml.sjtu.edu.cn/TADB/browse.php).

While persisters form stochastically, their numbers rise sharply as population density increases, reaching 1% in stationary-phase cultures (Keren I. et al., 2004). This indicates a deterministic component of persister formation. A deterministic component was recently identified in *E.coli* in a study of persister formation under conditions of DNA damage (Dorr T. et al., 2010; Dorr T. et al., 2009): fluoroquinolones induce the SOS response, including the LexA repressor. This protein was shown to regulate also the transcription of the TisAB TA module (Vogel J. et al., 2004). A knock-out mutant of *tisB* had a sharply reduced level of persisters, showing that they are mainly produced in a TisB-dependent manner when the SOS response is activated. TisB is an antimicrobial peptide that apparently forms an ion channel in the membrane, leading to a drop in proton motive force (PMF) and ATP levels, which would shut down important functions and lead to a dormant state (Dorr T. et al., 2010).

Persisters and multidrug tolerance

Persisters are tolerant to various bactericidal antibiotics, a property called multidrug tolerance (MDT). Although mechanisms involved in MDT are not clearly understood, recent studies have shown that there may be multiple mechanisms of MDT.

These mechanisms include reduced production in persisters of reactive oxygen species (ROS) influenced by the levels of antioxidant enzymes (Kim J.S. et al., 2011), inhibition of macromolecules by toxin-antitoxin modules (Gerdes K. et al., 2012), increased suppression of cellular metabolism mediated by PhoU (Li Y. et al., 2007) and the presence of defects in trans-translation pathway that confer a broad defect in MDT (Li Y. et al., 2007).

Although antibiotic tolerance in persisters is thought to be phenotypic, it is possible that under some conditions, antibiotic tolerant persisters may acquire mutations and develop genetic resistance. (Zhang Y. et al., 2014)

Persisters and Stress

Persisters are tolerant not only to antibiotics but usually to other stresses and, because of that, mutants are often tested to check their susceptibility to stresses in evaluating persisters-defective mutants. For example, *phoU* and *sucB* mutants defective in persisters production are susceptible to not only antibiotics but to a variety of stresses. (Li Y. et al., 2007)

It is also true that stressful condition can slow and inhibit bacterial growth facilitating persisters production. Nutrient depletion has been show to induce drug-tolerant persisters, as in the case of carbon starvation mediated persister formation that happens through activation of the ppGpp-SpoT metabolic TA module. However, persisters generated by transient nutrient depletion seem to lack the sustainable MDT phenotype of persisters generated in stationary-phase cultures. (Fung D.K. et al., 2010)

Heat, acidic Ph, oxidative stresses are all condition showed to induce persisters formation. Interestingly, bacterial persisters can tolerate antibiotics by reducing the number of hydroxyl radicals. Defects in the stringent response genes *relA* and *spoT* are known to cause decreased antibiotic tolerance but recently this phenotype has been shown to be mediated through reduced production of antioxidant defense enzymes such as superoxide dismutase and catalase. (Bink A. et al., 2011)

Of most importance for persisters formation is the inactivation of the enzymes involved in hydrogen sulfide (H_2S) production, that make the bacteria highly sensitive to various antibiotics due to the loss of H_2S antagonism on hydroxyl radicals generated by antibiotics. (Shatalin K. et al., 2011)

Low concentrations of antibiotics, such as ciprofloxacin which can cause reactive oxygen species and disrupt membrane potential via the *tisB* toxin, can induce persisters formation. (Dorr T. et al., 2009)

In general, the block of protein synthesis caused by the various stresses previously described, seems to correlate with persisters production. (Kwan B.W. et al., 2013)

Antibiotics and Oxidative stress

Current antimicrobial therapies fall into two general categories: bactericidal drugs, which kill bacteria with an efficiency of >99.9%, and bacteriostatic drugs, which merely inhibit growth (Li. J. et al., 2013).

The bactericidal antibiotic killing mechanisms are currently attributed to the class-specific drug-target interactions. Recently, however, new findings led to the identification of a new mechanism of killing, independent from drug-target interactions and common to all the major classes of bactericidal antibiotics. In fact, it has been demonstrated that the three major classes of bactericidal antibiotics (beta-lactams, aminoglycosides and fluoroquinolones) use a common mechanism of killing by stimulating the production of hydroxyl radicals in lethal quantities for the bacterial cells.

More in detail (**Figure 4**), all the various classes of antibiotics converge towards this common mechanism of killing via a metabolic response that involves the tricarboxylic acid cycle (TCA). The primary drug-target interactions stimulate oxidation of NADH via the electron transport chain that is dependent upon the TCA cycle. Hyper-activation of the electron transport chain induces a sharp increase in superoxide production. This event leads to the destabilization of the Fe-S intracellular clusters and the release of Fe^{2+} , the necessary substrate for the Fenton reaction to proceed. Activation of the Fencton reaction causes the release of hydroxyl radicals and thus cell death. (Pankey G.A. et al., 2004)

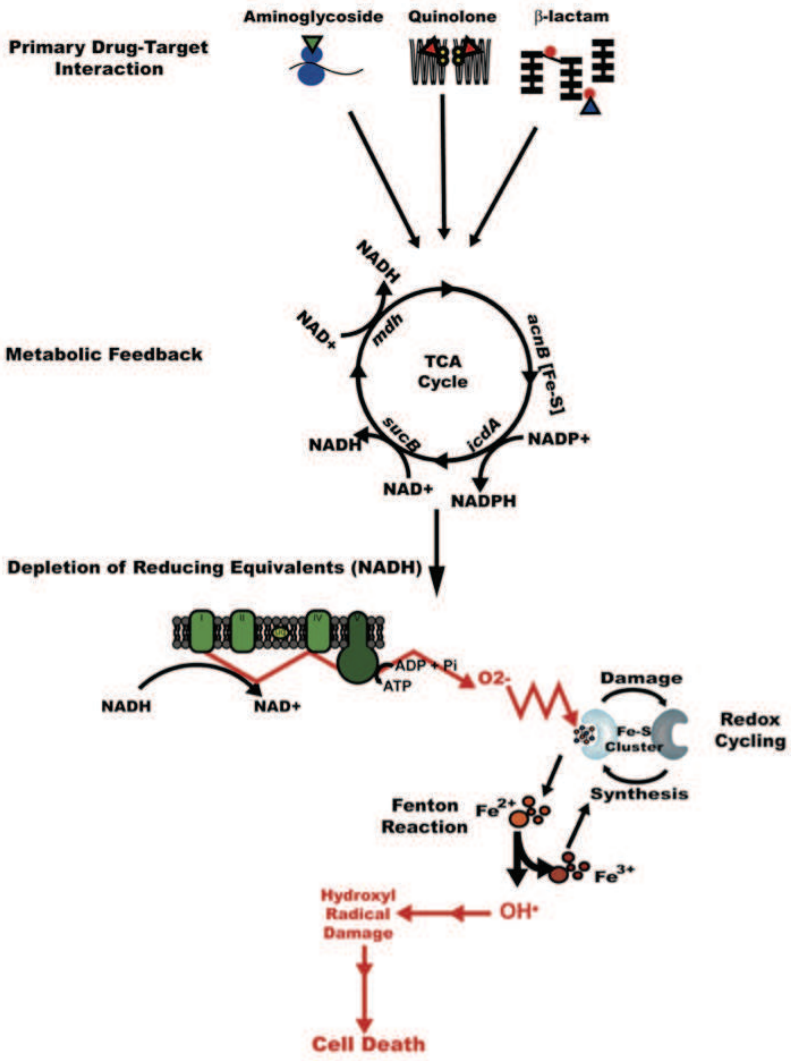

Figure 4: Antibiotics bind to their targets, disrupting normal cellular metabolism, including the tricarboxylic acid (TCA) cycle. This depletes intracellular NADH, which is paired with a commensurate rise in the production of reactive oxygen species such as peroxide and superoxide. These reactive oxygen species interact with Fe^{2+} via Fenton chemistry to generate highly toxic oxygen radicals, which react with DNA and proteins, resulting in death of bacteria. (Kohanski et al., 2007)

Oxidation of the guanine nucleotide pool

Elevated levels of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals (OH^*), within prokaryotic cells potentiate cell death. As stated before, because elevated intracellular levels of OH^* can damage DNA, lipids and proteins, generalized oxidation catastrophe could result in cell death. However, recently, J.Foti *et al.* (Foti J.J. et al., 2012) have found that cell death is predominantly elicited by specific oxidation of the guanine nucleotide pool and its subsequent use in nucleic acid transactions.

The nucleotide pool is an important target of ROS and guanine is particularly susceptible to oxidation because of its low redox potential (Haghdoost S. et al., 2006; Neeley W.L. & Essigmann J.M., 2006). One of the most intensively studied major products of guanine oxidation is 7,8-diihydro-8-oxoguanine (8-oxo-guanine) (Neeley W.L. & Essigmann J.M., 2006). Its deoxyribonucleotide, 8-oxo-dG, is potentially mutagenic because of its ability to form base pairs with both cytosine and adenine.

In *E.coli,* it has been shown that DinB, a translesion DNA polymerase, can use 8-oxo-deoxyguanosine triphospate (8-oxo-dGTP) as the incoming nucleotide, pairing it with either deoxycytidine or deoxyadenosine (dC or dA), with a preference for dA. So, DinB overproduction causes cell death by incorporating more 8-oxo-dG than the cells can tolerate, resulting in the generation of double strand breaks (DSB) and consequent activation of the SOS response mechanisms seen before.

A very important machinery used by cells to minimize the deleterious effects caused by the oxidation of the guanine nucleotide pool, is that of the GO system, composed by a set of three proteins: MutM, MutY, MutT.

It has been shown, in *E.coli*, that when MutT (which hydrolyzes 8-oxo-dGTP to 8-oxo-deoxyguanosine monophosphate dGMP) is co-overproduced, it minimizes the deleterious effects of oxidized guanine, eliminating the cytotoxicity related to DinB overproduction. (Foti J.J. et al., 2012)

Although the absolute levels of 8-oxo-dG present in DNA are too low to cause chromosomal fragmentation (Rotman E. & Kuzminov A., 2007), a change in the levels of 8-oxo-dGTP and/or a change in the ratios of the particular DNA polymerase operating in a cell could result in closely spaced 8-oxo-dG nucleotides. Closely spaced DNA lesions are potentially problematic, because the proximity of individual DNA lesions can alter the cell's ability to repair damage (Ward J.F. et al., 1987). Thus, in principle, incomplete base excision repair by MutM and MutY glycosylases acting at closely spaced dC:8-oxo-dG and dA:8-oxo-dG pairs, could result in the generation of a lethal DSB. DSB formation by

either of these mechanisms could be suppressed by MutT overproduction. In principle, the occurrence of a closely spaced dC:8-oxo-dG and 8-oxo-dG:C pair as a consequence of direct oxidation of DNA could also result in DSBs, but these would not be suppressible by overproduction of MutT.

To conclude, it has been demonstrated, in *E.coli,* through generation of *ΔdinB*, *ΔmutY, ΔmutT, ΔmutY, ΔrecA and ΔrecB* mutants that, in conditions where high amount of hydroxyl radicals are found inside the cells, like for example under stress caused by antibiotics, the combined action of these repair systems lead to an increase in the incorporation of 8-oxi-dG during DNA replication and therefore to the generation of lethal DSBs.

Furthermore, it has also been suggested that the cytotoxicity of aminoglicosides may result from erroneous translation due to the incorporation of 8-oxi-guanine in the newly synthesized RNA.

Metabolic pathways and persisters

Persistence in *M.tuberculosis* requires the coordinated expression of numerous virulence determinants, including those involved in intermediary and secondary metabolism, cell wall processes, stress responses and signal transduction pathways.

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A full list of genes which have been reported in literature to be involved in *M.tuberculosis* persistence are shown in **Table 2**. In the following paragraph we will focus, particularly, on two important sigma factors: SigE and SigB.

Gene	***Name***	**Function**	**Knockout/expression studies**
Rv0467	***icl***	Isocitrate lyase	Reduced persistence in mice
Rv1832	***gcvB***	Glycine decarboxylase	Increased expression during hypoxia
Rv1161-4	***narGHJI***	Nitrate reductase	Reduced persistence in mice
Rv1736c	***narX***	Fused nitrate reductase	Increased expression during hypoxia and in human granulomas
Rv1737c	***narK2***	Nitrate/nitrite transport	Increased expression during hypoxia
Rv0470c	***pcaA***	Cyclopropane synthase	Reduced persistence in mice
Rv0126	***treS***	Trehalose synthase	Prolonged time-to-death in mice
Rv0981	***mprA***	Two-component regulator	Reduced persistence in mice
Rv3132c	***dosR***	Two-component regulator	Increased lethality in mice
Rv3764/5c	***tcrY***	Two-component regulator	Increased lethality in mice
Rv1032c	***trcS***	Two-component regulator	Increased lethality in mice
Rv1027/8c	***kdpDE***	Two-component regulator	Increased lethality in mice
Rv3286c	***sigF***	RNA polymerase σ factor	Prolonged time-to-death in mice
Rv3223c	***sigH***	RNA polymerase σ factor	Reduced pathology, prolonged time-to-death in mice
Rv1221	***sigE***	RNA polymerase σ factor	Prolonged time-to-death in mice
Rv3416	***whiB3***	Transcriptional regulator	Reduced pathology, prolonged time-to-death in mice
Rv2583c	***relA***	(p)ppGpp synthase	Reduced persistence in mice
Rv2031c	***hspX***	α-crystallin	Increased expression during hypoxia and in mice
Rv0353	***hspR***	HspR repressor	Reduced persistence in mice

Rv0353	***hspR***	HspR repressor	Reduced persistence in mice
Rv0350	***hsp70***	Heat shock protein 70	
Rv1908c	***katG***	Catalase-peroxidase	Reduced persistence in mice

Table 2: List of the genes implicated in *Mtb* persistence.

Sigma factors

The *Mycobacterium tuberculosis* genome contains genes encoding 13 putative sigma factors. Sigma factors regulate gene expression by binding to the RNA polymerase core enzyme to confer different promoter specificity. Those encoded by *sigA* and *sigB* belong to the family of the principal sigma factors whilst others belong to the ECF (extracytoplasmic function) family and are widespread in several bacterial species.

Most of these sigma factors seem to be involved in regulating bacterial interactions with the extracellular environment, adaptation to stress and, in some cases, bacterial virulence.

Two of the most important sigma factors known to be involved in response to the various kind of stress *Mtb* has to face during infection and those on which we focused in this work are: SigE and SigB.

SigE, although highly conserved in mycobacteria, is not essential for in vitro growth (Jensen-Cain D.M. & Quinn F.D., 2001). Manganelli et al. (2001) defined the role of SigE in *M.tuberculosis* response to oxidative stress, testing the relative resistance of H37Rv (wild-type), ST28 (mutant) and ST29 (complemented) strains to various oxidative compounds such as hydrogen peroxide, cumene hydroperoxide (an organic peroxide) and plumbagine, using a disk diffusion assay on agar plates. They showed that the ST28 strain (mutant) was more sensitive than the WT to all the three compounds and that the WT phenotypes were restored in ST29 (complemented) strain (**Table 3**).

	H37Rv	ST28	ST29
H_2O_2 5 mmol[b]	2.0 ± 0.2[c]	2.5 ± 0.1	2.0 ± 0.1
Cumene hydroperoxide 350 nmol[d]	3.8 ± 0.1	4.5 ± 0.1	3.7 ± 0.2
Plumbagine 50 nmol	1.0 ± 0.07	1.5 ± 0.06	1.0 ± 0.06

Table 3: Sensitivity of H37Rv, ST28 and ST29 to various oxidative stresses. (Manganelli et al., 2001)

Furthermore, they showed that a *sigE* mutant was more sensitive than the wild-type to heat shock and surface stresses (Manganelli R. et al., 2001). Moreover, it was also defective in the ability to grow inside both human and murine inactivated macrophages and was more sensitive than the wild-type strain to the killing activity of activated murine macrophages.

SigE is a key element for *M.tuberculosis* stress response.

In a transcriptional profile of the *sigE* mutant a total of 38 genes showed a reduced level of expression and most of them were involved at various degrees in pathways related to the central metabolism. Among these, of great meaningfulness were those involved in electron transport (*ctaE, qcrA, qcrB*), acyl-CoA biosynthesis (*aceE and fadD29*), glutamine biosynthesis (*glnA1*) mycolic acid biosynthesis (*bpC2, fbpB, fabD* and *acpM*) and *sodA*, the gene encoding the superoxide dismutase.

SigB, closely related to the primary sigma factor SigA in terms of amino acid sequence, (Doukhan L. et al., 1995) is induced by different stresses (Manganelli R. et al., 1999) and is positively regulated by three extracytoplasmic function sigma factors, SigE, SigH and SigL (Dainese E. et al., 2006; Manganelli R. et al., 2002; Manganelli R. et al., 2001).

In *M. tuberculosis*, the response to surface stress is regulated by SigE and the response to oxidative stress and heat shock is regulated by SigH; SigB is a component of both regulons (Manganelli R. et al., 2002; Manganelli R. et al., 2001).

The fact that *sigB* expression is included in several regulatory pathways suggests that it may play a central role in the *M. tuberculosis* stress response.

Comparison of the transcriptional profiles of the *sigB* mutant and the wild-type strain after treatment with SDS revealed 72 genes downregulated in the *sigB* mutant. Among the genes downregulated in the *sigB* mutant were many genes related to the cell envelope stress response and also several encoding transcriptional regulators. The analysis of the transcriptional profile of the *sigB* mutant under oxidative stress induced by diamide treatment indicated 40 genes under SigB regulation (**Table 4**).

Type of stress	Category	No. of regulated genes
Cell envelope	Hypothetical proteins	25
	Cell wall-associated genes	20
	Regulatory proteins	13
	Intermediate metabolism	5
	Virulence, detoxification, adaptation	4
	Information pathways	4
	Lipid metabolism	1
	Insertion sequences and phages	1
Oxidative	Hypothetical proteins	15
	Intermediate metabolism	12
	Regulatory proteins	7
	Virulence, detoxification, adaptation	4
	Cell wall-associated genes	3

Table 4: distribution in functional categories of SigB regulated genes. (P.A. Fontan et al., 2009)

The complexity of gene regulatory networks in *M.tuberculosis* persistence

Most events that occur inside the cells require complex gene expression interactions and regulatory networks. Gene regulatory networks include complicated gene interactions and consist of many nodes (genes) and links among nodes (positive and negative regulation). A gene regulatory network based on the genes previously reported to be implicated in *M.tuberculosis* persistence is provided in **Figure 5**.

The network depicted in the figure indicates that sigma factors and two component systems may play an essential role during *Mtb* persistence. Two-component systems initially respond to various enviromental stresses within the granuloma. Histidine kinase sensors proteins transmit stress signals to cytoplasmic response regulators to control the expression of sigma factors which in turn mediate expression of other genes. The signal transduction pathway "*mprB/A-sigE-relA*" is a perfect example of this (Sureka K. et al., 2007). In this gene regulatory network there are several genes encoding PE/PPE/PE-PGRS protein family members which are required for virulence and persistence of *M.tuberculosis*. In addition, ion transport genes, such as nitrate/nitrite transport gene *narK2*, sulfate transport genes *cysA*, *cysW* and *cysT*, and phosphate transport gene *pstS1* are present.

There is also a large number of Isoniazid related resistance genes such as the *katG* catalase-peroxidase, an enzyme that converts isoniazid to the active form to inhibit the biosynthesis of the mycolic acid of cell wall and the *kasA* gene encoding the 3-oxoacy-(acyl carrier protein) synthase, a key enzyme involved in the biosynthesis of mycolic acid (mutations in the *kasA* gene are related to isoniazid resistance). (Wang X. et al., 2011)

Looking at the network, it is possible to note the presence of a complex regulatory network involving several sigma factors. For example SigE, SigH, and SigF are able to positively regulate *sigB*, while SigB auto-regulate/amplify the expression of its own structural gene suggesting that the complex interplay of sigma factors converges at sigB. The expression of other sigma factors is unchanged after *sigB*

induction, thus suggesting that SigB functions as an end regulator in the sigma factors nework. (Lee J.H. et al., 2008)

The deletion of *sigB* in *M. tuberculosis* results in higher sensitivity to SDS, heat shock, oxidative stress and hypoxic conditions and overexpression of *sigB* significantly upregulates *katG, kasA, whiB2*, and *ideR*. (Fontan P.A. et al., 2009)

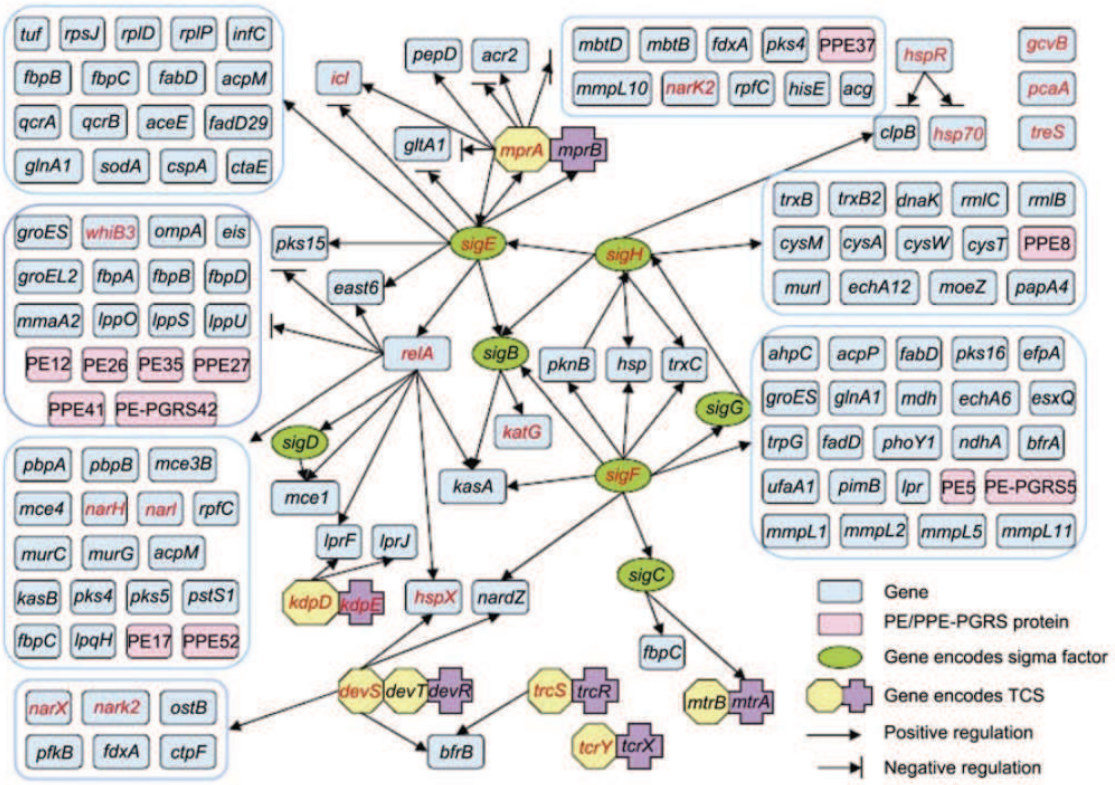


Figure 5: Gene regulatory network based on the gene and regulators reported to be implicated in *M.tuberculosis* persistence. (Wang X. et al., 2011)

Aim of the work

It has been previously shown that persistence is a phenomenon that plays a fundamental role in *M.tuberculosis* infection, as it is responsible for the relapse of the disease and for the need of a lengthy treatment.

Following some hypothesis that sigma factors may play an important role in persisters production in response to stresses caused by antibacterial drugs, in this work we decided to characterize an important sigma factor, SigE, to investigate its role in the response to stress caused by antimicrobials. This sigma factor was already known to play an important role in heat shock, oxidation and surface stresses that bacteria have to face in the course of an infection. However, nothing was known on its role during exposure to high concentration of antibiotics.

Taking into account all of these considerations, aims of this research project were: i) to define the role of SigE in *Mtb* drugs susceptibility; ii) to define the role of SigE in persister cells formation upon exposure to high concentrations of different classes of antibiotics; iii) to verify if other sigma factors have a role in persisters production or in *Mtb* drug suseptibility; iv) to shed light on the molecular pathways used by the pathogen to escape the killing mediated by antimicrobial drugs.

The results we obtained are promising and lay the foundations for the use of SigE as a molecular target for the development of new and more effective therapies against *M.tuberculosis*.

MATERIALS AND METHODS

1. Strains used in this work

A list of the bacterial strains used in this study is shown in **Table 5.**

All procedures with live *M. tuberculosis* were carried out under biohazard safety level 3 conditions. Antibiotics were added as appropriate: kanamycin (Sigma) was used at 20 µg ml^{-1}, hygromycin (Life Technologies) at 50 µg ml^{-1}, streptomycin (Sigma) at 20 ug ml^{-1}**.**

Name	Description
H37Rv (WT)	*M. tuberculosis* parental strain
TB218	*M.tuberculosis sigE* knock-out (KO) mutant
TB1	*M.tuberculosis sigL* knock-out (KO) mutant
ST82	*M.tuberculosis sigB* knock-out (KO) mutant
TB195	*M.tuberculosis sigG* knock-out (KO) mutant
TB341	*M.tuberculosis* WT complemented P_{hsp60} *mutT1*
TB343	*M.tuberculosis* WT complemented P_{hsp60} *mutT2*
TB345	*M.tuberculosis* WT complemented P_{hsp60} *mutT3*
TB347	*M.tuberculosis* WT complemented P_{hsp60} *mazG*
TB349	*M.tuberculosis ΔsigE* complemented P_{hsp60} *mutT1*
TB351	*M.tuberculosis ΔsigE* complemented P_{hsp60} *mutT2*
TB353	*M.tuberculosis ΔsigE* complemented P_{hsp60} *mutT3*
TB355	*M.tuberculosis ΔsigE* complemented P_{hsp60} *mazG*
TB425	*M.tuberculosis ΔsigE* complemented P_{mpy64} *clgr*
TB426	*M.tuberculosis ΔsigE* complemented P_{mpy64} *sigB*
TB427	*M.tuberculosis ΔsigE* complemented $P_{rv1818c}$ *clgr*
TB428	*M.tuberculosis WT* complemented P_{mpy64} *clgr*
TB429	*M.tuberculosis WT* complemented P_{mpy64} *sigB*
TB430	*M.tuberculosis WT* complemented $P_{rv1818c}$ *clgr*
TB446	*M.tuberculosis WT* complemented *pMV-10-25 (P_{hsp60}-gfp)*
TB447	*M.tuberculosis WT* complemented *pMV-2-33 (P_{mpy64}-gfp)*
TB448	*M.tuberculosis WT* complemented *pMV-4-36 ($P_{rv1818c}$-gfp)*
TB449	*M.tuberculosis ΔsigE* complemented *pMV-10-25*

	(P_{hsp60}-gfp)
TB450	*M.tuberculosis ΔsigE* complemented *pMV-2-33 (P_{mpy64}-gfp)*
TB451	*M.tuberculosis ΔsigE* complemented *pMV-4-36 ($P_{rv1818c}$-gfp)*
TB184	*M.tuberculosis ΔsigE* complemented *pEL107 (SigE small isoform – P3 promoter)*
TB185	*M.tuberculosis ΔsigE* complemented *pEL108 (SigE large isoform – P1,P2,P3 promoter, with mutagenesis of the first two codons of the small SigE isoform)*
TB186	*M.tuberculosis ΔsigE* complemented *pEL109 (SigE WT – positive control)*
EC1161	*E.coli – DH5α pDAV10 (PCR-BluntII-TOPO / mazG)*
EC1162	*E.coli – DH5α pDAV11 (PCR-BluntII-TOPO / mutT1)*
EC1163	*E.coli – DH5α pDAV12 (PCR-BluntII-TOPO / mutT2)*
EC1164	*E.coli – DH5α pDAV13 (PCR-BluntII-TOPO / mutT3)*
EC1165	*E.coli – DH5α pDAV14 (pMV10-25 / mutT1)*
EC1166	*E.coli – DH5α pDAV15 (pMV10-25 / mutT2)*
EC1167	*E.coli – DH5α pDAV16 (pMV10-25 / mutT3)*
EC1168	*E.coli – DH5α pDAV17 (pMV10-25 / mazG)*
EC1169	*E.coli – DH5α pDAV18 (PCR-BluntII-TOPO / Rv2745c)*
EC1170	*E.coli – DH5α pDAV19 (PCR-BluntII-TOPO / sigB)*
EC1171	*E.coli – DH5α pDAV20 (pMV2-33 / clgr)*
EC1172	*E.coli – DH5α pDAV21 (pMV2-33 / sigB)*
EC1173	*E.coli – DH5α pDAV22 (pMV4-36 / clgr)*

Table 5: List of the bacterial strains used in this study.

2. Media used for the growth of *M.tuberculosis*

7H9 and 7H10 are the media of choice for the growth of *M.tuberculosis*, respectively in liquid and solid conditions. *M. tuberculosis* strains were cultured in 7H9 medium containing 0,5% glycerol and supplemented with 10% bovine serum albumin – dextrose – catalase (ADC), 0,05% Tween 80. 7H10 medium supplemented with ADC and glycerol was used as the solid medium. All of the *M. tuberculosis* strains were grown at 37°C in standing condition.

7H9:

Ingredients	Grams/Litre
Ammonium Sulfate	0.5
Disodium Phosphate	2.5
Monopotassium Phosphate	1.0
Sodium Citrate	0.1
Magnesium Sulfate	0.05
Calcium Chloride	0.0005
Zinc Sulfate	0.001
Copper Sulfate	0.001
Ferric Ammonium Citrate	0.04
L-Glutamic Acid	0.5
Pyridoxine	0.001
Biotin	0.0005
Final pH 6.6 +/- 0.2 at 25°C	

7H10:

Ingredients	Grams/Litre
Ammonium Sulfate	0.5
L-Glutamic Acid	0.5
Disodium Phosphate	1.5
Monopotassium Phosphate	1.5
Sodium Citrate	0.4
Magnesium Sulfate	0.025
Calcium Chloride	0.0005
Zinc Sulfate	0.001
Copper Sulfate	0.001
Ferric Ammonium Citrate	0.04
Pyridoxine Hydrochloride	0.001
Biotin	0.0005
Malachite Green	0.00025
Agar	15.0

2. **Media used for the growth of *E.coli* strains**

Luria-Bertani (LB) is an excellent medium for the growth of *E.coli* strains. Its main constituents are yeast extract (that is mainly a carbon source and contains other essential cofactors), bacto-tryptone (hydrolized of casein and thus excellent source of nitrogen) and the sodium chloride whose addition is required for both the achievement of the appropriate osmolarity of the medium and to supplement specific elements (Na^+ and Cl^- ions).

Prior to use the medium has been autoclaved at 121°C, 1 atm, for 20 minutes. *E. coli* strains were grown under agitation (180 rpm) in Luria-Bertani (LB) broth or on LB broth agar.

Final composition g/L:

Tryptone	10
Yeast extract	5
NaCl	10
pH	7,5

3. Chemical reagents

Where not otherwise specified, the different chemical reagents used in these experiments (buffers, salts, etc..) were prepared as previously described (Sambrook, J. et al. 2001).

4. Enzymatic digestions and ligation reactions

Where not otherwise specified, all the enzymatic digestions and ligation reactions were performed according to the standard protocols indicated by supplier companies.

5. Vectors construction

All the *mutT, sigB and Rv2745c (clgr)* genes were amplified using genomic *M.tuberculosis* H37Rv DNA as a template. Pcr amplification reactions were performed using a high-fidelity polymerase (*Pfu* from Agilent or *Q5*^{Tm} from NEB) with primers showed in **Table 6**.

Oligonucleotides have been designed to include a restriction site both at 5' and 3' ends of the genes, for subsequent digestion and cloning in the final vector. Particularly, we added a *NheI* restriction site before the initial codon of the gene and a *XbaI* or *BamHI* restriction site after the last codon.

Hence, fragments obtained have been cloned inside the PCR-BluntII-TOPO (Invitrogen) and sequenced to verify the absence of mutations.

Plasmids pMV10-25 (carrying P_{hsp60} promoter), pMV2-33 (carrying P_{mpy64} promoter) and pMV4-36 (carrying P_{1818c} promoter) (Delogu et al. 2004a e 2004b) were digested with the NheI/XbaI or NheI/BamHI (for the pMV4-36) enzymes to extract the *gfp* and to insert our *mutT, sigB and clgr* genes under control of the different promoters, thus obtaining the final vectors electroporated in both *ΔsigE and WT Mtb* strains. At the end, all the genes were inserted downstream of the desidered expression promoters (P_{hsp60} in pMV 10-25, P_{mpt64} in pMV 4-36 and $P_{rv1818c}$ in pMV 2-33).

M.tuberculosis strains containing the plasmids pEL107, pEL108, pEL109 (*sigE* small and big isoforms) were already available in the laboratory.

Name	Gene	Sequence
RP1541	*Fw – mutT1*	GCTAGCgtgtcgatccagaactcgtc
RP1542	*Rev- mutT1*	TCTAGAttaggcccgcacgttggcgg
RP1543	*Fw – mutT2*	GCTAGCatgctgaatcagatcgtggt
RP1544	*Rev – mutT2*	TCTAGActaacagcgacggtggacat
RP1545	*Fw – mutT3*	GCTAGCttgcccagttgcccgcctgc
RP1546	*Rev – mutT3*	TCTAGAttacagcagcgaactgatccgcc
RP1549	Fw - mazG	GCTAGCatgattgtcgtcctggtcga
RP1550	*Rev- mazG*	TCTAGAttatcgcatgcctttacgtt
RP1637	Fw - Rv2745c	GACTGCTAGCatggcggctt
RP1638	Rev – Rv2745c	TCGATCTAGAttaggccaccgc
RP1640	*Fw - sigB*	TATTGCTAGCatggccgatgca
RP1641	*Rev - sigB*	ATTATCTAGAtcagctggcgta
RP1670	*Rev – Rv2745c - BamHI*	TCGAGGATCCttaggccaccgc
RP1671	*Rev – sigB - BamHI*	ATTAGGATCCtcagctggcgta

Table 6: primers used in this study for amplification of *mutT, mazG, sigB* and *clgr* genes.

6. Persisters assay in *M.tuberculosis*

Frozen stock cultures of both WT and *ΔsigE* mutants were grown on solid medium 7H10/ADN. The obtained cultures were then grown standing for 2 weeks in 5 ml of liquid medium 7H9/ADN starting from an OD_{540} of 0,05.

The fourteenth day a sample of the cultures was collected and 1) washed, resuspended in half of the initial volume and refreshed (20ul) in 2ml of fresh liquid media without drug 2) serially diluted and plated for subsequent colony count. After that, a 25X MIC concentration of the drug to test was added to the remaining cultures.

At different time-points (3 days, 6 days, 10 days) samples of the cultures (600ul) were taken and then again diluted (20ul) in 2ml of fresh media and the growth followed with a spectrophotometer (until the new cultures reached an OD_{540} of 0.1), in order to check the delay (days) in growth of the *ΔsigE* mutant compared to the WT (a delay in growth indicates a possible lower production of persisters cells) and plated for colony-count.

A summary of the procedure is schematically illustrated in **Figure 6**.

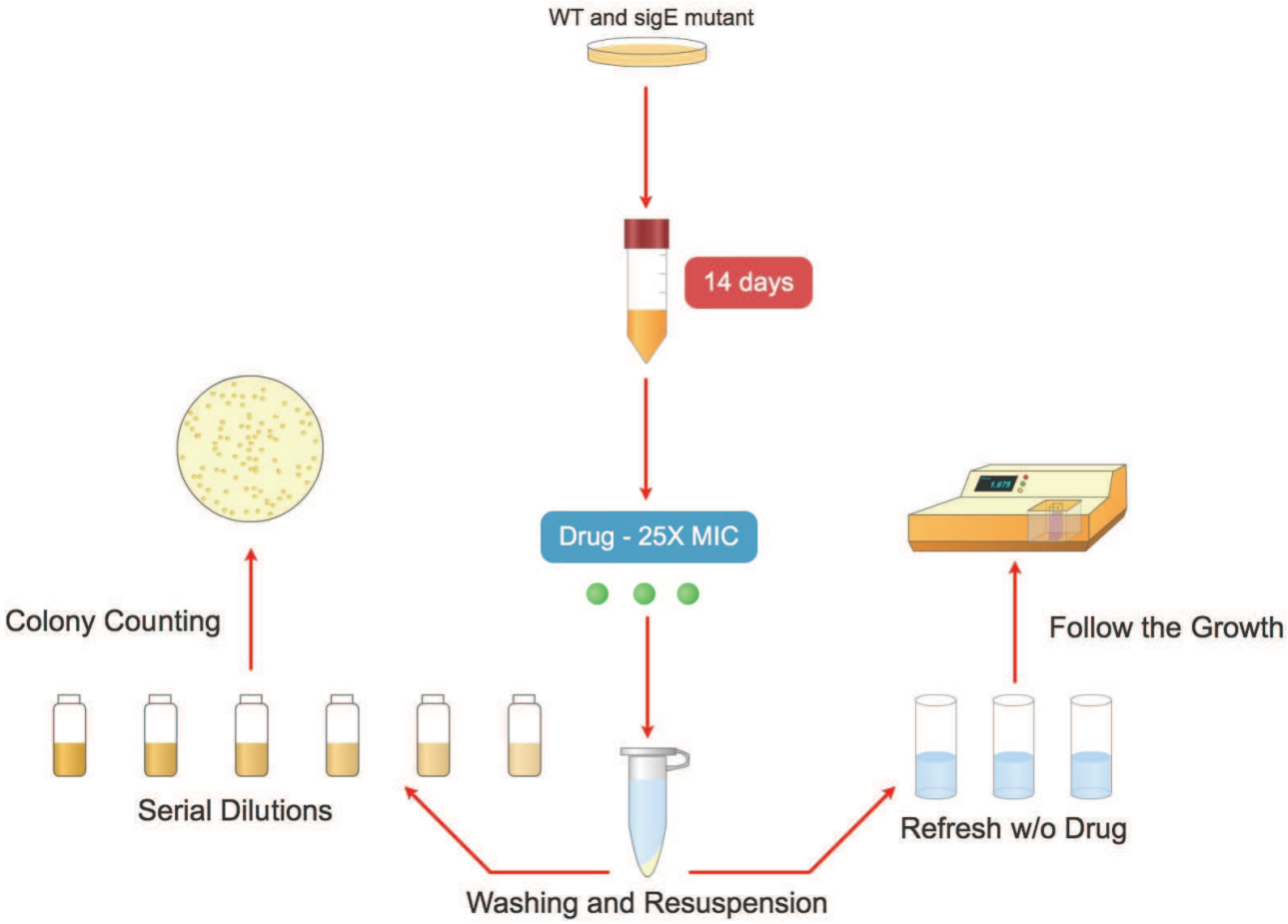


Figure 6: Schematic representation of the assay used to evaluate production of persister cells.

7. Disk diffusion assay

Mycobacterium tuberculosis strains were grown to early exponential phase to an OD_{540} 0,3. Aliquots of 100ul containing $3x10^6$ cfu were spread on 7H10 plates. Paper disks (6.5mm in diameter; Schleicher and Schuell) containing 10ul of the antibiotic at a defined concentration were placed on top of the agar. The diameters of the zones of inhibition were measured after 14 days of incubation at 37°C.

8. MIC evaluation using the Alamar Blue Assay (resazurine assay)

Frozen stock coltures of the parental strain (WT) and the mutant (*ΔsigE)* were grown on solid medium 7H10/ADN. Subsequently, a pre-colture was carried out in 2ml of liquid medium (7H9/ADN) starting from an OD_{540} of 0,05.

Coltures were then grown up to a mid-exponential phase (OD_{540} 0,6-0,8) and then diluted to an OD of 0,01.

In microplates suitable for fluorescence reading (96-well FluoroNunc™ black flat bottom plates) serial dilutions of the antibiotic to be tested were made in 7H9/ADN liquid medium.

Subsequently, a quantity of $5x10^4$ bacteria (50ul) was then taken from the OD 0,01 cultures and inserted in the wells of the microplate that contains the antibiotic.

The plates thus obtained were left to incubate sealed for 1 week at 37°C.

After incubation, Alamar-Blue (Invitrogen) was added to each well and the plates, after another 1 day of incubation a 37°C (necessary for the fluorescence reaction to develop), were read with a microplate reader (Tecan Infinite 200 Pro) to determine the relative fluorescence.

For each strain we used a positive control (cells without antibiotic), to determine the maximum fluorescence that could be obtained, and a negative control (medium plus antibiotic without cells).

RESULTS

A *sigE* null mutant of *M.tuberculosis* is more sensitive to several drugs

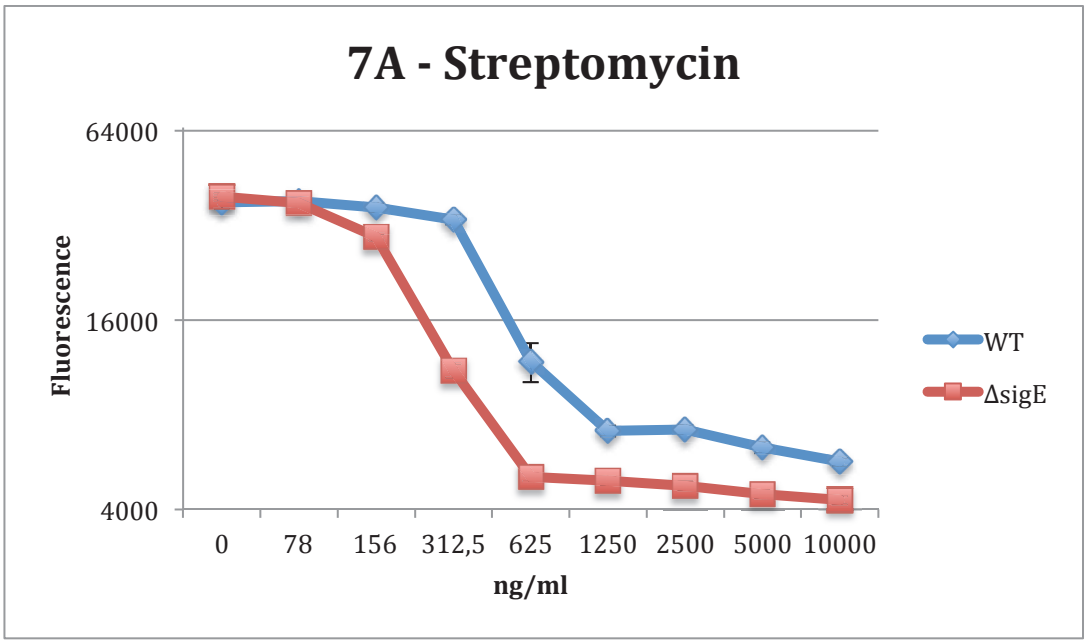
Sigma factors play a central role in *Mtb* persistence as indicated in the gene regulatory network described before. Particularly, they allow the tubercle bacilli to respond and survive the various stresses it has to face during infection.

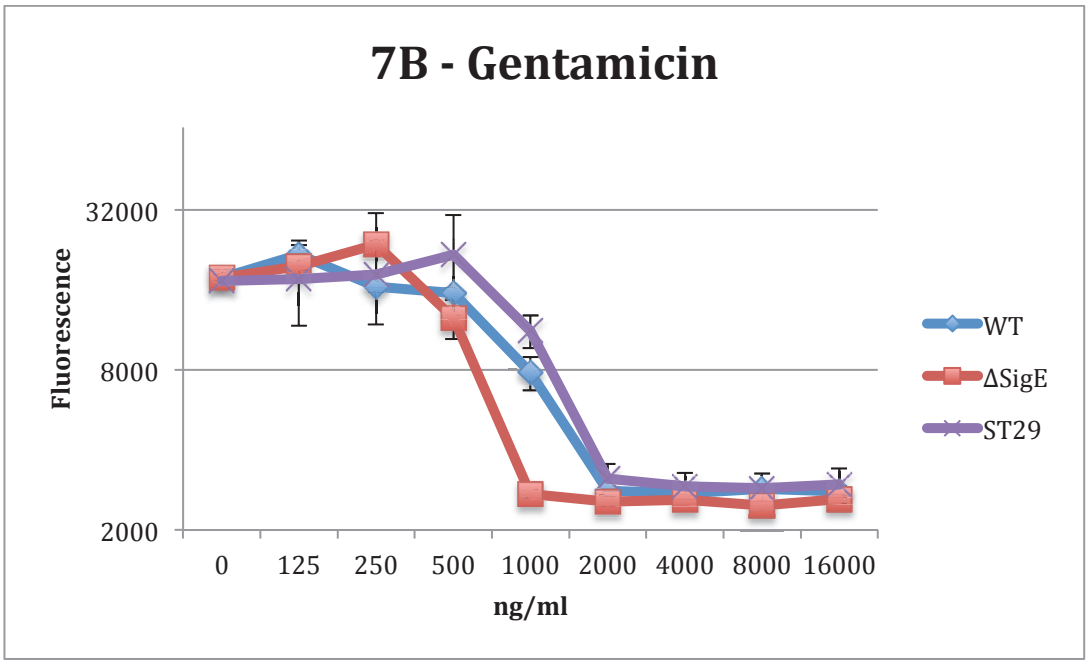
SigE represents one of the most important players in *M.tuberculosis* stress response and plays a fundamental role in the adaptation to heat, oxidative and surface stresses. (Manganelli et al., 2001)

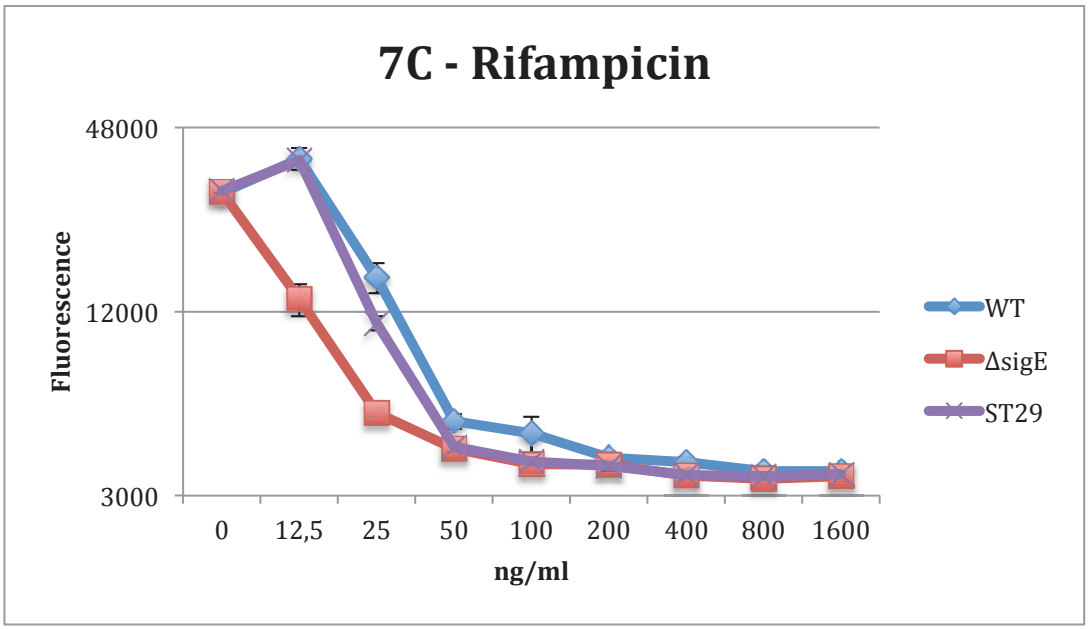
During antibiotic treatment bacteria are subjected to several kind of stresses, such as oxidative and surface stresses, depending on the mechanism of action of the drug itself.

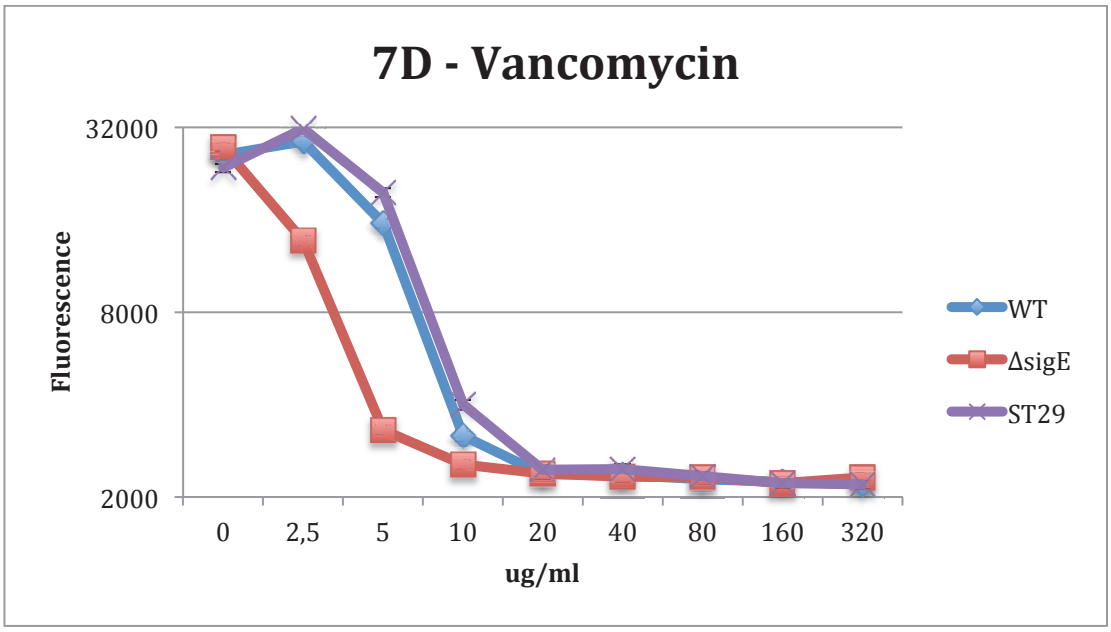
To determine if SigE plays also a role in the adaptation to stress caused by antibiotics, we subjected an *Mtb sigE* null mutant (TB218), its parental strain H37Rv (WT) and a complemented strain (ST29) in which a wild-type copy of the *sigE* gene was reintroduced in an ectopic locus of the genome, to treatment with increased concentrations of several drugs using the resazurine assays (see material and methods).

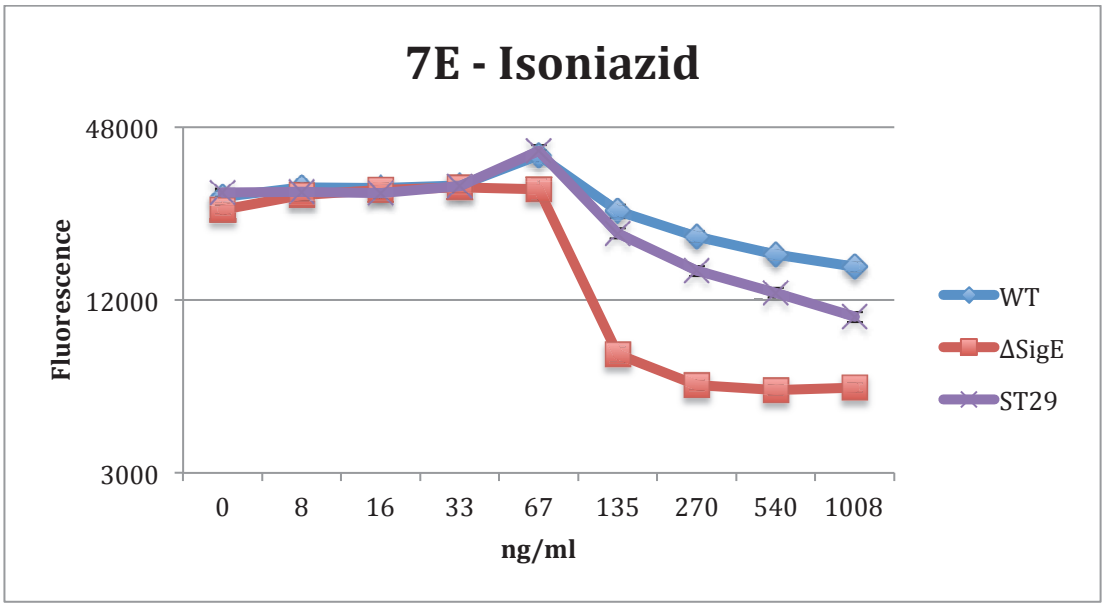
As shown in **Figure 7** the *sigE* mutant was more sensitive than the WT (parental strain) and ST29 (complemented strain) to vancomycin, rifampicin, streptomycin, gentamicin, isoniazid and ethambutol drugs, while its sentivity to the fluoroquinolone ciprofloxacin drug was the same as that of the H37Rv**.**

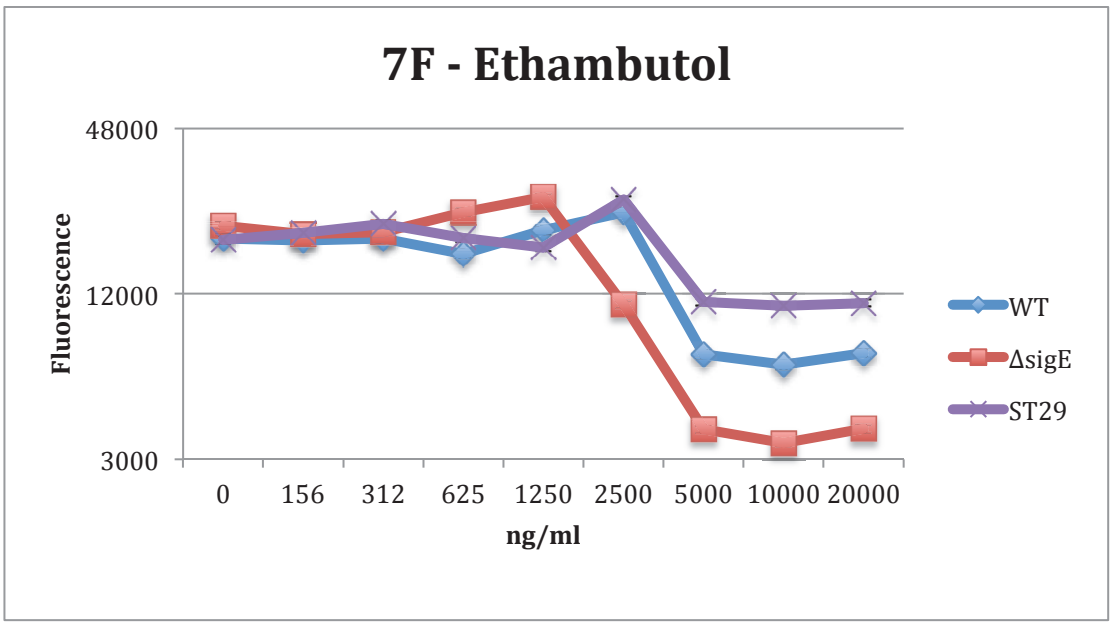

7A - Streptomycin
64000
16000
4000
Fluorescence
0
78
156
312,5
625
1250
2500
5000
10000
ng/ml
WT
ΔsigE


7B - Gentamicin
32000
8000
2000
Fluorescence
0
125
250
500
1000
2000
4000
8000
16000
ng/ml
WT
ΔSigE
ST29


7C - Rifampicin
48000
12000
3000
Fluorescence
0
12,5
25
50
100
200
400
800
1600
ng/ml
WT
ΔsigE
ST29


7D - Vancomycin
32000
8000
2000
Fluorescence
0
2,5
5
10
20
40
80
160
320
ug/ml
WT
ΔsigE
ST29


7E - Isoniazid
48000
12000
3000
Fluorescence
0
8
16
33
67
135
270
540
1008
ng/ml
WT
ΔSigE
ST29



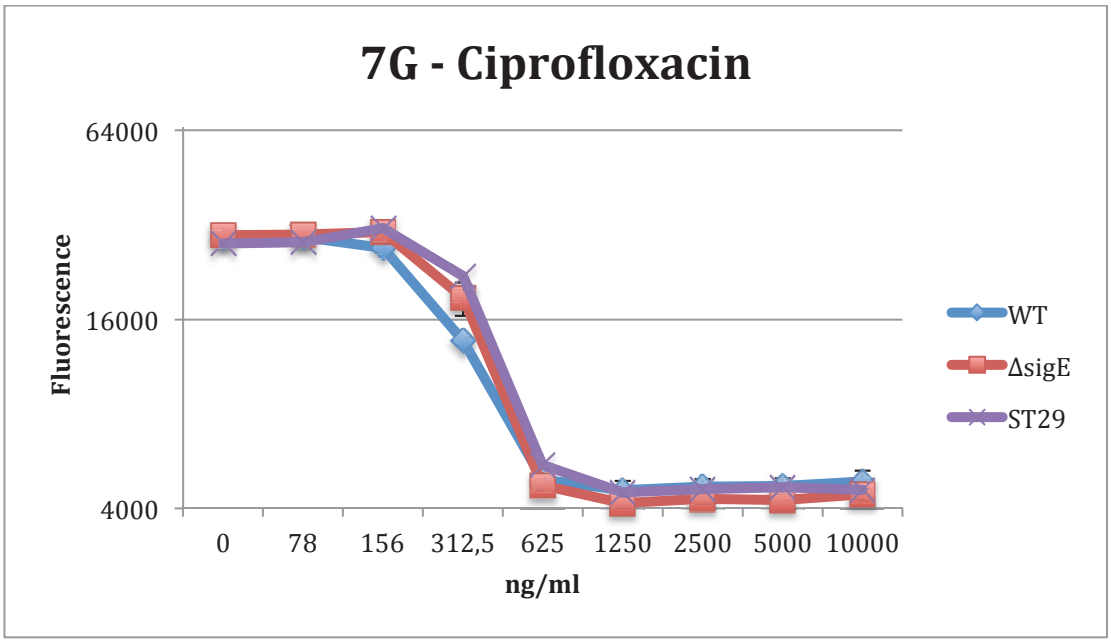


Figure 7: MIC evaluation of the *M.tuberculosis sigE null* mutant (*ΔsigE*), H37Rv (WT), ST29 (complemented strain) with the drugs (7A) Streptomycin, (7B) Gentamicin, (7C) Rifampicin, (7D) Vancomycin, (7E) Isoniazid, (7F) Ethambutol, (7G) Ciprofloxacin. Data were obtained using the Alamar Blue assay. All the experiments were performed at least twice.

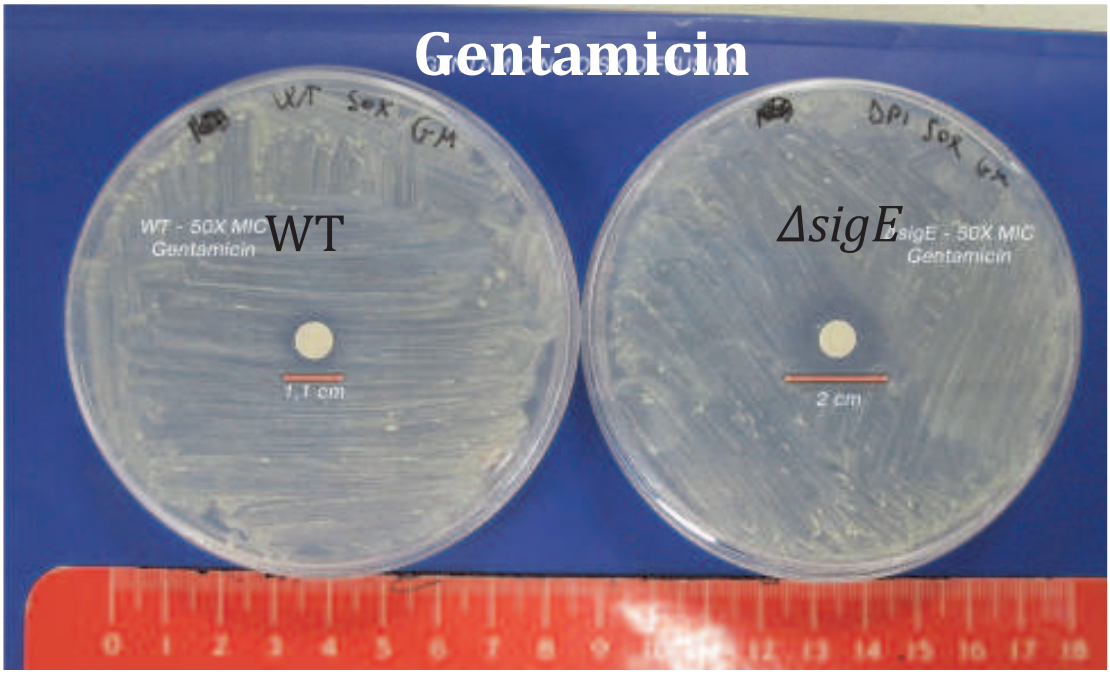
As visible in **Figure 7**, the wild-type basal level of drug resistance was fully restored with the complemented strain, thus demonstrating that the increase in sensitivity observed in the mutant was indeed due to the absence of a functional *sigE* copy in the genome.

Interestingly, with isoniazid and ethambutol drugs that share a similar mechanism of action (isoniazid acts by inhibiting the synthesis of micolic acids while ethambutol disrupts arabinogalactan synthesis

thus inhibiting cell wall biosynthesis), the inhibition profile obtained after treatment of the *sigE* mutant was different from that obtained with other drugs. In this case, the difference was in the amount of fluorescence drop and not in the concentration at which the fluorescence started to drop.

It is likely that the killng kinetic of the *sigE* mutant with these two drugs is different from that of WT.

To confirm these results, we also tested the different sensitivity of the *ΔsigE* mutant with a disk diffusion assay. In **Figure 8** results for the gentamicin and rifampicin drugs are shown. In both cases, the higher sensitivity of the *sigE* mutant was confirmed.



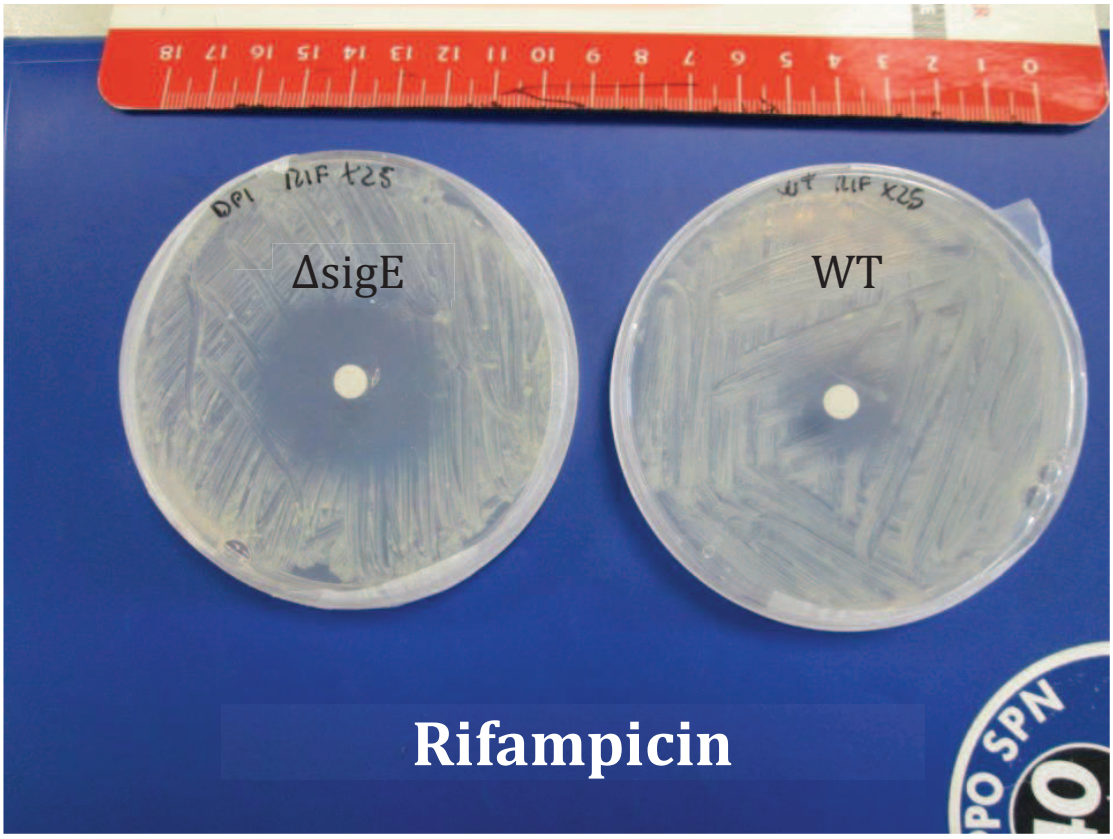


Figure 8: Disk diffusion assay for gentamicin and rifampicin drugs. A 25X – 50X MIC concentration of the drug to test has been used (see material and methods for details). Disk diffusion assays have also been performed for Streptomycin, Isoniazid, Ethambutol, Vancomycin and Ciprofloxacin drugs (data no shown). All the experiments were performed at least twice.

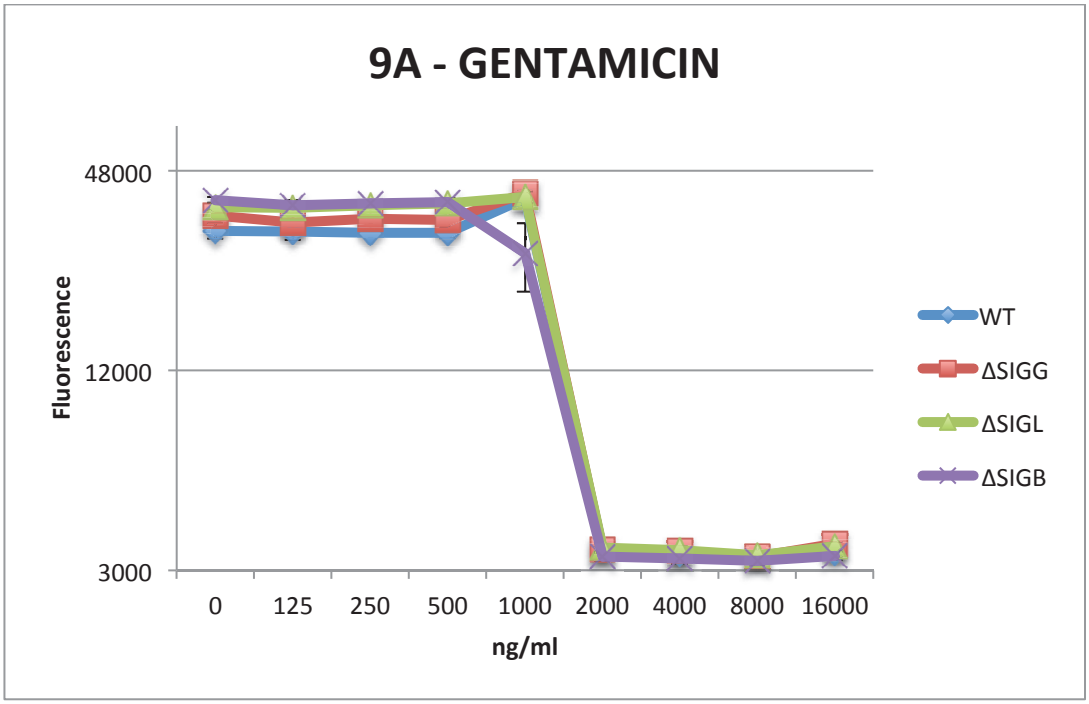
SigL, SigG and SigB have minor effects on drug sensitivity

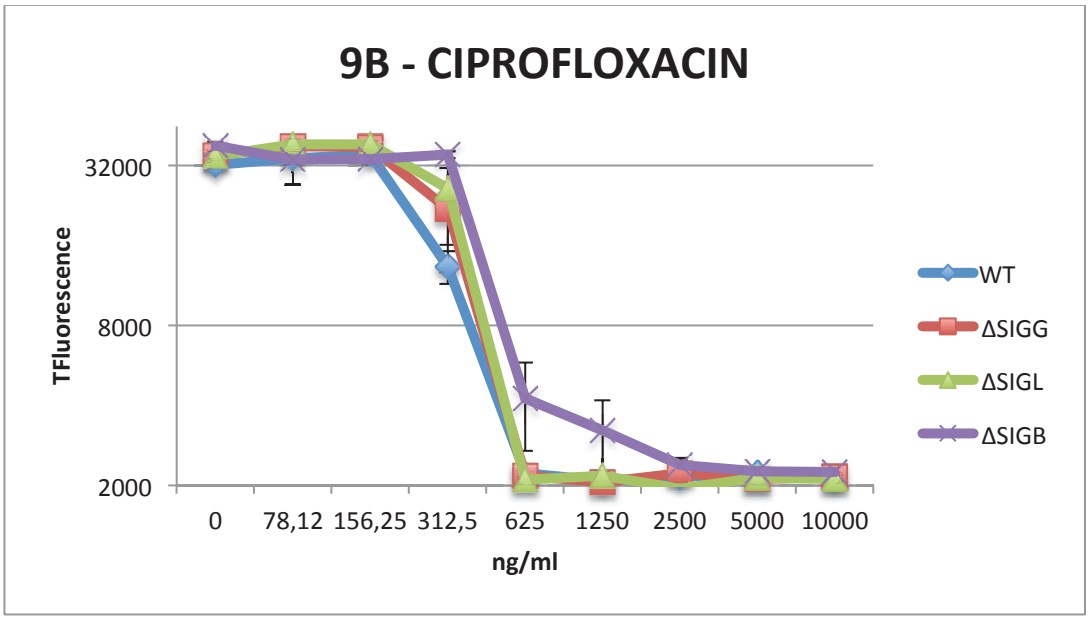
As previously shown, other sigma factors like SigB and SigH may be also involved in response to oxidative and enviromental stresses.

Furthermore, because of the interplay in sigma factors regulation and the strict relantioships among them in the genes regulatory network pathway, to address if the drug sensitivity phenotype observed is a common feature shared among sigma factors mutants or if it is a unique feature of the *sigE* mutant, we decided to investigate other sigma factors mutants.

For this purpose, we exposed H37Rv derivatives, in which *sigL, sigG* or *sigB* were deleted, to increasing concentrations of the same drugs used for the *sigE* mutant. Results of the MIC determination with the Alamar Blue assay for the strains TB1 (*sigL* mutant), ST82 (*sigB* mutant), TB195 (*sigG* mutant) are showed in **Figure 9**.

As illustrated in the charts, the *sigL, sigG* and *sigB* mutants did not show any difference in antibiotic sensitivity compared to their parental strains, while the *sigB* mutant showed an increased sensitivity to isoniazide and ethambutol. Interestingly, also in this case the inhibition profile was the same as that observed for the *sigE* mutant.





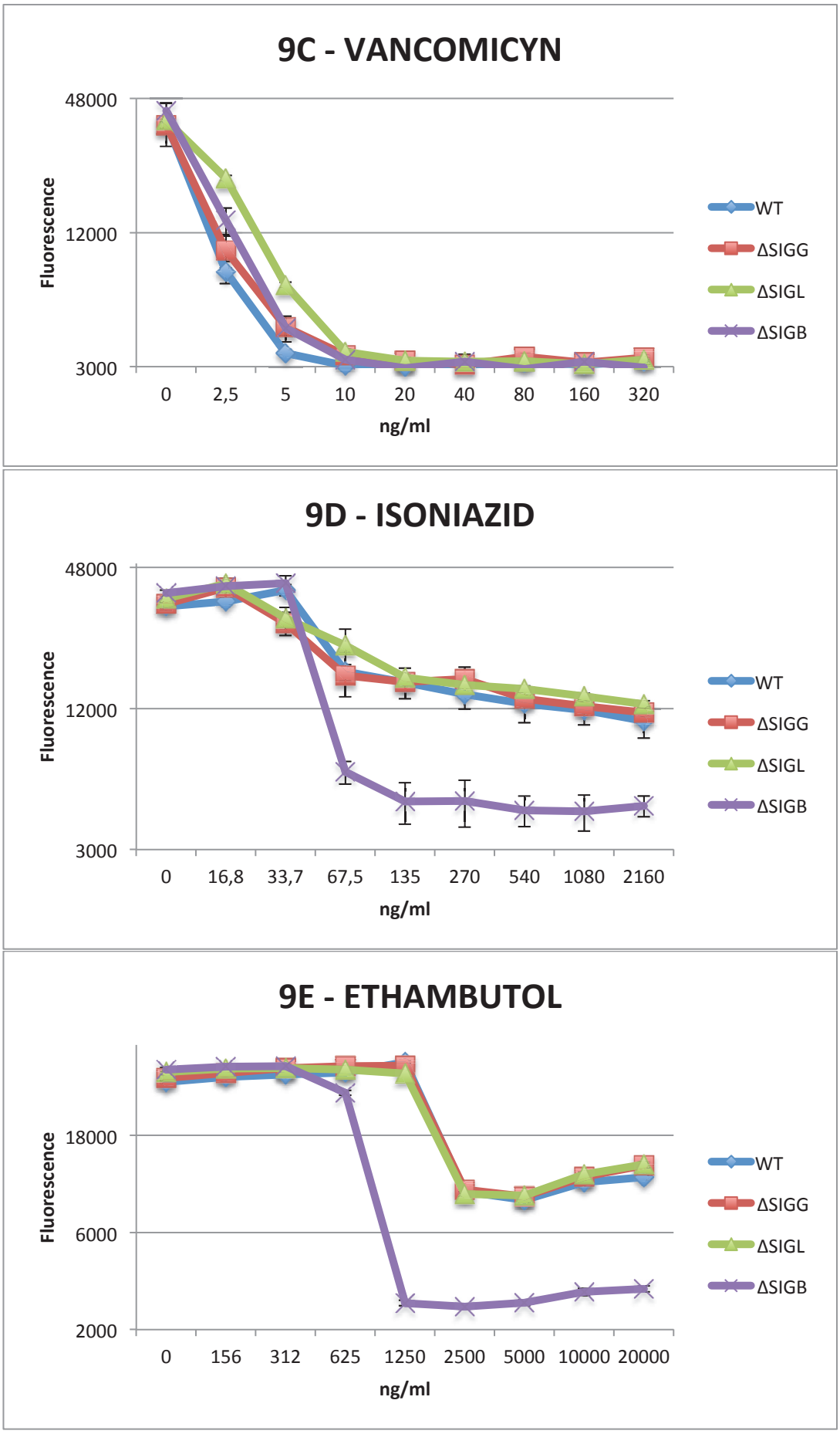


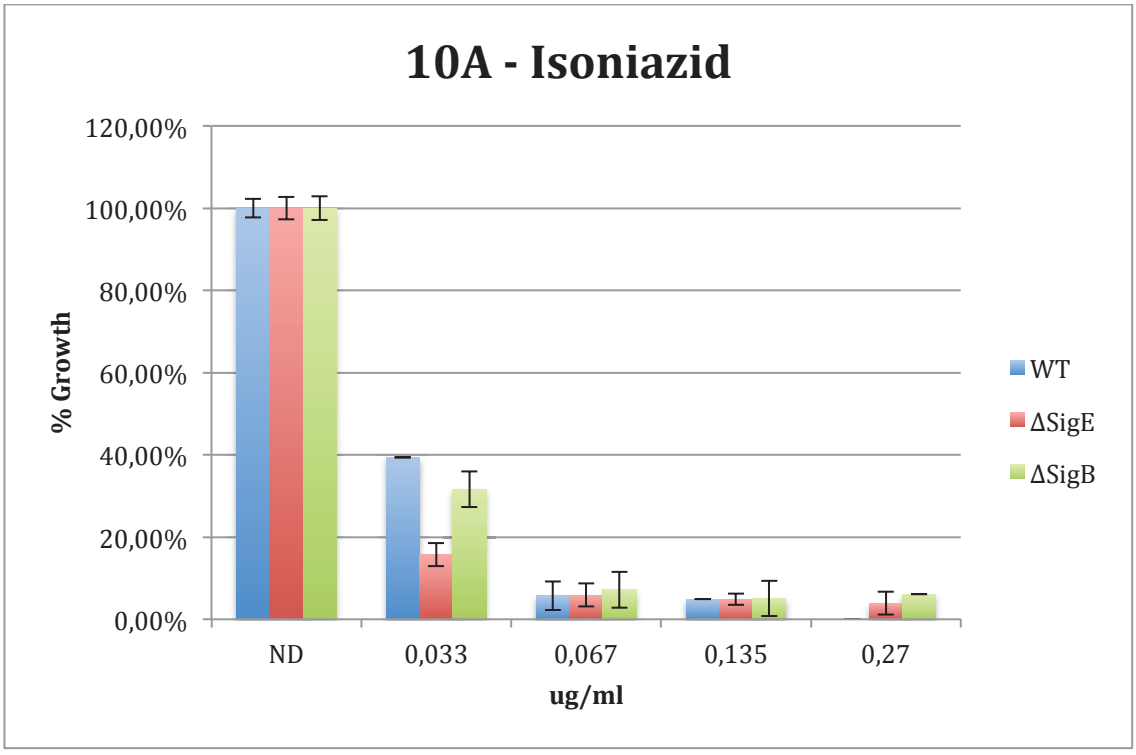
Figure 9: MIC evaluation of the *sigG, sigL, sigB* mutants and H37Rv parental strain with the drugs (9A) Gentamicin, (9B) Ciprofloxacin, (9C) Vancomycin, (9D) Isoniazid, (9E) Ethamnutol. Data were obtained using the Alamar Blue Assay. All the experiments were performed at least in double.

To further investigate the impact of the *sigE* and *sigB* deletion on isoniazid and ethambutol sensitivity, we performed two additional experiments.

In the first one, the mutants and their parental strains were grown for 7 days with increasing concentrations of the two drugs and their growth followed by optical density. Results of this experiment are shown in **Figure 10**, expressed in $\%OD_{540}$ increase.

This experiment confirmed that while both mutants were more susceptible to ethambutol, only the *sigE* mutant showed an increase in sensitivity to isoniazid.

Since the Alamar Blue assay is an indirect indicator of cell viability, we decided to test if the differences observed in the MIC experiments were due to a higher degree of killing of the two drugs against the mutants or if they were just reflecting a different metabolic state of the mutants respect to the wild-type. For this reason, we performed an additional experiment in which, to measure bactericidal activity of both drugs. Results of this experiment are shown in **Figure 11**.



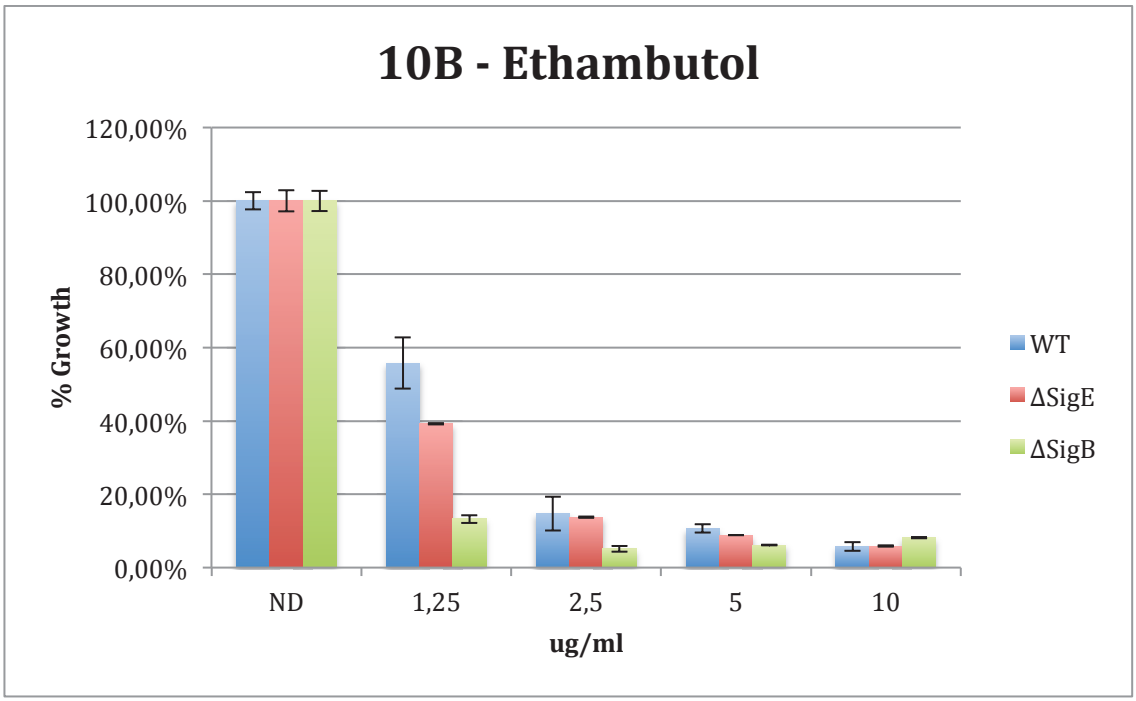
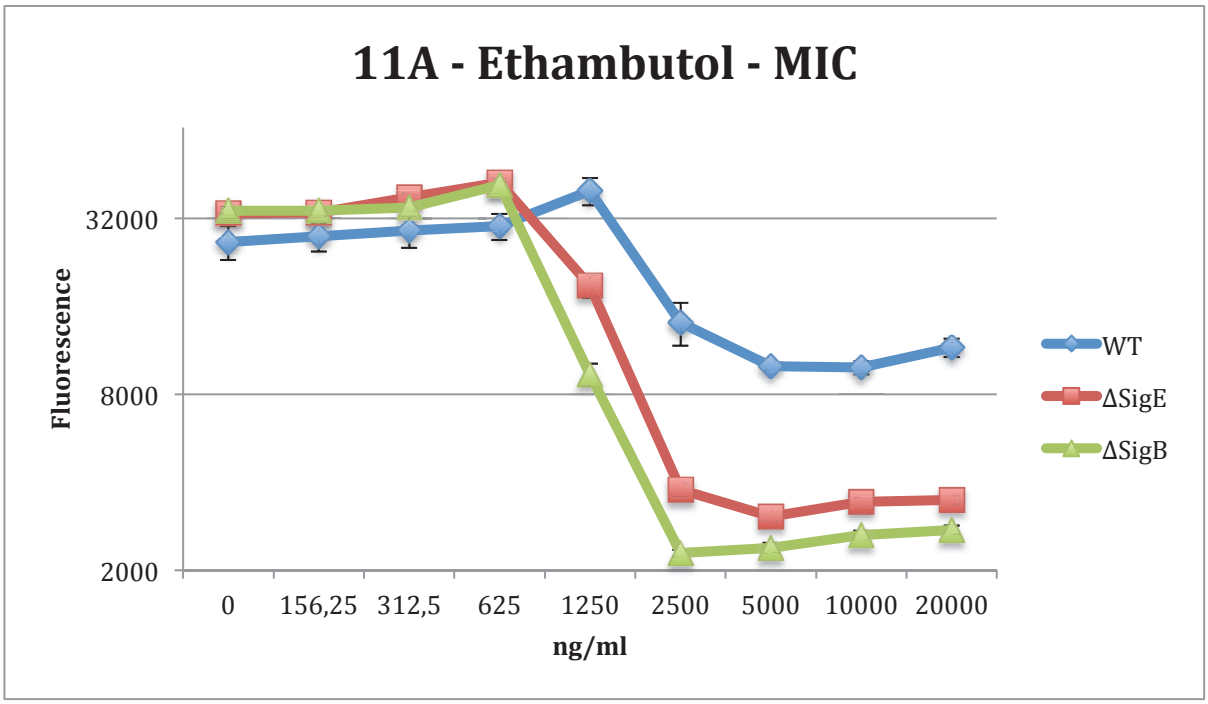
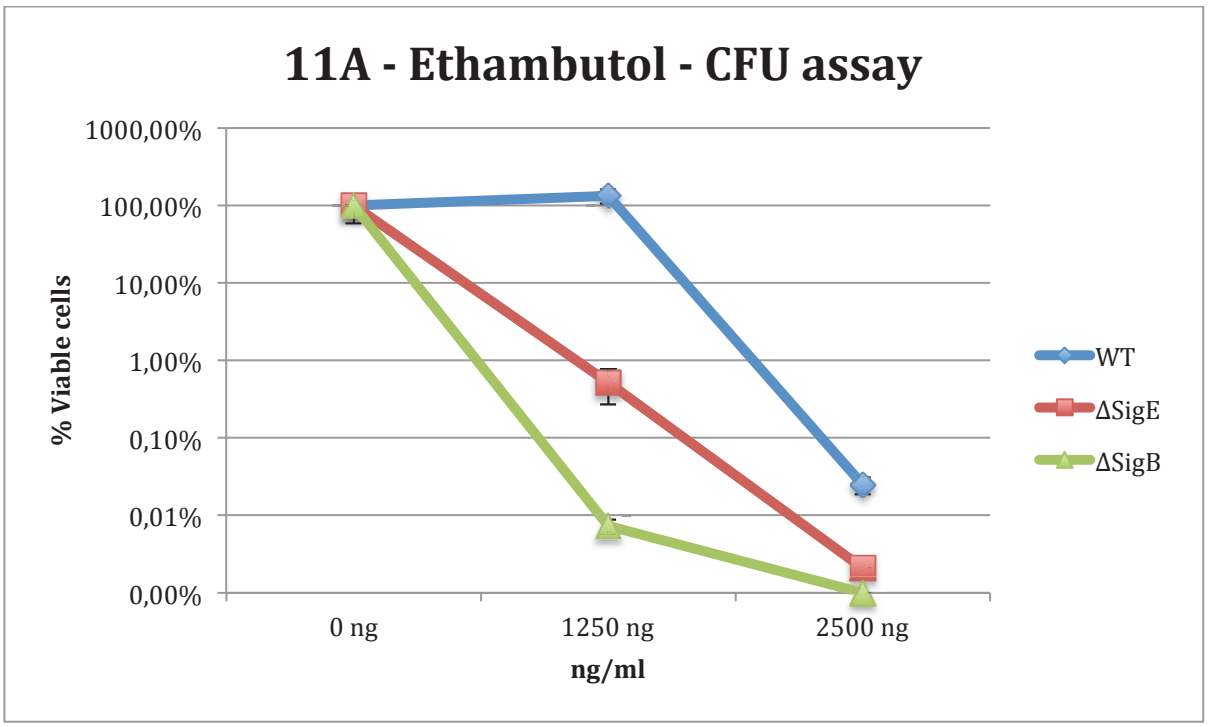
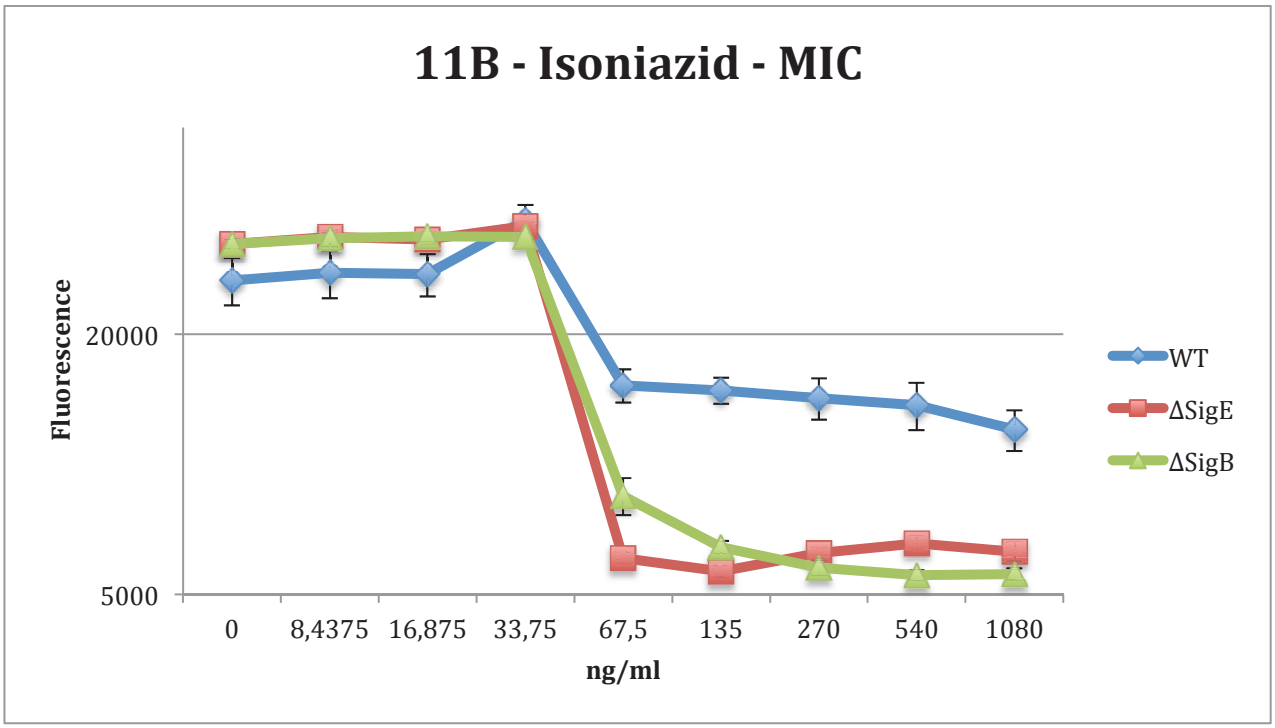


Figure 10: % growth of the *sigE, sigB* mutants compared to the parental strain (H37Rv WT) at increasing concentrations of the drugs A) Isoniazid, B) Ethambutol. Both mutants are more sensitive to ethambutol, while only the *sigE null* mutant shows an increased sensitivity to isoniazid.


11A - Ethambutol - MIC
Fluorescence
32000
8000
2000
0
156,25
312,5
625
1250
2500
5000
10000
20000
ng/ml
WT
ΔSigE
ΔSigB


11A - Ethambutol - CFU assay
% Viable cells
1000,00%
100,00%
10,00%
1,00%
0,10%
0,01%
0,00%
0 ng
1250 ng
2500 ng
ng/ml
WT
ΔSigE
ΔSigB



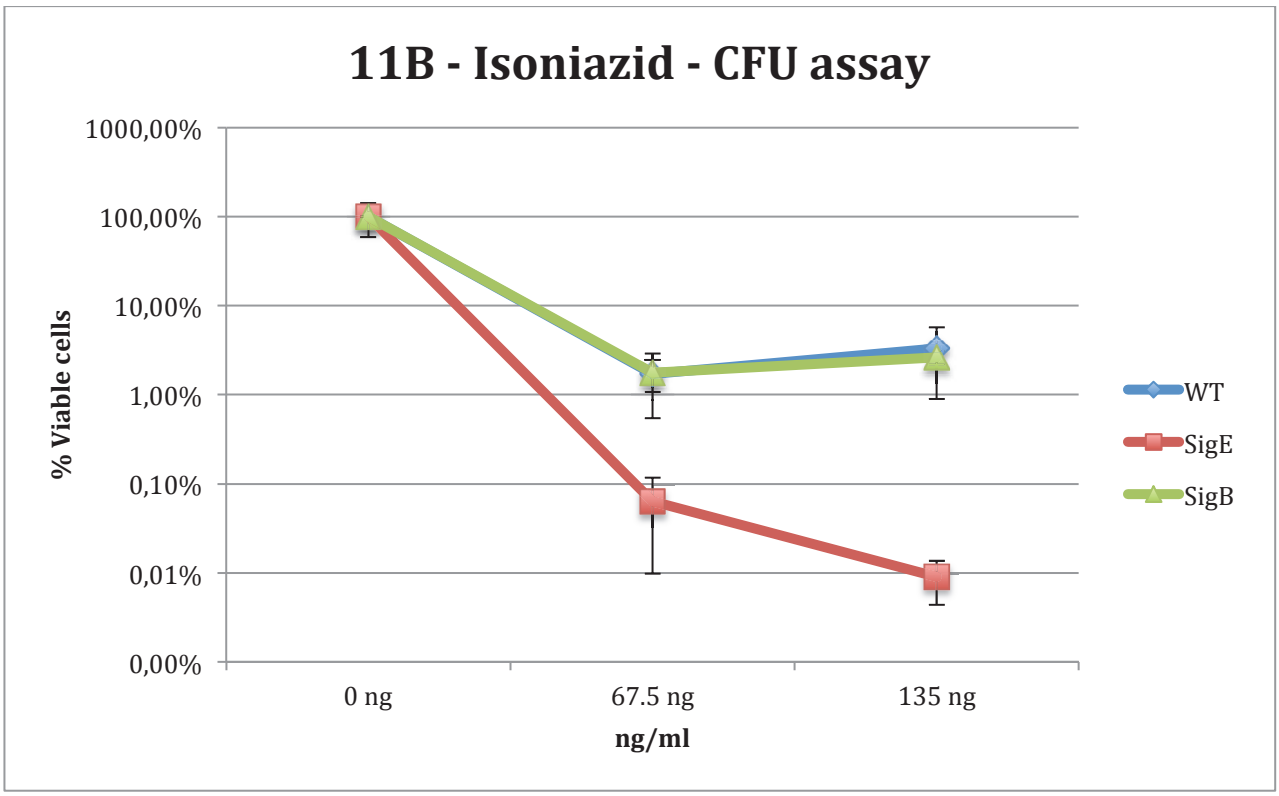


Figure 11: MIC and MBC of the *sigE* and *sigB* mutants for the two drugs A) Ethambutol and B) Isoniazid. While both mutants are killed with high efficiency by ethambutol, only the *ΔsigE* is killed more efficiently by isoniazid.

This experiment confirmed that while both mutants are more sensitive than the parental strain (WT) to ethambutol, showing a 20 folds decrease in the number of viable counts vs WT strain, only the *sigE* mutant is be killed with higher efficiency by isoniazid, showing a decrease in viable counts of about 100 folds respect to WT and *sigB* mutant.

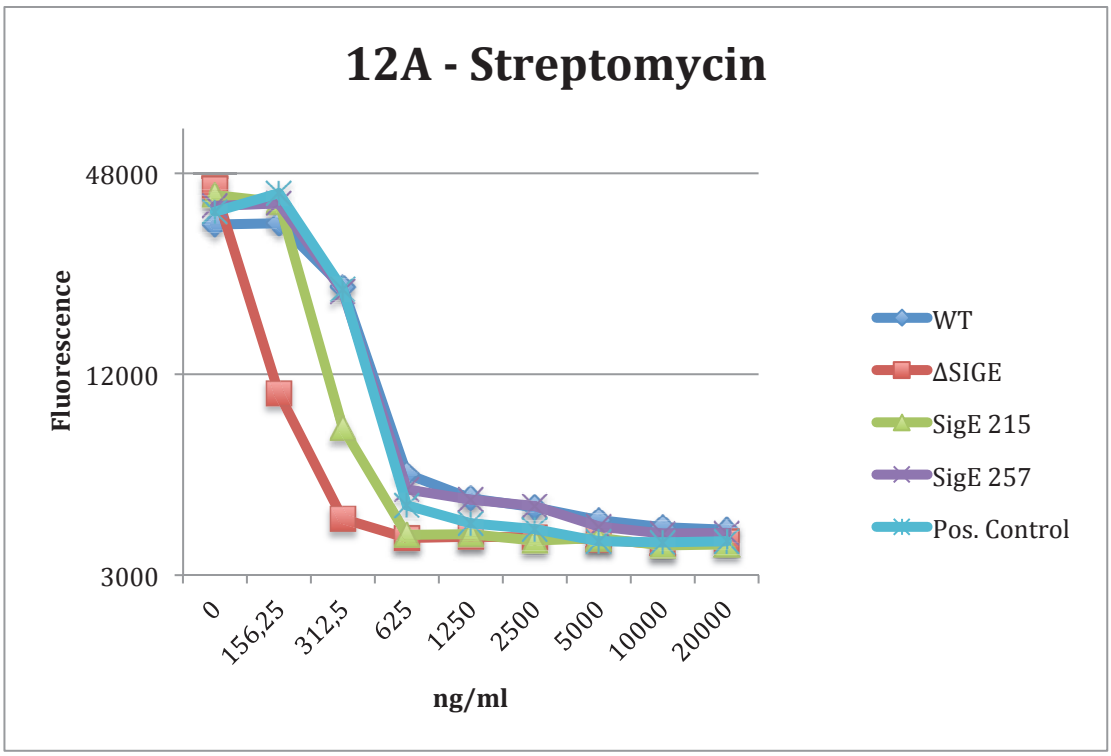
Different SigE isoforms have the same effect on drug sensitivity

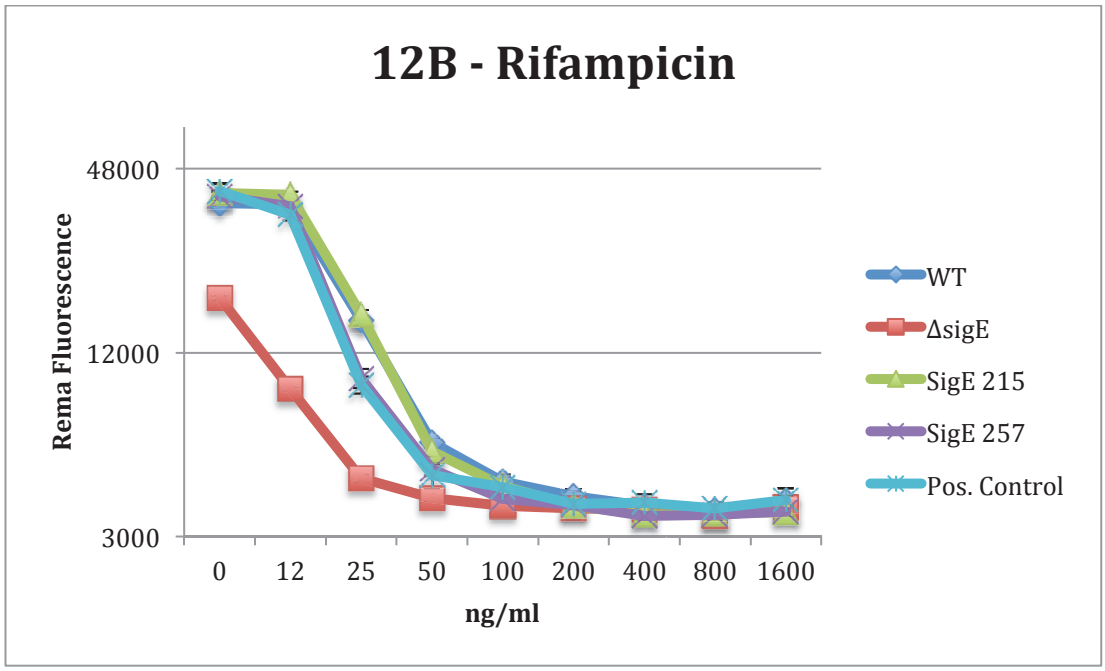
σ^E is subject to an extremely complex regulation at transcriptional, translational and post-translational levels. Its structural gene is transcribed by three promoters (P1–P3) (Donà V. et al., 2008). P1 is active under normal physiologic conditions, being responsible for the basal level of *sigE* expression. P2 is induced upon surface stress and at alkaline pH, and is activated by the two component system MprAB (He H. et al., 2006) whose transcription is itself under σ^E transcriptional control, resulting in a first positive feedback loop. The last *sigE* promoter (P3) is transcribed by σ^H-RNAP in response to oxidative stress. Interestingly, its transcriptional start site is located 62 base pairs within the *sigE* open reading frame. Translation of mRNAs originating from P3 results in the production of two smaller σ^E isoforms of 218 and 215 aa. (Donà V. et al., 2008)

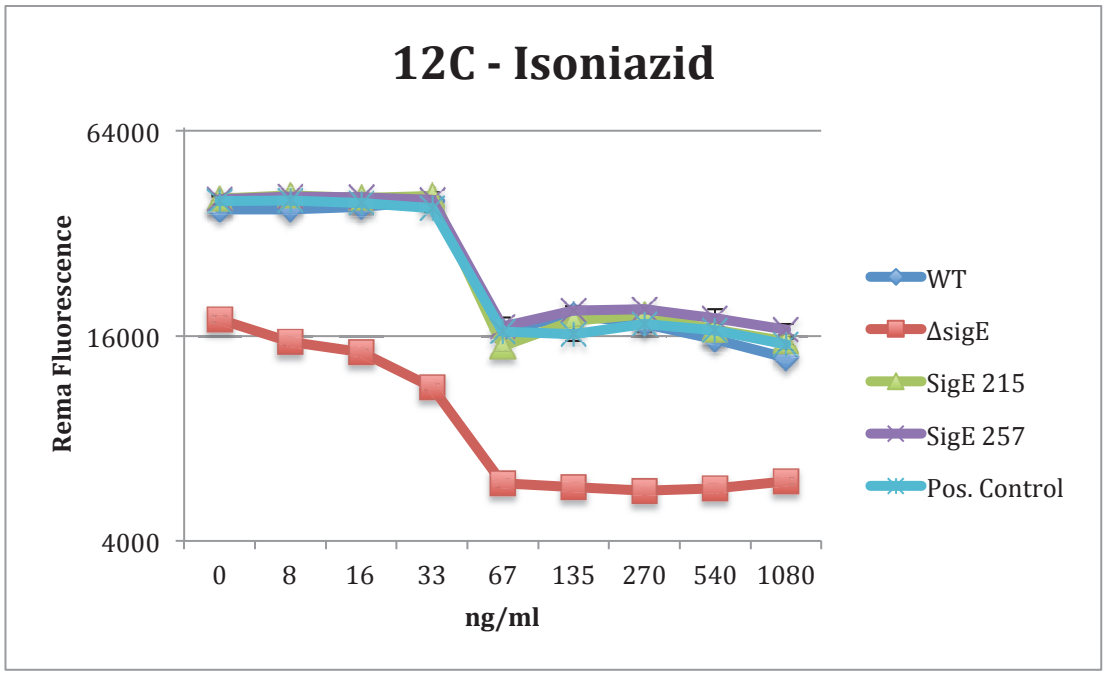
It is not known whether these two smaller isoforms, which are produced following oxidative stress and heat shock, and the largest isoform (257 aa), which is produced following surface stress, have the same physiological role. (Manganelli R. & Provvedi R., 2010)

To further characterize the role of SigE in the phenotypes observed, we decided to test, with increasing concentrations of the same drugs used in the previous experiments, derivatives of the *ΔsigE* mutant complemented with both isoforms of the protein (Provvedi and coll., unpublished data).

Results of the MIC experiments for the drugs streptomycin, rifampicin and isoniazid are shown in **Figure 12**.


12A - Streptomycin
48000
12000
3000
Fluorescence
0
156,25
312,5
625
1250
2500
5000
10000
20000
ng/ml
WT
ΔSIGE
SigE 215
SigE 257
Pos. Control


12B - Rifampicin
48000
12000
3000
Rema Fluorescence
0
12
25
50
100
200
400
800
1600
ng/ml
WT
ΔsigE
SigE 215
SigE 257
Pos. Control



As it is shown in the charts, except for streptomycin where the small isoform shows only "partial" complementation, with all the other drugs tested both isoforms and the positive control were able to restore the basal level of resistance.

Role of SigE in *M.tuberculosis* persisters formation

As previously described, one of the major problems in the control of a *M.tuberculosis* infection is the presence of multi-drug tolerant cells not killed by antibiotics, responsible for the relapse of the disease and causing the need for a long-term therapy with different drugs.

It has been demonstrated that a small part of a bacterial population during normal growth, either in a stochastic (casual way) or deterministic manner (in response to enviromental stimuli), enters into a non-replicating dormancy state, in which it becomes tolerant to high levels of bactericidal antibiotic concentrations.

Although this phenomenon is not yet fully understood, it has been hypothesized that sigma factors and two component systems may play an essential role during *Mtb* persistence. (Wang X. Z. et al., 2011)

SigE is subjected to a very complex regulatory network (**Figure 5**) which includes several feed forward regulatory loops, regulation by two component systems and other sigma factors.
This complex regulation may result in bistability and SigE has been proposed as one of the major *Mtb* bistable switches responsible of persisters formation. (Sanyal S. et al., 2013)

For this reason we decided to investigate the impact of SigE deletion on persisters formation after drug treatment.
Using the persisters assay we have developed (see materials and methods), we exposed late stationary phase bacterial cultures of H37Rv, the *sigE* mutant and its complemented strain to a concentration 25 times higher than the MIC for the drugs vancomycin, gentamycin, streptomycin, rifampicin, ethambutol, isoniazid and ciprofloxacin. At different times after exposure to drug (0, 3, 6 and 10 days) an aliquot of each culture was collected, washed and diluted in fresh media without drug and the days needed to reach an OD_{540} of 0.1 was determined.

As shown in **Figure 14**, while in the absence of drug and in the presence of ciprofloxacin, all strains needed the same time to resume growth, after treatment with all of the other drugs the *sigE* mutant needed more time to resume growth suggesting a reduced number of persisters in its cultures.

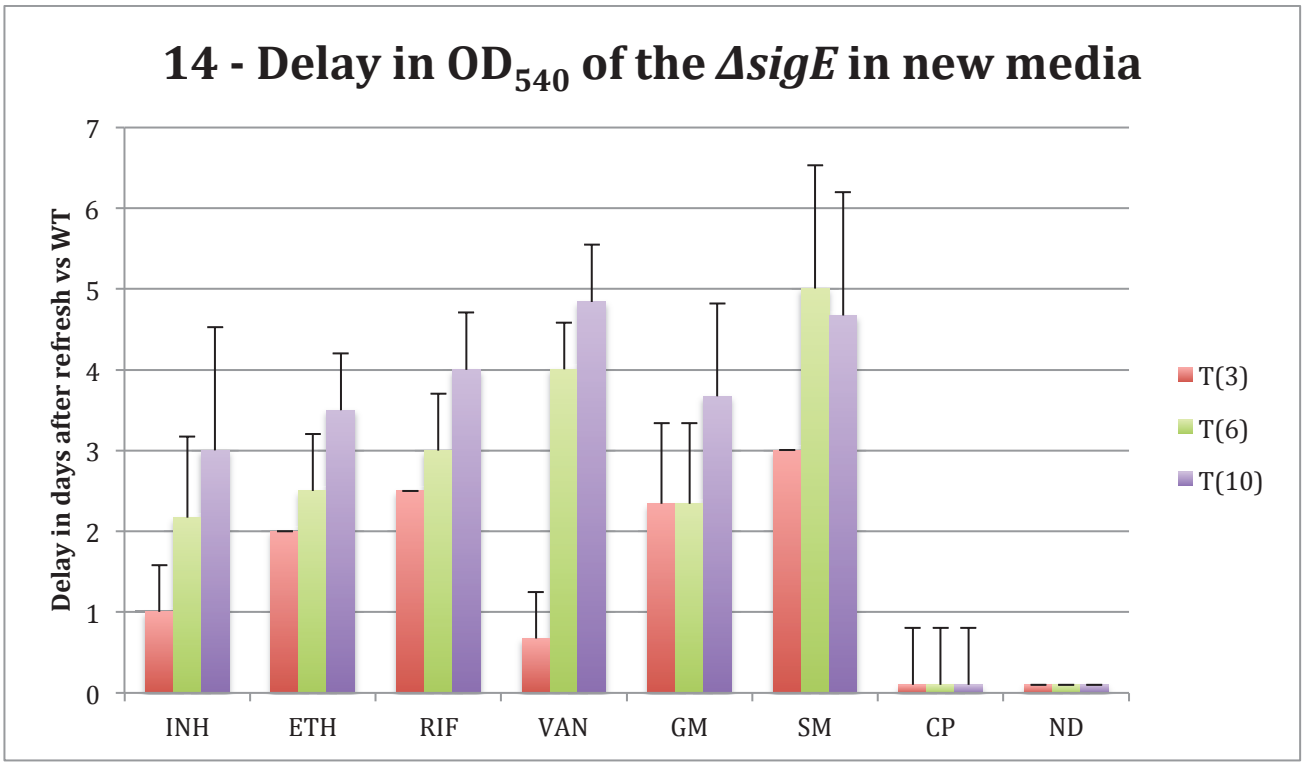
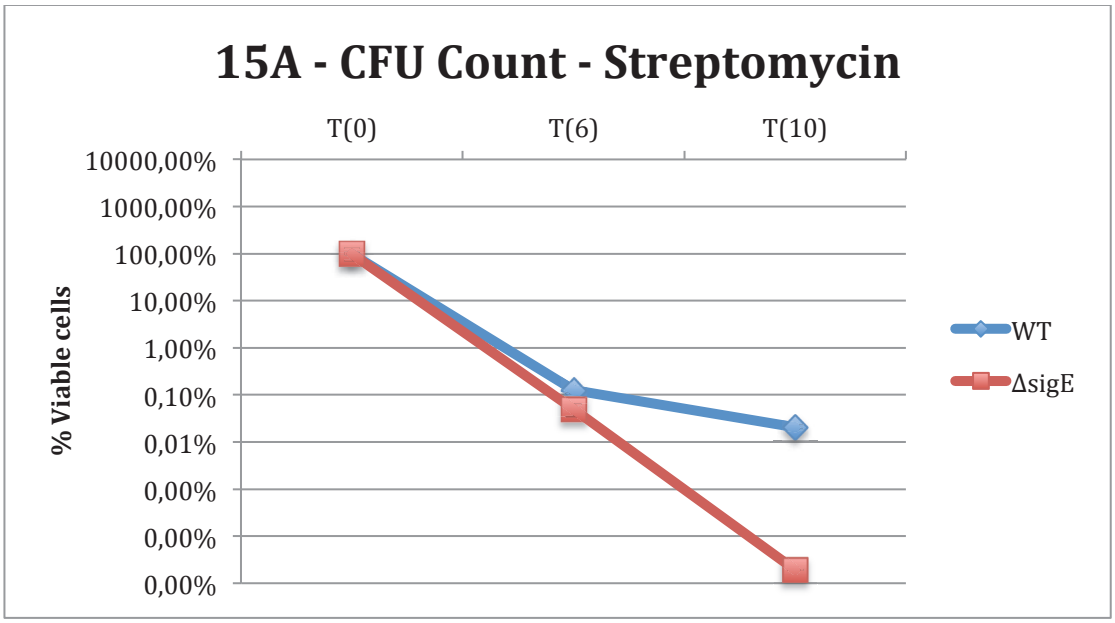


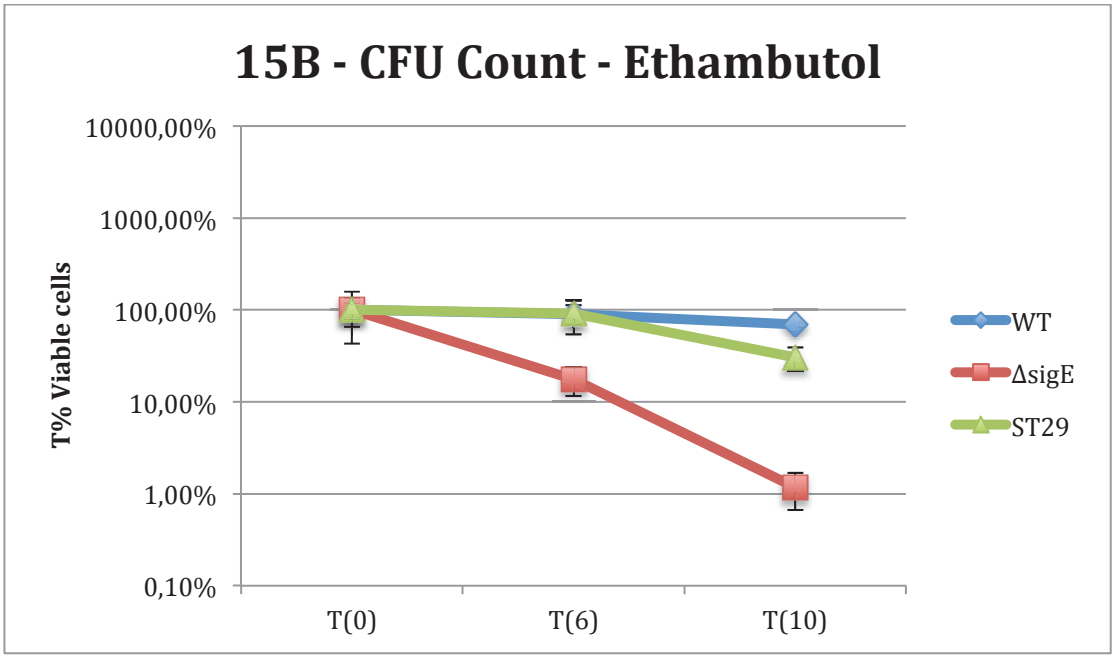
Figure 14: Delay in growth of the *sigE* mutant vs the parental strain, when cultures were exposed to a high concentration (25X MIC) of the various antibiotics. A delay in the growth of the mutant may indicate a possible lower number of persister cells.

To confirm that the differences observed were indeed due to the presence of a smaller number of persister cells in the *sigE* mutant cultures, we also followed the drop of viable counts in the cultures treated with streptomycin and ethambutol.

As it is possible to observe from **Figure 15**, while the H37Rv with the streptomycin follows a typical biphasic pattern in the CFU assay (fast drop in the first few days followed by a plateau phase in which the drop in the number of viable cells is lower), indicating the presence of persister cells, the counts for the *sigE* mutant continued to decline at the same rate for the whole experiment (10 days), reaching a number that is 1000 fold lower than that of the wild-type strain.

When bacteria were treated with ethambutol, a bacteriostatic drug, as expected the mortality of wt and complemented strains was very low, if any. However, the *sigE* mutant showed a decrease in viable counts of about 100 folds. As a control we also followed viable counts in cultures not exposed to drugs. In this case all strains behaved in the same way.


15A - CFU Count - Streptomycin
T(0)
T(6)
T(10)
10000,00%
1000,00%
100,00%
10,00%
1,00%
0,10%
0,01%
0,00%
0,00%
0,00%
% Viable cells
WT
ΔsigE


15B - CFU Count - Ethambutol
10000,00%
1000,00%
100,00%
10,00%
1,00%
0,10%
T% Viable cells
T(0)
T(6)
T(10)
WT
ΔsigE
ST29

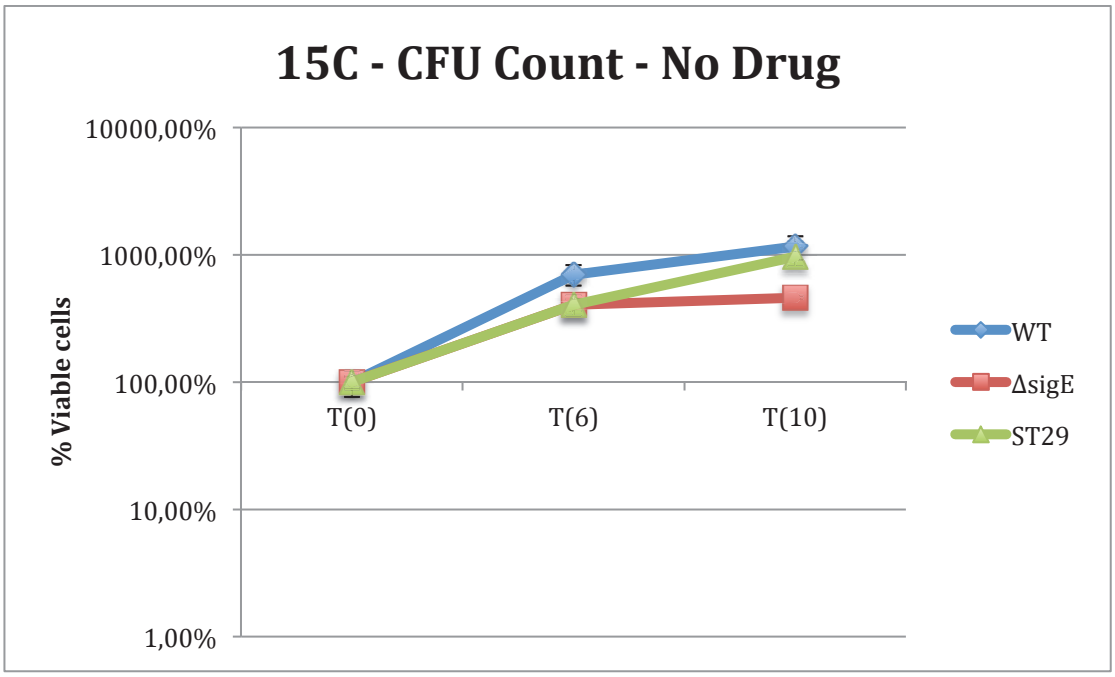


Figure 15: CFU counts for the wt, *sigE* mutant and complemented strain (ST29) exposed to a 25X MIC concentration of the drugs (15A) Streptomycin, (15B) Ethambutol, (15C) No Drug in the persister assay.

Role of SigB in *M.tuberculosis* persisters formation

It is known that *sigB*, is induced by different stresses (Manganelli R. et al., 1999) and is positively regulated by three extracytoplasmic function sigma factors, SigE , SigH and SigL (Dainese E. et al., 2006; Manganelli R. et al., 2002; Manganelli R. et al., 2001).

SigB levels are decreased in a *sigE* mutant during logarithmic growth, SDS-mediated stress and hypoxia and deletion of *sigB* in *M. tuberculosis* results in higher sensitivity to SDS, heat shock, oxidative stress and hypoxic conditions.

Furthermore, it has been demonstrated that expression of other sigma factors is unchanged after *sigB* induction, thus suggesting that *sigB* functions as an end regulator in the sigma factors nework (Lee J. H. et al., 2008).

For this reason, we decided to investigate if also SigB, like its cognate SigE, may have a role in persisters formation under stress conditions like those caused by high concentration of bactericidal antibiotics.

We therefore performed the same assay used to investigate the impact of *sigE* deletion in persisters formation and described previously. Results for the *sigB* mutant are shown in **Figure 16A** for the streptomycin, rifampicin, isoniazid and ethambutol.

Results have been confirmed by CFU counts for streptomycin, rifampicin and ethambutol (**Figure 17**).

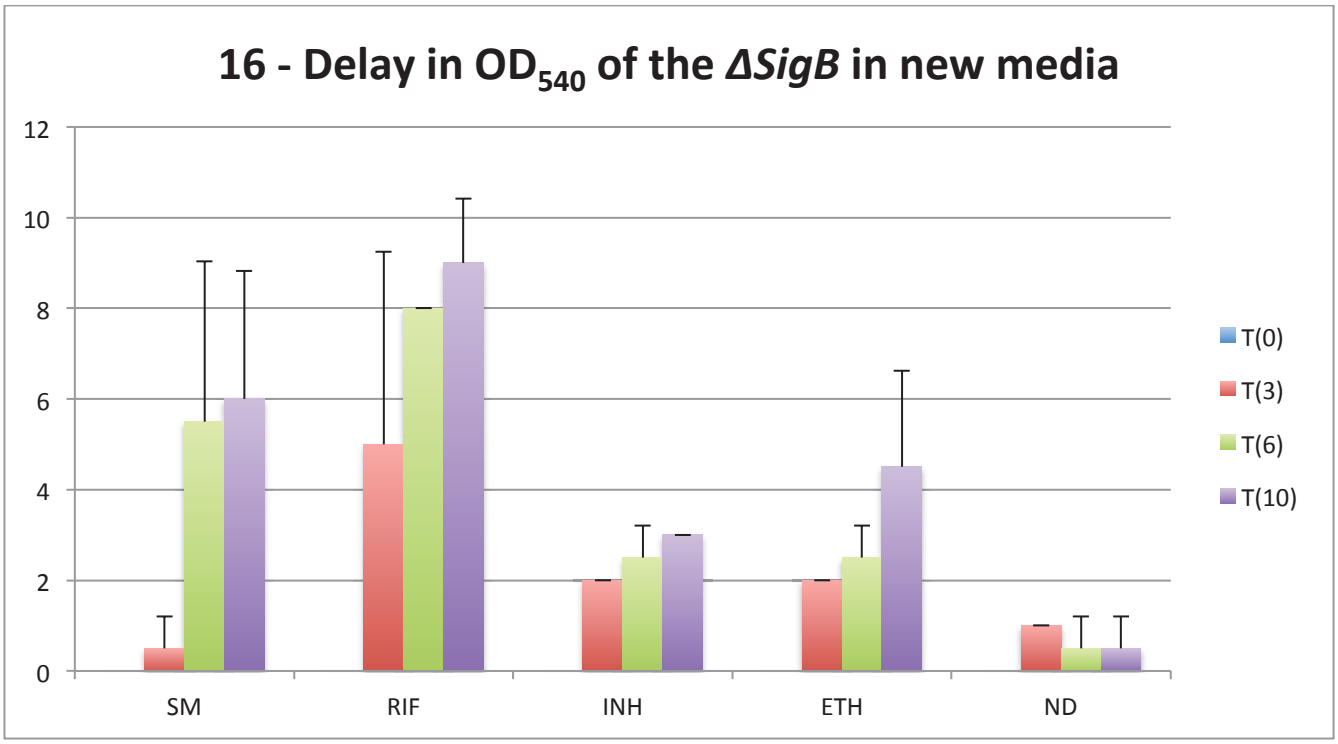
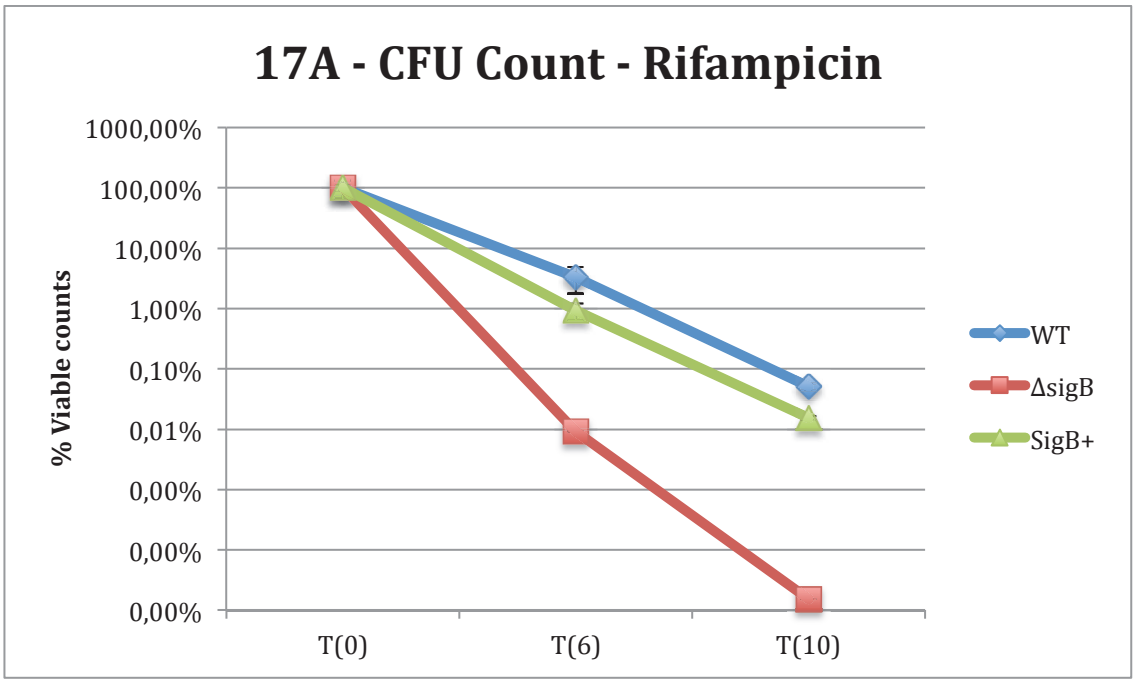
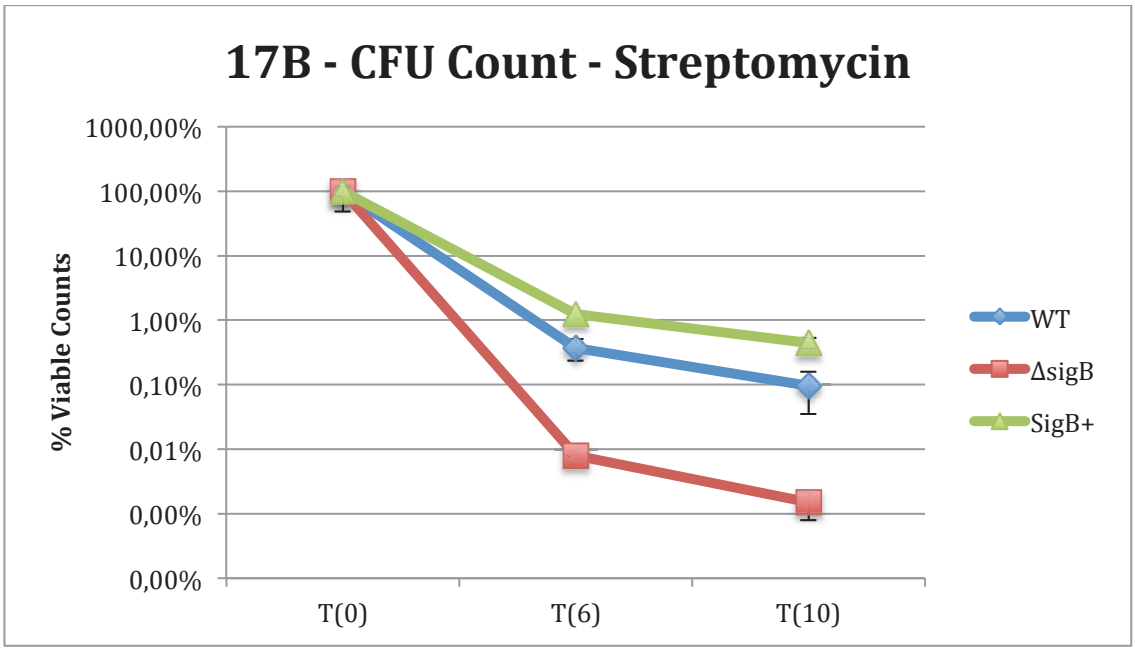


Figure 16: Delay in growth of the *sigB* mutant vs the parental strain, when cultures were exposed to a high concentration (25X MIC) of the various antibiotics. A delay in the growth of the mutant may indicate a possible lower number of persisters cells.


17A - CFU Count - Rifampicin
% Viable counts
1000,00%
100,00%
10,00%
1,00%
0,10%
0,01%
0,00%
0,00%
0,00%
T(0)
T(6)
T(10)
WT
ΔsigB
SigB+


17B - CFU Count - Streptomycin
% Viable Counts
1000,00%
100,00%
10,00%
1,00%
0,10%
0,01%
0,00%
0,00%
T(0)
T(6)
T(10)
WT
ΔsigB
SigB+

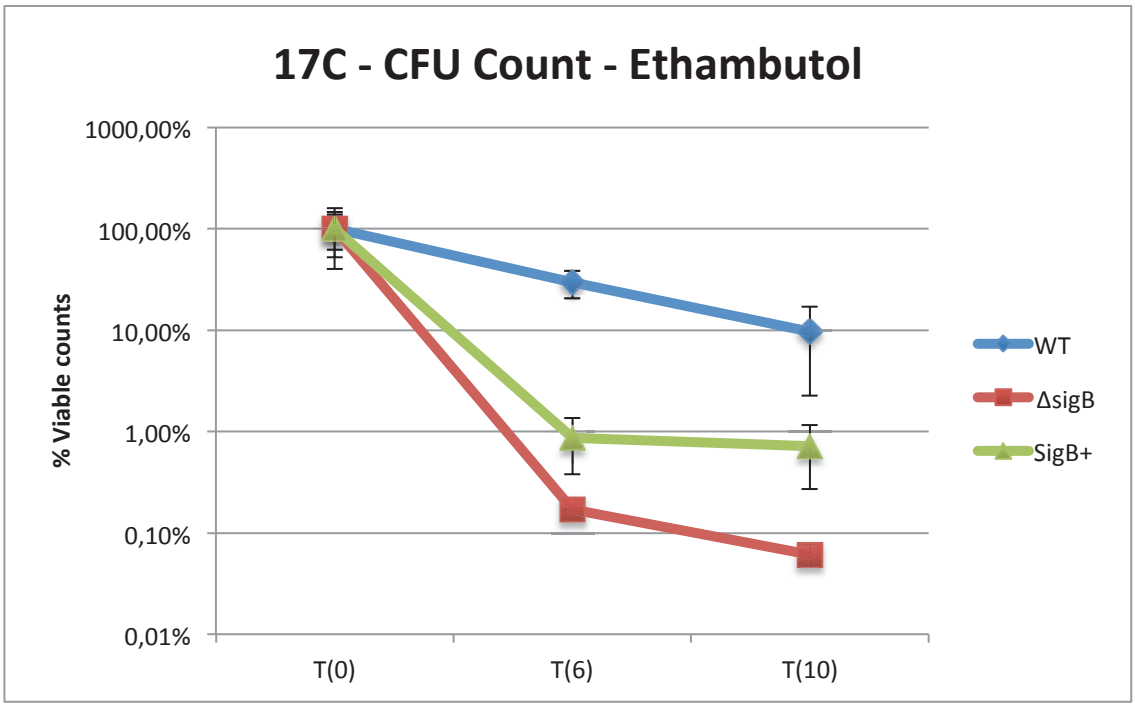


Figure 17: CFU count for the wt, *sigB* mutant and complemented strain (ST82) exposed to a 25X MIC concentration of the drugs (17A) Rifampicin, (17B) Streptomycin, (17C) Ethambutol in the persisters assay.

As it is possible to observe from the charts, even with the *sigB* mutant we were able to observe a decrease in the number of persister cells when the bacterial population was exposed to high concentration of the drugs. This is not surprising since the regulation of *sigB* by SigE during stress conditions and the interplay which occurs between sigma factors in the gene regulatory network.

Interestingly, with the isoniazid drug we couldn't notice any major difference in the drop of viable counts between the *sigB* mutant and its parental strain (data no shown), but this seems to be consistent with what we observed in the previous experiments on drug sensitivity.

DISCUSSION

Persisters continue to be an enourmous challenge for *M.tuberculosis* therapy, as they are responsible for relapse of the disease and for the need of a lenghty treatment.

Numerous metabolic pathways and gene interactions are involved in persisters formation and thus coordinated expression of different virulence determinants, including those involved in intermediary and secondary metabolism, cell wall processes, stress response and signal transduction pathways, is required for persisters to develop.

Particularly, as shown in **Figure 5**, sigma factors and two component systems may play an essential role in this phenomena.

In this work, we focused on two important sigma factors, SigE and SigB, trying to shed light on their role in *M.tuberculosis* persistence.

SigE is subjected to a very complex regulatory network and recently it has been proposed as one the major bistable switches responsible for persisters formation (Sanyal S. et al., 2013). Furthermore, SigE is one the most important players in *Mtb* stress response and plays a fundamental role in adaptation to heat, surface and oxidative stress (Manganelli R. et al., 2001).

It is known that during antibiotic treatment bacteria are subjected to various kind of stresses such as oxidative and surface stresses.

To test if SigE plays a major role in the adaptation to stress caused by antimicrobials we subjected a *sigE null* mutant to increasing concentrations of different drugs such as streptomycin, gentamicin, (aminoglycosides), rifampicin (inhibition of protein synthesis), isoniazid, ethambutol (cell wall inhibition) and ciprofloxacin (fluoroquinolone), using the Alamar Blue assay for determination of the MIC (see materials and methods).

With basically all the drugs tested the *sigE mutant* was more sensitive than the parental (WT) and complemented strains (ST29) to lower concentrations of the drugs. (**Figure 7**)

Interestingly, with isoniazid and ethambutol the inhibition profile we obtained was different from what observed with other antibiotics, as we did not notice any difference in the MIC for the mutant, but instead we noted a difference in the amount of fluorescence drop that was higher with the mutant respect to the WT.

Futhermore, we were not able to recognize any major difference in sensitivity between the mutant, parental (WT) and complemented strains (ST29) when ciprofloxacin was used.

Whether these different behaviors may be attributable to the different mechanisms of action of the drugs tested remains to be investigated. However, it is clear from what we observed that the *sigE* mutant shows a 2-4 fold decrease in MIC value when exposed to drugs that act by inhibiting the protein synthesis, while a different killing kinetic seems to occur when the bacillus is exposed to drugs that act by inhibiting the cell wall synthesis.

Because sigma factors regulation is subjected to very complex gene interactions and there are interconnections between the sigma factors themselves, in a way in which some of them can regulate others (as it is known for *sigB* for example, being regulated by *sigE, sigH* and *sigF*), we decided to test some others sigma factors mutants and particularly *sigL, sigG* and *sigB*, using the same assay and drugs with which we tested the *sigE* mutant.

While we did not observe any difference in antibiotic sensitivity for the mutants compared to their parental strains (**Figure 9**), the *sigB* mutant showed an increased sensitivity to isoniazid and ethambutol.

Interestingly, also in this case the inhibition profile was the same as that observed for the *sigE* mutant.

Thus, to further investigate the impact of *sigE* and *sigB* deletion on isoniazid and ethambutol sensitivity, we performed two additional experiments.

In the first one we exposed the mutants and their parental strains to increasing concentrations of the two drugs and we followed their growth by optical density. Results of this experiment confirmed that

both mutants were susceptible to the ethambutol drug, while only the *sigE* mutant showed an increased in sensitivity to the isoniazid. (**Figure 10**)

In the second one, we performed an MBC assay to determine if the differences observed in MIC experiments were due to a higher degree of killing of the drugs against the mutants. This experiment confirmed that while both mutants were more sensitivity to ethambutol, showing a 20-fold decrease in the number of viable counts respect to the parental strain (WT), only the *sigE* mutant was more sensitive to the isoniazid as no difference was observed between the *ΔsigB* and the WT.

Why there is such a different response between the two mutants with two drugs that share a similar mechanism of action it is not clear and certainly need to be investigated more in depth.

Relying on the results we obtained, we can speculate that the molecular pathway that involves *sigE* is more important in the response to stress caused by isoniazid probably because of the *kasA* and *kasG* genes, that may be under direct control of the "*mprA/B – sigE – relA*" pathway. This is consistent with the fact that *sigB* expression is regulated by SigE, since *sigB* contains a sigE-regulated promoter, so the effect we observed in both mutants with the ethambutol drug may be attributable to the fact that *sigB* may function as an end regulator in the response to stress caused by ethambutol, while for other drugs other pathways are required and some of them may require *sigE* as an intermediate regulator.

However, the differences we observed in drug sensitivity with the *sigE* mutant seem to be specific of this sigma factor and not shared among the others we tested.

sigE is transcribed by three promoters, namely P1-P3. P1 is active under normal physiologic conditions and is responsible for the basal level of *sigE* expression, while P2 is induced upon surface and alkaline stress and P3 upon oxidative stress. (Donà V. et al., 2008). Interestingly, translation of mRNAs originating from P3 results in the production of two smaller σ^E isoforms.

Unfortunately, it is not clear if these two smaller isoforms, which are produced following oxidative stress and heat shock, and the largest one have the same physiological role in *M.tuberculosis*.

For this reason, we decided to subject derivatives of the *ΔsigE* mutant, complemented with both small and large isoforms of the protein, to the same MIC experiments carried out before with the *sigE* and *sigB* mutants. Our results showed that, except for streptomycin, for which we obtained a "partial" complementation of the basal phenotype with the *ΔsigE* derivative containing the small isoform, for all the other drugs we couldn't notice any major difference in the antibiotic susceptibility phenotype between the two isoforms, making us believe they are both essential for the bacteria to respond to all the various kind of stresses generated by antibiotic exposure.

Furthermore, following some evidences (Foti J.J. et al., 2012) that oxidation of the guanine nucleotide pool is the leading cause of death by bactericidal antibiotics and that *mutT* gene (a nucleotide sanitizer) was able to mitigate the effects of this conditions in *E.coli*, it would be of great interest to investigate if a similar mechanism may exist in *Mtb* and if this may be correlated with the susceptibility phenotype we observed with the *sigE* mutant.

For this reason, to better characterize the sigE regulatory pathway involved in the sensitivity phenotype observed, the next step in our research will be to complement our *sigE* mutant with *mutT Mtb* orthologs and with two important transcriptional regulators downstream of SigE: SigB (*Rv2710*) and Clgr (*Rv2745c*), in order to verify if, in the absence of a functional *sigE* gene, their expression is sufficient to restore the basal wild-type phenotype.

Persistence is a major problem in facing *M.tuberculosis* infection because of the presence of multi-drug tolerante cells not killed by antibiotics, that are responsible for reactivation of the disease and for the lenghty treatment (6 months of chemoterapy).

Although this phenomenon is not yet fully understood, it has been hypothesized that sigma factors may play a major role in contributing to it.

SigE, particularly, has been proposed as one of the major *Mtb* bistable switches responsible of persisters formation.

For this reason, using a *sigE* null mutant, we investigated the contribute of this sigma factor on persisters production following prolonged exposure to high concentrations of antibiotics.

Using the persisters assay we have optimized, we were able to quantify the amount of persister cells produced by the *sigE* mutant compared to the parental strain.

Our results clearly indicate a role for SigE in persisters production following exposure to high concentration of antimicrobials. For most of the drug we tested, except ciprofloxacin for which the total amount of persister cells was the same between the mutant and parental strain, we obtained a lower number in viable counts for the mutant versus the wild-type and complemented strains, up to reach 1000 fold lower counts with streptomycin.

Furthermore, because *sigB* is induced by different stresses and its expression during stress response is regulated by other sigma factors, including SigE, we decided to test, using the same assays, if also SigB, like its cognate SigE, may have a role in persisters formation.

Also in this case, we were able to observe a decrease in persister numbers for the *sigB* mutant, when the bacterial population was exposed to high concentration of the drugs for a long time. This was not surprising, since the interplay between these two sigma factors in gene regulatory networks pathway and since *sigB* expression is modulated by SigE. We suspect the *sigB* may be the end regulator of the sigma factors cascade in some of the regulatory pathways that lead to persister cells generation.

In conclusion, in this work we successfully demonstrated, using some simple as effective approaches, how the sigma factors SigE and SigB have a role in response to stresses caused by antibiotics. Particularly, while SigE seems to have a broader role in response to stress caused by many different

classes of antibacterial drugs, SigB seems to only have a role in response to stress caused by antibiotics that act on the cell wall synthesis.

Moreover, we demonstrated that both sigma factors have a role in the pathway that leads to the production of persisters cells in response to high concentration of antimicrobials, and this makes them excellent targets to develop new innovative drugs that can shorten the treatment regimen of a *M.tuberculosis* infection.

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