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Characterization of the activation process of the sigma factor $\boldsymbol{\sigma E}$ regulatory network in *Mycobacterium tuberculosis* under stress conditions

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ABSTRACT (Italiano)

SigE (σ^E) è uno dei 13 fattori sigma codificati dal genoma di *Mycobacterium tuberculosis*. Esso ha un ruolo fondamentale nella virulenza ed è implicato in una circuito di regolazione molto complesso che coinvolge altri regolatori tra cui il sistema a due componenti MprAB, il regolatore pleiotropico ClgR e il fattore anti-sigma RseA.

Attraverso esperimenti di real-time PCR, la dinamica di trascrizione di diversi geni *sigE*-dipendenti è stata studiata: *sigE*, *sigB* la cui trascrizione è dovuta a SigE sia in condizioni fisiologiche che sotto stress, *rseA* e *clp,* codificante per una proteasi responsabile della degradazione del complesso SigE-RseA dopo la fosforilazione da parte di PknB.

Il circuito di regolazione di SigE è stato analizzato in ceppi mutanti simulando le condizioni a cui i bacilli tubercolari sono esposti durante il processo di infezione.

I risultati ottenuti dimostrano il ruolo fondamentale di MrpAB e ClgR nel permettere una risposta efficace in condizioni di stress superficiale infatti la loro presenza è necessaria per indurre la trascrizione di *sigE*. L'effetto dell'attività di SigE è visibile nella dinamica trascrizionale di *sigB* che, per essere indotto, necessita sia del sistema a due componenti MprAB sia di ClgR. A pH acido, l'assenza di MprAB e ClgR ha portato ad una risposta allo stress ritardata. In entrambe le condizioni, invece, il fattore anti-sigma RseA deve essere fosforilato per attivare una risposta completa mediata da *sigE*.

Questi risultati forniscono una visione più chiara del meccanismo di adattamento allo stress di *M. tuberculosis* e consentono una migliore comprensione della fisiologia di questo potente patogeno.

ABSTRACT

SigE (σ^E) is one of the 13 sigma factors encoded by the *Mycobacterium tuberculosis* chromosome, it is implicated in a very complex regulatory network involving other regulators such as the two component system MprAB, the pleyotropic regulator ClgR and the anti-sigma factor RseA, and its role is of prime importance for virulence.

Through real-time PCR analysis, the dynamic of transcription of several *sigE* dependent genes was studied: *sigE* itself*, sigB* whose transcription is due to SigE both in physiological and under stress conditions*, rseA* and *clp*, encoding a protease responsible for the degradation of the SigE-RseA complex after its phosphorylation by PknB.

The SigE regulatory network was analyzed in mutant strains under different stress conditions that mimic the challenging environments to which tubercular bacilli are exposed during the infection process.

The data strongly support the fundamental role of both MrpAB and ClgR to act out an efficient stress response under surface stress; indeed their presence is necessary to induce the expression of *sigE*. The effect of SigE activity could be seen in the transcriptional dynamic of *sigB* that requires the two-component system and ClgR to be induced and sustained.

In acidic pH, the absence of MprAB and ClgR led to a delayed stress response. In both cases, the anti-sigma factor RseA must be phosphorylated to activate a full *sigE*-mediated response.

These results provide a clearer insight into the mechanism of adaptation to specific stress in *M. tuberculosis* and allow a better understanding of the physiology of this powerful pathogen.

1. INTRODUCTION

1.1 Mycobacteria

The family Mycobacteriaceae belongs to the order Actinomycetales and consists of a single genus, *Mycobacterium*. *Mycobacterium sp*. are thin, slightly curved to straight, 0.3–0.5 µm in diameter and of variable length non-spore-forming, non-motile bacilli. The genus consists of more than 50 species, which can be found in water, food, soil, and vegetation (1). Mycobacteria typically are free-living environmental saprophytes and strict pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, form rare exceptions within the genus *Mycobacterium* (2).

Traditionally, mycobacteria are identified by phenotypic traits, such as morphological features, growth rates, preferred growth temperature, pigmentation, and biochemical profiles. It is critical to identify mycobacteria to the species level to address the clinical significance (non-pathogenic versus pathogenic species) (3). Practically, it is usefull to divide the genus in two different groups: rapid growers and slow growers mycobacteria. (4) The rapid growers were confirmed to be the most ancestral mycobacteria and forming colonies within 3–7 days while slow growers require more than 7 days on subculture to form colonies. Most of the major pathogenic mycobacteria are slow growers. Concerning pathogenicity, some of the non-tuberculous mycobacteria have also been shown to cause disease in humans especially in immunocompromised individuals. However not all pathogenic are slow growers, such as *M*ycobacterium *abscessus*, a commonly isolated rapidly growing non-tuberculous mycobacteria, that is the third most common cause of lung disease among this phylum (5). The most relevant species belonging to the slow growers mycobacteria are that of *Mycobacterium tuberculosis* complex (MTBC), a group of mycobacteria that comprises closely related species that cause tuberculosis (TB) in animals, including humans, such as *M. tuberculosis, Mycobacterium africanum, Mycobacterium bovis, Mycobacterium canettii, Mycobacterium microti, Mycobacterium pinnipedii* and *Mycobacterium caprae* that are genetically related (6).

In this study, two species were analysed and compared to each other: the rapid grower, non-pathogenic *Mycobacterium smegmatis*, and the pathogenic, slow grower, *M. tuberculosis.*

1.1.2 Mycobacteria cell envelope

The most important feature of all mycobacteria is the peculiar structure of the cell envelope that is composed by lipids and carbohydrates forming a permeability barrier against hydrophilic drugs, crucial for survival and virulence (Fig. 1) (7). The protective cell envelope is composed of the cytoplasmic membrane, cell wall, surface lipids and a capsule, and is populated by numerous proteins. As the mycobacterial cell envelope is the interface with the external environment, it is important to understand its organization and regulation, above all in the context of an *M. tuberculosis* infection. The mycobacterial cytoplasmic membrane contains standard membrane lipids such as the glycerophospholipids, but also has phosphatidylinositol mannosides (PIMs), and lipomannans (LMs) and lipoarabinomannans (LAMs). PIMs likely contribute to the low permeability of the mycobacterial cell envelope and intrinsic tolerance to antibiotics. PIMs, LMs and LAMs are critical for cell wall integrity and for immune manipulation. PIMs, which are exclusively found in actinomycetes, are phospho-myo-inositols attached to one to six mannose residues. These are anchored in the membrane with one to four fatty acid chains linked to various sites on a 1-phospho-2-mannose myo-inositol base (PIM1), making AcPIM1. PIMs with four mannose residues (AcPIM4) are thought to be transported from the inner leaflet to the outer leaflet of the cytoplasmic membrane. It was predicted that PIMs predominate in rapid growth mycobacteria. Peptidoglycan maintains cell shape and fortifies the plasma membrane against the osmotic pressure of the cytoplasm. It is composed of linear strands of alternating N-acetylmuramic acid and N-acetylglucosamine (GlcNAc) sugars crosslinked by peptides into a layered stucture. The serine/ threonine protein kinases (STPK) PknB is the master upstream regulator of peptidoglycan metabolism during active growth. The middle layer of the cell wall core is made of arabinogalactan sugars. Arabinogalactan polymers are connected to peptidoglycan with a rhamnose–GlcNAc linker disaccharide, in which the GlcNAc attaches to the N-acetylmuramic acid residues in peptidoglycan and the rhamnose binds to galactan polymers. The mycomembrane is composed of lipids, glycolipids and secreted proteins. Its outer leaflet is composed mostly of mycolic acids, which can be free or attached to trehalose sugar to make trehalose monomycolate (TMM) or trehalose dimycolate (TDM) (8). On the practical point of view, when cell walls are disrupted, for instance extracted with various solvents, the free lipids, proteins, LAM, and PIMs are solubilized, and the mycolic acid–arabinogalactan–peptidoglycan complex

remains as the insoluble residue. In simplistic terms, it can be considered that these lipids, proteins, and lipoglycans are the signaling, effector molecules in the disease process, whereas the insoluble core is essential for the viability of the cell and should be addressed in the context of new drug development (9).

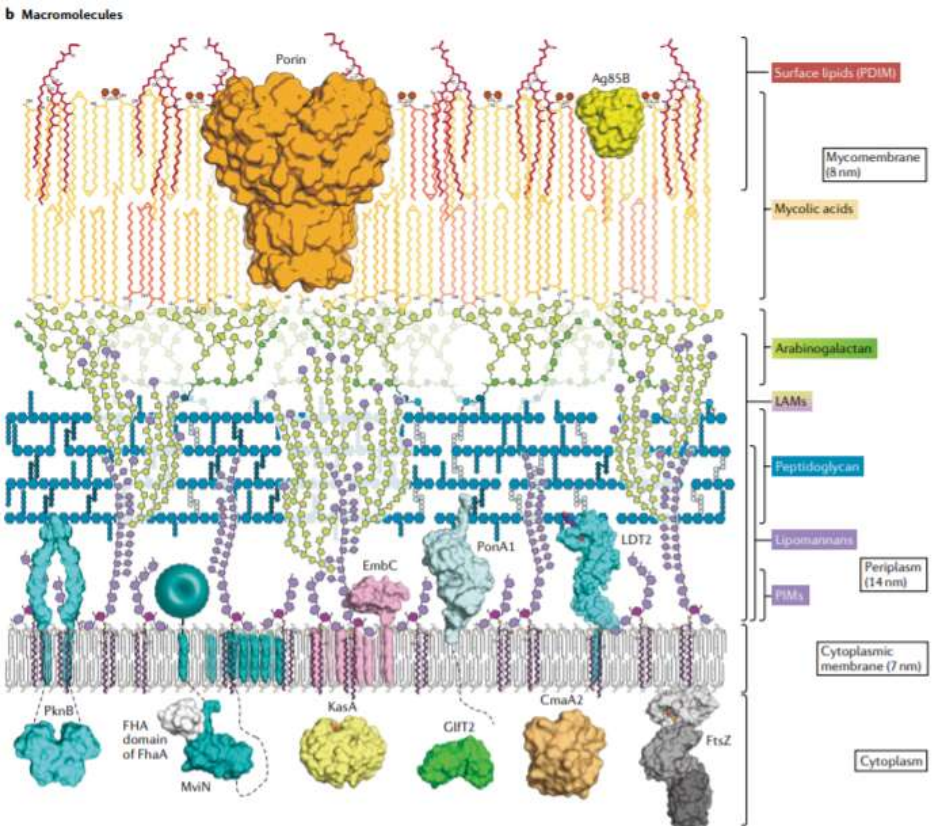


Fig. 1: Schematic representation of the cell envelope layers in Mycobacteria (9).

1.2 *Mycobacterium tuberculosis* and tubercular disease

Tuberculosis is a communicable disease that is a major cause of ill health and one of the leading causes of death worldwide. Until the coronavirus (COVID-19) pandemic, TB was the first cause of death from a single infectious agent, ranking above HIV/AIDS. About a quarter of the world's population is infected with *M. tuberculosis*. Globally in 2020, there were an estimated 1.3 million deaths (Fig. 2) (10).

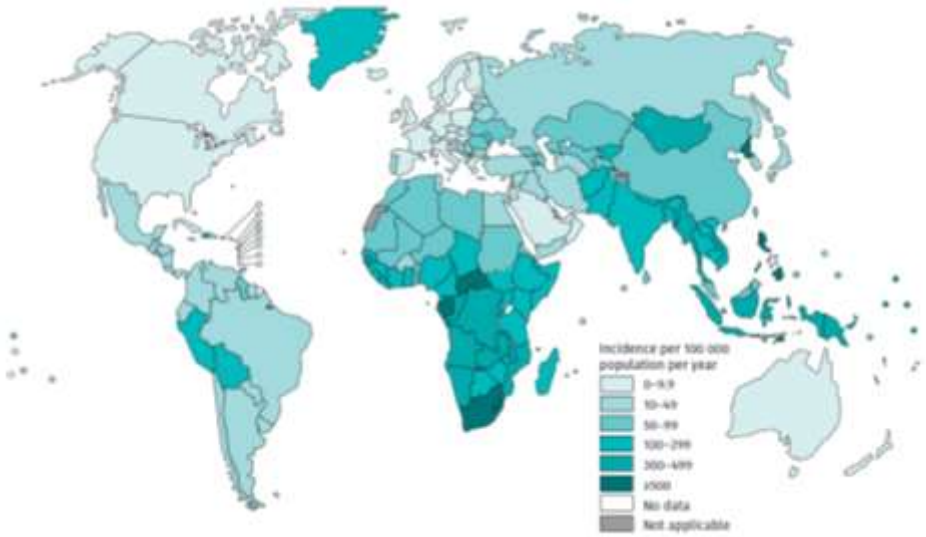


Fig. 2: Representation of tuberculosis cases relative to population size (the incidence rate) varied widely among countries in 2021. There were under 10 incident cases per 100 000 population in most high-income countries, 150–400 in most of the 30 high TB burden countries (10).

The genome of *M. tuberculosis* is rich in guanine and cytosine, composed by 4.411.532 base pair (bp) and more or less 4000 genes with 91% of coding activity. Most of them are involved in fatty acid metabolism to keep the integrity of the particular cell wall they are necessary to survive during infectious process or to persist better during latency.

The characteristic features of the tubercle bacillus include its slow growth, dormancy, complex cell envelope, intracellular pathogenesis and genetic homogeneity. The generation time of *M. tuberculosis* is typically ~24 hours (11). This contributes to the chronic nature of the disease, imposes lengthy treatment regimens and represents a formidable obstacle for researchers.

1.2.1 Clinical manifestation of tubercular disease

Pulmonar tuberculosis follows a general pattern in which the progression and resolution of the disease is divided into four stages. In the first stage, dating from 3 to 8 weeks after *M. tuberculosis* bacilli are inhaled and implanted in alveoli, the bacteria are disseminated by the lymphatic circulation to regional lymph nodes in the lung, forming the so-called primary complex. The second stage, lasting about 3 months, is marked by hematogenous circulation of bacteria to many organs including other parts of the lung; at this time in some individuals, acute and sometimes fatal disease can occur in the form of tuberculosis meningitis or miliary (disseminated) tuberculosis. Inflammation of the pleural surfaces can occur during the third stage, lasting 3 to 7 months and causing severe chest pain, but this stage can be delayed for up to 2 years. The last stage, where the disease does not progress, may take up to 3 years. In this stage, more slowly developing extrapulmonary lesions can appear in some individuals. However, most humans who are infected with TB do not exhibit progression of the disease. One-third of exposed HIV-negative individuals become infected, and of this number 3 to 5% develop TB in the first year. An additional 3 to 5% of those infected develop TB later in their lives. Immunosuppressed people can also be newly infected with *M. tuberculosis* and in many cases show rapid progression to active disease (12).

From a cellular viewpoint, *M. tuberculosis* is internalized by alveolar macrophages that provide a niche for *M. tuberculosis* replication. Macrophages, while protecting the hosts from invading mycobacteria, also facilitate the establishment of *M. tuberculosis* infection and allow it to stay in the latent stage of infection. The intracellular replication and the spreading of *M. tuberculosis* to adjacent pulmonary lymph nodes and other extrapulmonary tissue sites occur via lymphatics and blood circulation prior to the establishment of adaptive immune responses. The activation of adaptive immunity is dependent on the close interaction between dendritic cells and $CD4^+$ T cells, while macrophages facilitate both the innate and adaptive immune defense mechanisms. Activation of macrophages, together with the elicitation of adaptive immune responses result in phagosome-lysosome fusion and production of cytokines such as IFN-α, TNF-α, IFN-β, IL-1β, IL-6 and IL-12 can potentially cause significant lung inflammation and tissue destruction. Active tuberculosis, developed through either reinfection with *M. tuberculosis* or reactivation from latent infection is manifested by active replication of *M.*

tuberculosis and host tissue damage, leading to necrosis and cavitation. It remains largely unknown if the dynamic equilibrium between host immune system and *M. tuberculosis* in immunocompetent hosts is proficuos, or if it causes deterioration in host tissues due to exaggerated inflammatory responses promoting tissue damages in active tuberculosis state (13).

At the cellular level, the infection can be contained in a special structure called granuloma (Fig. 3) that is a compact, organized aggregate of mature macrophages that arises in response to a persistent stimulus (14). *M. tuberculosis* can go through a diversity of intra and extracellular locations, a factor, which contributes to the complexity of tuberculosis disease, is indeed that different bacterial subpopulations are present during the infection, and there are numerous barriers that prevent the penetration of the different drugs to reach their targets.

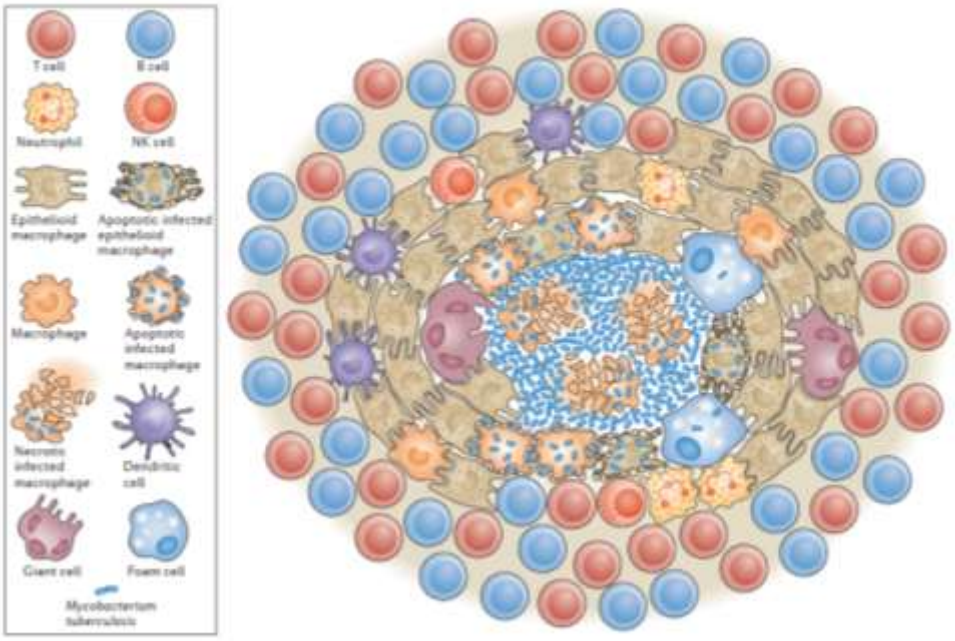


Fig. 3: Schematic rappresentation of granuloma: bacteria are most commonly present in the central necrotic areas and different cell types populate the granuloma, such as neutrophils, dendritic cells, B and T cells, natural killer (NK) cells, fibroblasts and cells that secrete extracellular matrix components (14).

1.2.2 Diagnosis

Despite the availability of advanced tools and techniques, early and accurate case diagnosis of TB remains a challenge. The choices of the screening and diagnostic tools for clinicians depend on the purpose and availability of the tests in the areas (15).

To diagnose *M. tuberculosis* infection, it is possible to carry out a microbiological or direct diagnosis, useful in cases of active disease, or an immunological or indirect diagnosis, which, however, does not distinguish latent from active infection. Using the direct method, the presence of mycobacteria is searched for in biological samples with microscopic and cultural examinations.

The most common direct method for diagnosing TB worldwide is sputum smear microscopy. This method is simple, inexpensive and quick, but their main drawbacks are low specificity and sensitivity. Acid-fast staining using Ziehl–Neelsen stain of sputum smear microscopy is a widely used method for the detection of acid-fast mycobacteria for TB diagnosis. However, this method cannot differentiate *M. tuberculosis* from other acid-fast bacilli, has low sensitivity and requires high concentration of bacilli in the sputum (16). The presence of tubercular bacilli has to be subsequently confirmed with culture examination.

Among indirect techniques, Mantoux tuberculin skin test was the most common test in the past. The reaction to intracutaneously injected tuberculin is an example of a delayed cellular hypersensitivity reaction. T-cells sensitized by prior infection are recruited to the skin injection site where they release lymphokines. These lymphokines induce induration through local vasodilation, edema, fibrin deposition, and recruitment of other inflammatory cells to the area. A person who has been exposed to the bacteria is expected to mount an immune response at the skin level containing the bacterial proteins.

The Mantoux test is technically difficult to administer and read, so false readings may occur if the tester has insufficient skill. The Food and Drug Administration, for this reason, approved a novel diagnostic test (QuantiFERON-TB GOLD, made by Cellestis, Inc.) for TB. The blood test detects the presence of *M. tuberculosis* infection by measuring interferon-gamma harvested in plasma from whole blood incubated with the *M. tuberculosis*-specific antigens, ESAT-6 and CFP-10 (17).

The use of molecular methods continues to transform the rapid diagnosis and treatment of patients with mycobacterial infections. The line probe assay technology (hybridization on strips) revolutionized tuberculosis molecular diagnosis. This began with the commercial availability of DNA probe technology, which could confirm the identification of the most commonly encountered *Mycobacterium* species, including *M. tuberculosis* complex, in positive cultures. This was followed by laboratory-developed species-specific and broad-range PCR assays, which have been used both on direct specimens and on aliquots from positive cultures. Most recently, a newly approved assay (i.e., Xpert MTB/RIF; Cepheid, Sunnyvale, CA) has been released and it detects *M. tuberculosis* complex and key genetic determinants of rifampicin resistance. The introduction of this assay allows the replacement of the traditional acid-fast bacillus smear with molecular methods, as well as the more rapid detection of and appropriate treatment for MDR-TB (18).

Early identification of people with symptomatic TB, not only allows therapy to be administered before serious damage occurs, but also helps prevent the spread of the bacteria to other people.

1.2.3 Treatment

Four hypothetical populations of organisms may exist in a patient with tuberculosis:

- actively growing organisms, usually present extracellularly;
- slow, intermittently growing organisms in an unstable part of the lesion;
- organisms surviving under microaerobic conditions in a low environmental pH, either in inflammatory lesions or within phagolysosomesof macrophages;
-completely dormant organisms surviving under anaerobic conditions (19).

The shortest required duration of treatment is 6 months. The standard regimen today comprises the combination of isoniazid, rifampicin, pyrazinamide and ethambutol followed by isoniazid and rifampicin for a further 4 months. Approximately 85% of patients who take the six-month regimen will have a successful treatment outcome. Despite its effectiveness, the current treatment regimen of six months remains too long for many patients (10).

This regimen allows opportunities for interruptions in drug intake that may lead to the emergence of drug resistance, as well as creating a serious burden for both patients and

clinics. The second problem is the increasing prevalence of multidrug-resistant (MDR) strains of *M. tuberculosis* resistant to rifampicin and isoniazid, and sometimes to injectables and fluoroquinolones as well extensively drug-resistant (XDR). Any solution to these problems depends on an understanding of the two theoretical issues underlying the success of chemotherapy, namely the prevention of the emergence of drug resistance by the simultaneous use of two or more antibacterial agents, and the reasons for the very slow killing of all *M. tuberculosis* in the lesions (20).

1.2.4 Vaccination

BCG is the current vaccine against TB. It is the most administered human vaccine in the world, with more than 3 billion people vaccinated and over 200 million doses administered per year (10).

BCG is a live attenuated vaccine derived from *M. bovis*, the causative agent of tuberculosis in cattle (21). In adults, its efficacy against pulmonary disease is variable possibly because of environmental, operational, demographic, and genetic factors. For instance, prior exposure to environmental mycobacteria severely compromises protection afforded by BCG, and this is influenced by the extent of cross-recognition of antigens shared with the vaccine (22).

TB vaccine development programmes have largely been directed at replacing BCG. However, despite testing large numbers of vaccine candidates in small animal models, the degree of protection has been considerably lower than that observed with BCG (23). Even if the field of TB vaccine development has experienced significant hurdles, it is important to recognize the great progress made both in immunological understanding and in empirical learning from human clinical trials. An immunological understanding of the pathogenesis of *M. tuberculosis*, one of the principal barriers to designing an effective vaccine, has slowly but surely increased the possibility to have the knowledge we have today (24).

1.3 *Mycobacterium tuberculosis* and environmental condition

Bacteria growing in the same environment, and encoding the same genetic information, exhibit clear phenotypic heterogeneity. This heterogeneity is useful for long-term survival of the population under environmental stresses. This heterogeneity can be manifested as a simple two-state bistable population or a completely random heterogeneous population. As

a facultative intracellular pathogen, *M. tuberculosis* has developed a clever lifestyle to ensure its endurance within a host (25).

During infection, it is exposed to several different challenging environments, and the understanding of the stress-response mechanisms essential for its survival in these conditions is important to design new diagnostics and treatment strategies. The main environmental stresses encountered by the tubercular bacilli during infection are (26):

- Exposure to intermediate oxygen, nitrogen species and toxic substancesproduced by macrophages;
- pH reduction in the phagosome;
- Alveolar surfactant with anti-bacterial activity that destabilizes the organization of the mycobacterium wall lipids;
- Hypoxia in granulomas;
- Deficiency of nutrients.

In this study, the conditions that mimic surface stress and low pH are reproduced.

- Surface stress is one of the main conditions sensed by the bacteria during infection. Surface stress induced by sodium dodecil sulfate (SDS) damages the cell wall by alterating its functioning. SDS is an anionic detergent composed of charged head groups followed by hydrophobic tails consisting of hydrocarbon chains. It is known for its ability to break up membranes and denature proteins. In this project, its use is functional to reproduce the action of the alveolar surfactant and antimicrobial peptides on the mycobacterium wall. Due to surface stress, numerous wall components are damaged and the synthesis of mycolic acids is inhibited. The reduced biosynthesis of mycolic acids leads to an accumulation of fatty acids which are toxic at high concentrations. The sigma factor SigE regulates the expression of numerous genes involved in the detoxification of fatty acids and in restoring the biosynthesis of the wall components. The induction of *sigE*, in the presence of surface stress, is determined both by the activation of the two-component system MprAB and by the membrane serine-treonin kinase PknB (see below).

- *M. tuberculosis* can sense and adapt to acidic host environments during infection. The pathogen colonizes several compartments in human body that can range from mildly acidic phagosome to more strongly acidic area of lysosome (pH 4.5). The pH of macrophage in which *M. tuberculosis* resides is dependent by the activation state of the immune cells. Acidic pH stress reproduces the environment of the alveolar macrophages in which the bacilli reside. Inside granulomas, macrophages containing mycobacteria are activated by the action of INF-γ, produced by lymphocytes. The activation process consists of a series of modifications of the intracellular environment of the macrophage in order to kill the mycobacteria and stop the infection. Reactive nitrogen and oxygen species are produced, antimicrobial peptides are released, and fusion of the phagosomes, containing the bacilli, with the lysosomes occurs. Inside the phagolysosome there is a notable and sudden lowering of the pH up to values between 5.5 and 4.5. In this acidic environment, the mycobacterium is able to maintain the intracellular homeostasis and the cytoplasmic pH at a value of 7. The first barrier is represented by the cell wall which avoid the entry of protons (28). The involvement of sigma factors in acid pH stress is still poorly studied. Some studies report the interaction between SigE and PhoP, the response regulator of the PhoPR two-component system that is active in acid pH conditions and involved in virulence. Through protein-protein interaction studies, the physical interaction between SigE and PhoP have been shown to be required for the induction of some pH-inducible genes (Fig. 4). The acidic pH inducible genes are involved in the regulation of transcription, in lipid metabolism and in the production of membrane proteins including proteins with antigenic activity (29).

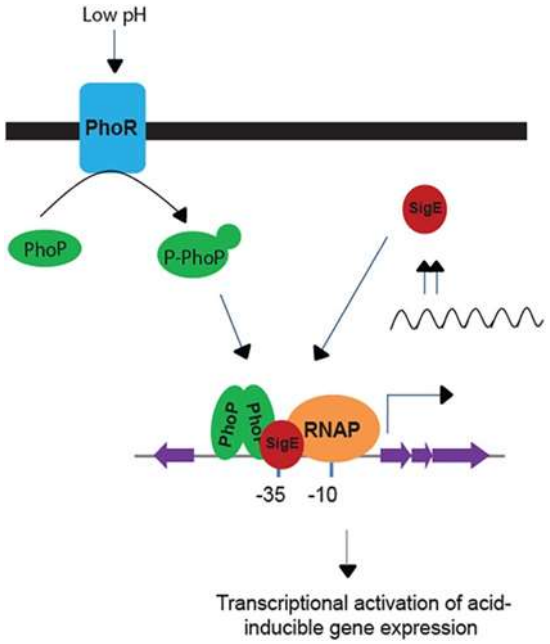


Fig. 4: Regulatory scheme of acid-inducible gene expression of *M. tuberculosis*. PhoP regulates acid-inducible gene expression both under normal condition and during acid stress, while SigE is recruited within the target promoter only during acid stress (29).

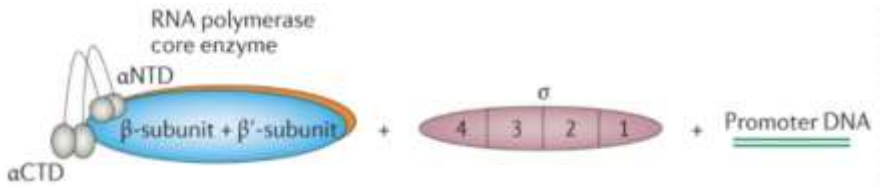
1.3.1 Transcriptional regulation in bacteria

The DNA-dependent RNA polymerase, that is composed by a stable complex known as *core,* mediates gene expression in bacteria. RNA polymerase (RNAP) core enzyme consists of one large β-subunit, one large β'-subunit, two α-subunits and the small ω-subunit. Each α-subunit consists of independently folded amino-terminal and carboxy-terminal domains that are joined by a flexible linker. The β-subunit and β'-subunit are assembled by binding to the N-terminal domains of the α-subunits, and form a cleft that contains the active site, whereas the ω-subunit is primarily a chaperone for the β'-subunit (30).

In bacteria, the initiation of transcription at promoters requires a dissociable subunit of RNA polymerase called sigma (Sig) that binds to the core to form the "holoenzyme". Bacterial promoters contain several discrete sequence motifs, including the –35 element, the extended –10 element, the –10 element and the discriminator region, which are recognized by sigma factors. Sigma factors play distinct roles at different stages of initiation including the direct recognition of promoter elements to form an initial "closed" complex, stabilisation of the "open" complex in which DNA around the transcription start site is melted, interaction with transcription activators, the stimulation of the early steps in RNA synthesis, and can influence

promoter escape (fig. 5) (31). All bacteria contain one predominant essential sigma factor, known as the housekeeping sigma factor, which is responsible for recognizing most promoters. These housekeeping sigma factors are composed of four structural domains connected to one another by flexible linkers.

In bacteria, responses to stress involve remodelling of cellular programs at both the transcriptional and translational levels. Implementing stress responses requires sensing and processing information that arrives from the internal and external environment in the form of biochemical and physical changes. Bacteria have evolved multiple stress responses that include two-component systems, protein-modifying and -degrading enzymes, molecular chaperones and accessory sigma factors.



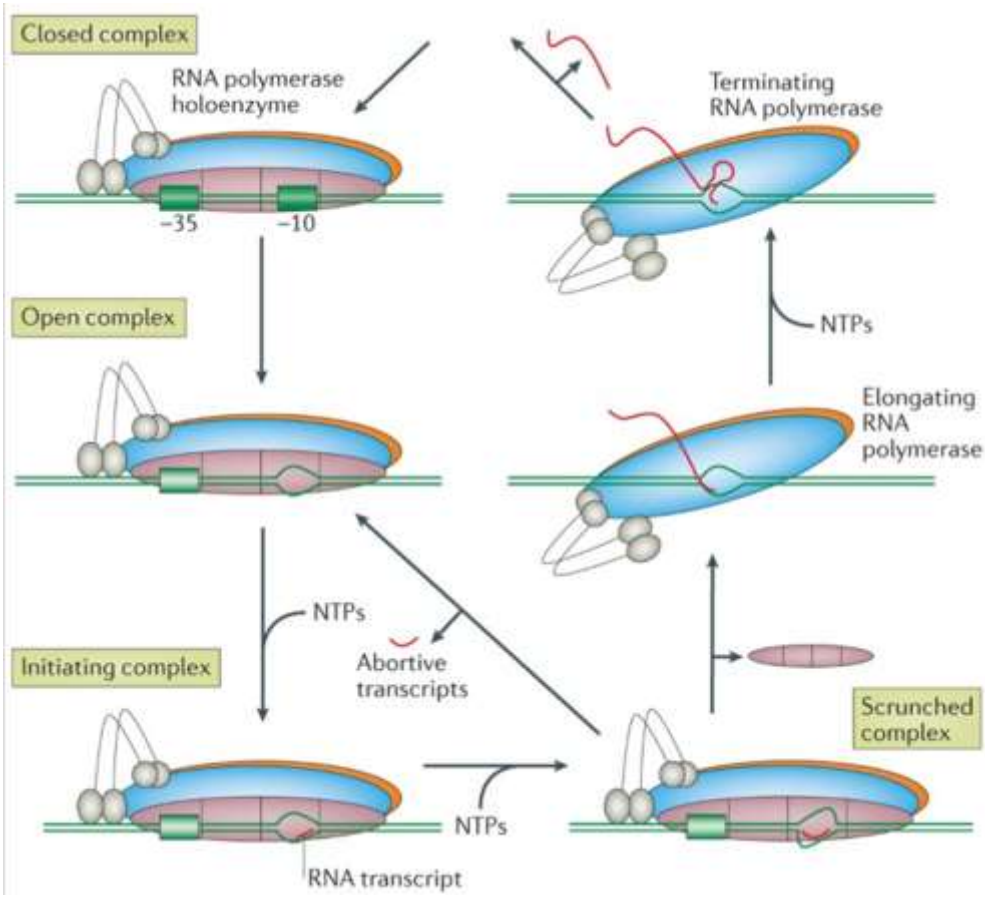


Fig. 5: Bacterial transcription cycle. RNA polymerase holoenzyme, which comprises the RNA polymerase core enzyme and a sigma factor, interacts with promoter DNA to form the closed complex. The closed complex transitions to the open complex in the region of the transcription start site. The addition of nucleoside triphosphates (NTPs) enables a further transition to the initiating complex, which synthesizes the RNA transcript. Initially, the template strand of the DNA is pulled into the initiating complex, which is a process known as 'scrunching'. The scrunched complex can be held at the promoter, which results in cycles of abortive initiation that only produce small RNA fragments. Alternatively, the RNA polymerase can escape the promoter to enter the elongation phase, leading to the release of the sigma factor and elongation of the RNA transcript. Transcription proceeds until the RNA polymerase encounters a transcriptional terminator, after which the RNA transcript is released and the polymerase dissociates from the DNA template to re-engage with a sigma factor and repeat the cycle (30).

1.3.2 Bacterial sigma factors

Bacterial sigma factors are classified into two families, Sig^{70}- and Sig^{54}-type factors, based on their distinct structures and mechanisms (32). Many, but not all, bacterial genomes carry one or two Sig^{54} genes, and these sigma factors are frequently involved in regulating the expression of genes involved in nitrogen metabolism. By contrast, all bacteria express at least one Sig^{70} family protein called housekeeping sigma factor that is responsible for the expression of most or all essential genes. All functional gene promoters in a genome are recognized by at least one sigma factor. Thus, sigma factors are the master regulators of all gene expression in bacteria, as they are required to initiate transcription from every gene (33).

The Sig^{70} – family can be further divided into four groups, depending on their function and structure (34)

- Group 1: composed of principal sigma sigma factors, which are essential genes.
- Group 2: only found in a limited number of bacterial species (*Proteobacteria*, *Cyanobacteria* and high-GC Gram-positive bacteria) and consist of primary-like sigma factors. Although closely related to Group 1, these sigma factors are normally not

essential under laboratory growth conditions. The best-characterized group 2 sigma factors are involved in the transcription of general stress response and stationary phase survival genes.

- Group 3: distantly related to principal sigma factors. Group 3 sigma factors fall into clusters comprising evolutionarily related proteins with similar functions, such as heat shock, sporulation or flagellar biosynthesis.
- Group 4: the largest and most heterogeneous collection of sigma factors (35), also described as extracytoplasmic function (ECF) sigma factors. They are often involved in the response to stress conditions, such as iron limitation, oxidative stress and surface stress. Moreover, several sigma factors from this category are important for virulence (36).

Regarding the structure, group I Sig^{70} consists of four folded domains (called Sig_1, Sig_2, Sig_3, Sig_4) connected by linker sequences of varying lengths (fig. 6). All of these domains either mediate protein-protein interactions with core RNAP, important interactions with promoter DNA, or catalyze mechanistically important steps in the transcription initiation process. Prior to the detailed structural information emerging from crystallographic analyses, peptide segments of sigma factor were delineated by regions (regions 1, 2, 3, and 4) and subregions based on functional roles and amino acid sequence conservation. These regional peptide designations are still useful for indicating contiguous, functionally important segments of amino acids within the folded domains. For example, amino acids within region 2.3 (a small component of Sig_2) are especially important for stabilizing the promoter open complex that is essential for the initiation of transcription of the 4 Sig^{70} domains, Sig_2 and Sig_4 are the most functionally important portions of the sigma factor. A segment of amino acids in domain Sig_4 forms a helix-turn-helix motif that recognizes and interacts with the –35 promoter element. This domain also forms a protein-protein interaction with the β subunit of core RNAP that is required for holoenzyme formation. Sig_2 likewise makes essential contact with core RNAP via the β' subunit and participates in essential functional interactions with the –10 element. The smallest Sig^{70} proteins, the ~20 kDa group 4 or ECF Sig factors, consist only of domains Sig_2 and Sig_4, highlighting the functional importance of these domains (31).

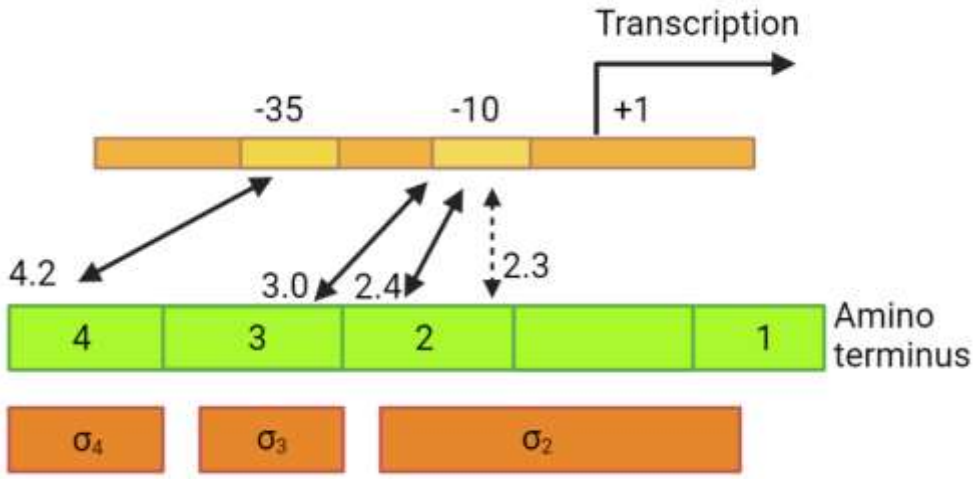


Fig. 6: Structural characteristics of *E. coli* σ^{70}. The protein sequence has been divided into four regions on the basis of sequence conservation with other members of the σ^{70} family. Residues from conserved regions 2 and 3 cooperate to mediate recognition of the -10 region and melting of the DNA. A residue in the amino-terminal part of region 3 (3.0) contacts the conserved TG motif in the extended -10 element of certain promoters that do not require a -35 region. Residues from an α helix in region 2 that corresponds to the conserved subregions 2.3 and 2.4 interact intimately with the -10 element. Subregion 2.3 is thought to interact primarily with single-stranded DNA in the open complex (dashed arrow). The three domains of the σ factor observed by X-ray crystallography (σ_2, σ_3 and σ_4) are indicated underneath the linear structure. Note that the protein domains correspond closely (although not precisely) with the regions assigned by sequence comparisons (modified from ref. 31)

Mycobacterial genomes encode only sigma factors belonging to the Sig^{70} family, and there is a lot of variability between different species depending on their lifestyle and ecological niches. The genome of *M. tuberculosis* encodes 13 sigma factors that represents the highest number of sigma factors among obligate pathogens (37) suggesting that *M. tuberculosis* can respond to diverse, complex stimuli (38). *M. smegmatis* is instead a saprophytic rapid-growing species and has been used to draw the basis of the mycobacterial genetics and as a surrogate host to study the virulence and regulatory pathways of *M. tuberculosis*. *M. smegmatis* presents 26 sigma factors. All the sigma factors encoded by *M. tuberculosis* genome are present in *M. smegmatis* except SigC, SigI and SigK, suggesting that these sigma factors could have a role in

the virulence. However, the specific sigma factors of *M. smegmatis* are probably involved in regulation of genes that are specific to this organism and to the adaptation of its lifestyle (39). Regulation of alternative sigma factor activity is usually complex, with multiple tiers of control to regulate both their expression levels and their activities (40).

Specifically, a sigma factor can be regulated at different levels:

- transcriptional regulation mediated by other sigma factors, two component-system or through a positive feedback activated by itself;
- post transcriptional regulation by non coding RNA;
- translational regulation in which mRNA structure could be modified by environmental conditions;
- post translational regulation: many sigma factors (particularly group 4) are negatively regulated by membrane or cytoplasmic proteins called anti- sigma factors. In the absence of a specific environmental cue, anti- sigma factors bind and sequester sigma factors from core RNAP. With the imposition of a specific physicochemical signal, anti-sigma factor function is abrogated through targeted proteolysis (41), structural modification (42), or the action of an anti-anti- sigma factor (43).

1.3.3 The ECF sigma factor SigE

In this study, we focused our attention on the regulatory system of SigE (σ^E), one of the best-characterized mycobacterial ECF sigma factors.

A clear indication that SigE is a critical node of the *M. tuberculosis* stress response derives from the intricate regulation of its gene expression and protein activity (Fig. 7).

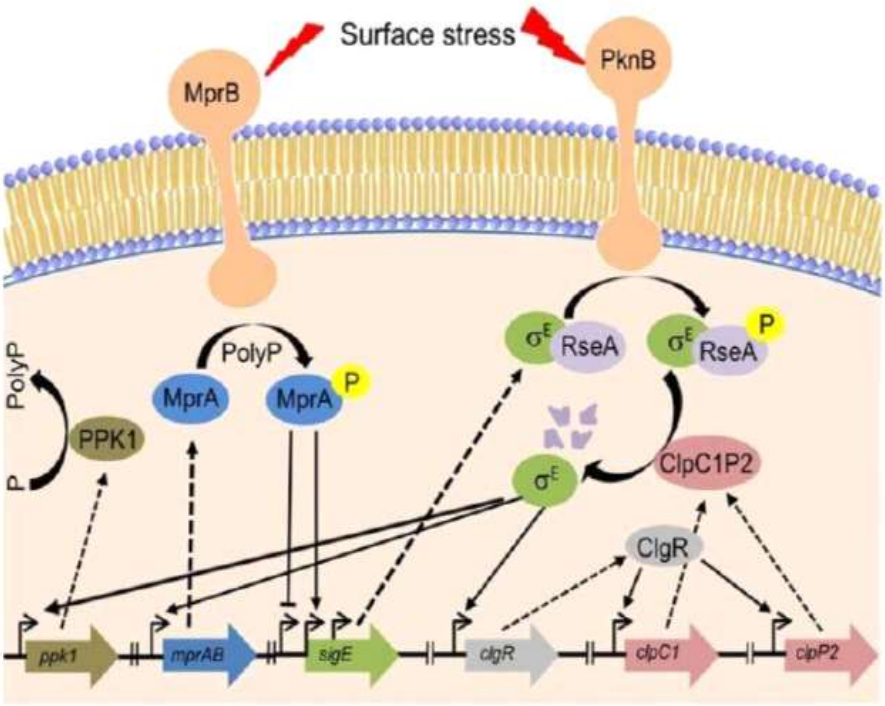


Fig. 7: Description of SigE regulatory network. Surface stress promotes PknB-dependent phosphorylation of RseA, leading to cleavage by ClpC1P2, which results in activation of the SigE regulon. SigE controls transcription of *clgR,* which in turn induces the *clp* regulon. An increase in ClpC1P2 levels leads to increased RseA degradation and a higher concentration of free SigE (positive feedback loop). SigE also controls transcription of *ppk1*: Increased PPK1 levels raise PolyP concentration, which controls s*igE* transcription through MprB-dependent phosphorylation of MprA (positive feedback loop). Also, a SigE-dependent promoter drives *mprAB* transcription (positive feedback loop). Finally, *sigE* is subject to autoregulation. Solid, dashed, and curved arrows represent transcriptional regulation, protein production, and catalytic reactions, respectively (54).

sigE structural gene is transcribed from three promoters:

1) The first one (P1) is constitutively active during growth and is probably under the control of the principal sigma factor SigA.

2) The second one (P2) is involved in conditions of surface stress mediated by SDS or vancomycin and is autoregulated by SigE. P2, does not have a canonical SigE consensus sequence and requires for its activation the two-component system MprAB, whose structural

genes are under SigE control. One of the two MprA binding sites upstream of P2 overlaps the P1 transcriptional start point, causing the downregulation of this promoter in stress conditions.

3) The third one (P3) is induced following oxidative stress and is under the control of SigH.

SigE can be present in *M. tuberculosis* in three different isoforms depending on the environmental conditions encountered from the bacteria. One of 257 aminoacids (aa), translated from mRNA transcribed from P1 or P2 in normal physiological conditions and after surface stress, and two nearly identical isoforms of 218 aa and 215 aa, translated from mRNA starting from P3 in conditions of oxidative stress.

sigE is subjected to at least two positive feedback loops: the first involving positive regulation of *mprAB* by SigE, leading to P2 activation, and the second involving the positive regulation of *ppk1* by *sigE,* resulting in higher polyphosphate intracellular levels and consequently a higher rate of MprA phosphorylation by MprB and P2 activation (44).

SigE activity is also regulated at the posttranlational level by the anti-sigma factor RseA. Its gene is located downstream of *sigE* but belongs to a different transcriptional unit that is constitutionally expressed. In conditions of surface stress, RseA is phosphorylated by the Ser/Thr kinase PknB, which contains PASTA (PBP and serine/threonine kinase-associated) domains that have been hypothesized to bind peptidoglycan to serve as cell wall stress sensors leading to its ClpC1P2-dependent proteolysis. Finally, another operon shown to require SigE for its induction after surface stress is that including *clgR* (third positive feedback loop), *rv2744c,* and *rv2745c*. The first gene encodes a global transcriptional regulator involved in one of the positive feedback loops regulating *sigE*. *rv2744c* encodes a protein highly homologous to PspA (phage shock protein A), a protein involved in homeostasis of the cell membrane while *rv2745c* encodes a transmembrane protein of unknown function (Fig. 8) (44).

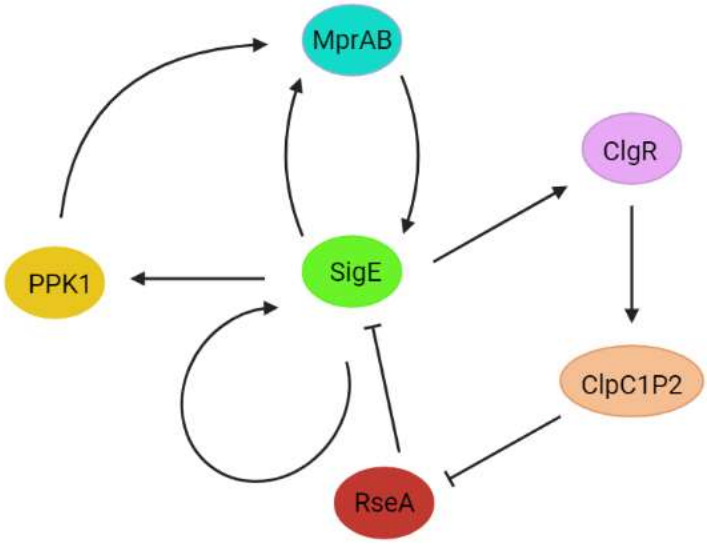


Fig. 8: Schematic rappresentation of *sigE* regulatory network.

Transcriptome profiling of a *sigE* mutant relative to the wild-type strain has also been used to identify genes that could be regulated by SigE. In the absence of stress, 38 genes were shown to be differentially regulated in the mutant and 23 genes were not appropriately induced during SDS-mediated surface stress. The most affected gene in these conditions was *sigB*, which was strongly repressed in the *sigE* mutant (44).

The gene encoding SigE is induced after exposure to various environmental stresses, such as heat shock and detergent-induced surface stress (37). A mutant of *M. tuberculosis* H37Rv lacking a functional *sigE* gene is more sensitive than the parental strain to detergent, high temperature, and oxidative stress. The genes whose expression during exponential growth require SigE include genes encoding proteins involved in translation, transcriptional control, mycolic acid biosynthesis, electron transport, and oxidative stress response. Interestingly, one of these genes is *sigB*, whose transcription under unstressed conditions is almost totally due to SigE. Since *sigB* is the only gene of this group to be preceded by an ECF Sig factor-like promoter, this suggests that at least some of the other 37 genes downregulated in the *sigE* mutant are in the SigB regulon (45). It was also shown that deletion of *sigE*, beyond rendering the bacterium sensitive to several environmental stresses, leads to an altered intracellular behavior in macrophages, with a decreased ability to arrest phagosome acidification and fusion with lysosomes. This altered intracellular trafficking might result in a

more efficient processing from macrophages, and ultimately determine the efficacy of this mutant strain as a vaccine (46).

Genes requiring SigE for SDS-mediated induction encode heat shock proteins, proteins involved in fatty acid degradation, transcriptional regulators (including SigB), and surface-exposed proteins with unknown function. The SigE-dependent induction of these genes after exposure to a detergent supports the hypothesis of their role in cell wall physiology and structure (47).

1.3.4 Regulators in SigE network

The focus of this study is the analysis of regulators involved in SigE circuit, specifically MprAB, ClgR and RseA.

The two-component system MprAB: Two-component regulatory systems are key players in bacterial responses to changing environments (Fig. 9). These systems act to integrate multiple stimuli into coordinated changes in global gene expression. Bacteria normally possess many two-component regulatory systems, which respond to specific signals and allow adaptive responses. Two-component systems are composed of a sensor histidine kinase (HK) and a response regulator (RR). Changes in the external environment result in activation of the HK, which autophosphorylates on a conserved histidine residue; the HK then mediates phosphotransfer to a conserved aspartate residue on the RR. Phosphorylation of the RR normally activates this protein, leading to DNA binding and promotion of transcription for a set of genes, termed the regulon. The MprAB system appears to be involved in the response to stress conditions, in particular those that affect the cell envelope. MprA controls the expression of the sigma factors *sigE* and *sigB* in response to sodium dodecyl sulfate exposure (48). The interaction between MprAB and SigE/B/H is complex, with many genes in the regulatory cascade being controlled by more than one of the regulators. SigE, for example, is controlled by both MprA and SigH. In *M. smegmatis*, the MprAB-SigE cascade is controlled by the availability of polyphosphate, suggesting this may be the source of the phosphoryl groups utilized by the sensor MprB under stress conditions. The two-component signal transduction system, *mprAB*, was also found to be required by *M. tuberculosis* for establishment and maintenance of persistent infection. In summary, MprAB is a two-component system that is

responsive to stress in *M. tuberculosis* and regulates the expression of sigma factors and other stress-responsive genetic determinants during growth under a variety of conditions. Continued study of these systems is essential to understand the complex and interconnected regulatory networks utilized by *M. tuberculosis* to survive environmental stress and to adapt during infection within the host (49).

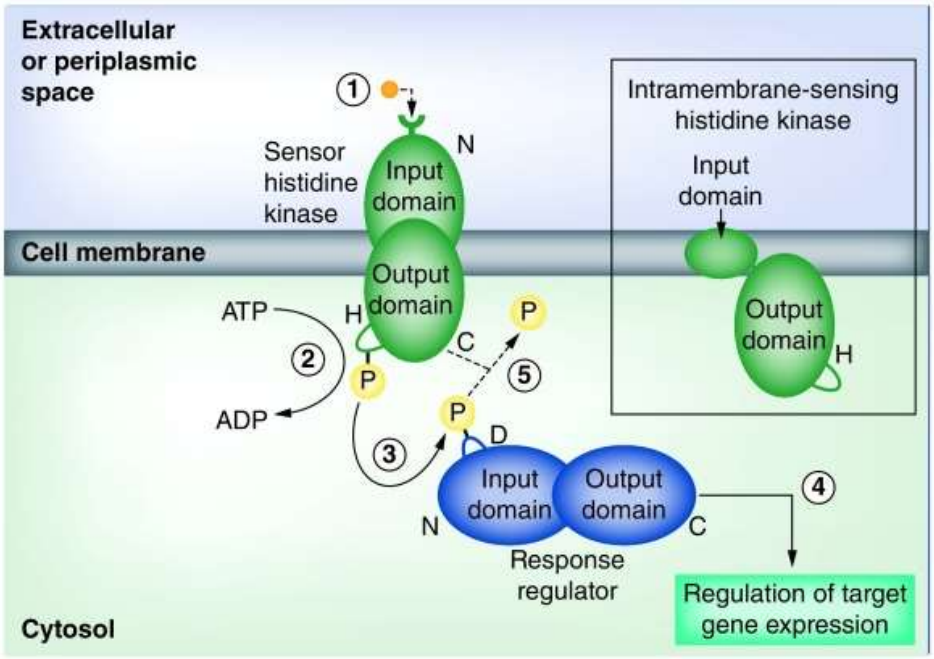


Fig. 9: Two-component signal transduction. (1) An extracellular ligand (orange) binds to the N-terminal receptor of the sensor histidine kinase (green), embedded in the cell membrane. Binding of the ligand causes (2) the C-terminal kinase domain to hydrolyze adenosine triphosphate and phosphorylate a histidine residue. (3) The phosphate (yellow) is then transferred to an aspartate residue on the N-terminal domain of the cytosolic response regulator (blue). (4) This phosphorylation event activates the response regulator's C-terminal output domain, which leads to global transcriptional changes. (5) In some cases, the sensor histidine kinase also functions as a phosphatase, and terminates the response regulator's activation by removal of the phosphate. Inset to right: intramembrane-sensing histidine kinases lack an extracytoplasmic sensory domain and have recently been shown to recruit other sensory proteins in order to function (50).

The pleiotropic regulator ClgR: The genome of *M. tuberculosis* encodes approximately 50 proteases, including two paralogues of ClpP and the associated ATPase chaperones ClpC and ClpX and the regulation of these proteolytic systems is extremely important. The high-GC actinomycetes appear to have adopted a highly conserved transcriptional activator ClgR (*clp* gene regulator), which has been shown to control *clp* genes. A homologue of ClgR exists in *M. tuberculosis* and its expression is highly upregulated in stress conditions such as heat shock and during macrophage infection; it also regulates expression of ClpP1/ClpP2 and ClpC1 in mycobacteria (51). The mycobacterial caseinolytic protease ClpP1P2 is a degradative protease that recently gained interest as a genetically and pharmacologically validated drug target for tuberculosis. The Clp protease complex is composed of a degradative chamber made of two different serine protease subunits, ClpP1 and ClpP2, encoded by the *clpP1P2* operon, which interacts with unfoldases involved in recognition and delivery of proteins into the degradation chamber (52). Both proteins are required for growth *in vitro* and in a mouse model of infection and depletion of either protein results in rapid death of the bacteria. The targets of ClpP proteolysis in *M. tuberculosis* are not well defined, although ClpP has been shown to degrade the phosphorylated SigE-specific anti-sigma factor RseA (51). By upregulating the *clp* regulon, ClgR likely facilitates its own transcription which is dependent on SigE (53). In addition, it appears that the MprAB signal transduction pathway would also influence the pleiotropic regulator transcription. The activation of polyphosphate kinase 1 (Ppk1) and MprAB under surface stress would induce *sigE* transcription, which in turn would activate *clgR* transcription (38).

The anti- sigma factor RseA**:** SigE activity is also regulated at the post-translational level by RseA, an anti-sigma factor belonging to the zinc-associated (ZAS) family (Fig. 10) (54). Anti-sigma factors are proteins that interact with specific sigma factors, preventing their binding to RNAP until a specific environmental stimulus is sensed.

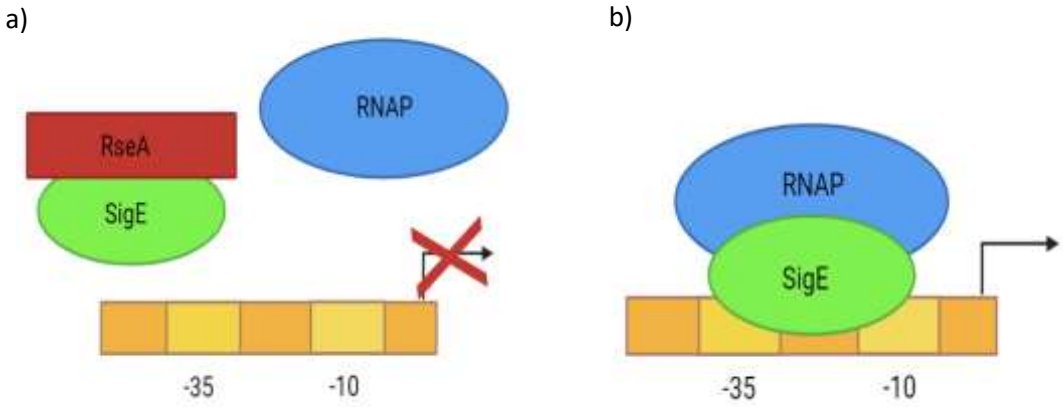


Fig. 10: Schematic representation of anti-sigma regulation. a) In physiological conditions, sigma factors and their specific anti-sigma factor (i.e. SigE/RseA) are bound in a stable complex preventing the link with RNA polymerase. b) Under specific stress conditions, this complex is disrupted allowing the transcription of specific set of genes.

Anti-sigma factors activity can be modulated by other proteins (anti-anti-Sigma factors), or by specific modifications including phosphorylation, intramembrane proteolysis, conformational changes induced by redox potential or temperature. Anti-sigma factors of the ZAS family are usually able to sense changes in redox potential through the reduction/oxidation of cysteine residues in the conserved HXXXCXXC motif. The modulation of RseA activity follows different pathways, depending on the environmental conditions encountered by the bacteria. First of all, it was demonstrate that, as expected for an anti-sigma factor of the ZAS family, RseA can bind SigE only under reducing conditions and that cysteine residues at positions 70 and 73 (part of the HXXXCXXC motif) are required for this interaction. It was shown that RseA undergoes phosphorylation-dependent proteolytic degradation in cells subjected to surface stress, but not oxidative stress or heat shock (55). Under surface stress imparted either by SDS or by exposure to sublethal doses of vancomycin, results in proteolysis of RseA on a single amino acid residue. $RseA_{MTB}$ has two putative threonine residues at positions 39 and 98. Mutation of threonine-39 to alanine prevented the phosphorylation of $RseA_{MTB}$ by PknB *in vitro*, whereas mutation of threonine-98 to alanine did not affect PknB-mediated phosphorylation of RseA. This proteolysis is dependent on PknB-mediated phosphorylation of RseA on threonine 39 (T39), followed by its interaction with ClpC1 and degradation by the ClpC1P2 proteolytic machinery (38).

2. AIM OF THE WORK

Mycobacterium tuberculosis is the causative agent of tuberculosis and it is one of the deadliest human bacterial pathogens; indeed it is responsible for more than one million death annually. Despite the relative decrease of that in last decade, the lack of an effective vaccine and the emergence of multi-drug resistant bacteria require to better understand the physiology of this pathogen.

Specifically, in our laboratory, one of the main research fields is about the role of sigma factors involved in the survival of bacteria in different environmental conditions. Adaptation involves modulation of global transcriptional profile in response to the changing environment found in the host.

One of the most important sigma factors that allows the survival at different stress conditions during the infectious process, and which is implied in virulence, is the alternative extracytoplasmic sigma factor SigE.

The aim of this work is to better characterize SigE regulatory network understanding its dynamics under surface stress, in phosphate deprivation and in acidic pH environment.

For this purpose, the role of different regulators implied in its circuit, MprAB, ClgR and RseA, was evaluated mimicking the conditions to which tubercular bacilli are exposed in the human body.

Specifically, the dynamic of transcription of several SigE-dependent genes *(sigE, sigB, rseA, clgR, clp)* in wild-type and mutant strains (*ΔmprAB*, *ΔclgR* and $RseA_{T39A}$) was evaluated along time. Firstly, *M. smegmatis*, a rapid growth, non-pathogenic, environmental mycobacterium, was used to set up the experiments and obtain preliminary results. Then, these data were corroborated in *M. tuberculosis*, the pathogenic species, providing a greater understanding of the regulatory mechanisms of Mycobacteria under stress conditions.

3. MATERIALS AND METHODS

3.1 Bacterial strains, media and growth conditions

Escherichia coli strain DH5α was grown in Luria broth (Difco) at 37 °C. When required, antibiotics were added at the following concentrations: kanamycin (Sigma-Aldrich) 50 μg/ml, hygromycin (Invitrogen) 150 μg/ml.

M. smegmatis was grown in either Middlebrook 7H9 liquid medium or Middlebrook 7H10 solid medium (Difco) supplemented with 0.2% glycerol (Sigma Aldrich), 0.05% Tween 80 (Sigma Aldrich). *M. smegmatis* liquid cultures were grown in shacking at 37°C. Plates were incubated at 37°C for 48 hours. *M. tuberculosis* H37Rv was grown in either Middlebrook 7H9 liquid medium or Middlebrook 7H10 solid medium (Difco) supplemented with 0.2% glycerol (Sigma Aldrich), 0.05% Tween 80 (Sigma Aldrich) and ADN (2% glucose, 5% bovine serum albumin, 0.85% NaCl). *M. tuberculosis* liquid cultures were grown in roller bottles at 37 °C. Plates were incubated at 37 °C in sealed plastic bags. When required, antibiotics were added at the following concentrations: kanamycin 20 μg/ml and hygromycin 50 μg/ml.

To perform the experiments under acidic pH conditions, the cultures were grown in Sauton medium (3.67 mM K_2HPO_4, 4mM $MgSO_4$, 30mM L-Asparagine, 0.18 mM Ferric Ammonium Citrate, 5mM Citric Acid, 4mM Glycerol, 0.1 ml 1% Zinc Sulfate, 0.05% Tween 80). The pH of the minimal medium was adjusted to 4.5 with 10M NaOH and antibiotics were added when required.

All the strains tested in this work were listed in Table 1 and Table 2.

M. smegmatis strains	*Relevant genotype or description*	*Reference*
mc²155	parental strain	Lab collection
MS133	mc²155 *ΔclgR*	Lab collection
MS266	mc²155 *ΔmprAB*	Lab collection
MS265	mc²155 *ΔsigE_ΔrseA::sigE::rseA$_{T36A}$*	Lab collection

Table 1: List of *M. smegmatis* strains used in this work. *(clgR = MSMEG_2694; mprA=MSMEG_5488; mprB = MSMEG_5487; sigE = MS_5072; rseA = MSMEG_5071)*

M. tuberculosis strains	*Relevant genotype or description*	*Reference*
H37Rv	parental strain	Lab collection
TB522	*H37Rv ΔclgR*	This work
TB552	*H37Rv ΔmprAB*	This work
TB509	H37Rv *ΔsigE_ΔrseA::sigE::rseA$_{T39A}$*	Lab collection

Table 2: List of *M. tuberculosis* strains used in this work *(clgR = Rv2745c; mprA=Rv0981; mprB = rv0982; sigE = rv1221; rseA = rv1222)*

M. tuberculosis was handled and cultivated in a biosafety level 3 (BL3) laboratory.

3.2 DNA manipulation

All recombinant DNA techniques were performed according to standard procedures using *E. coli* DH5α as the initial host. DNA restriction and modifying enzymes were obtained from New England Biolabs and used according to the manufacturer's recommendations.

3.2.1 Construction of a *ΔclgR* mutant in *Mycobacterium tuberculosis*

An unmarked *ΔclgR* deletion mutant was constructed in *M. tuberculosis* according to the published pNIL-pGOAL method schematically represented in fig 11 and 12 (56). Two DNA regions, one upstream and one downstream *clgR* were amplified by PCR and sequentially cloned into p1NIL as DraI/StuI and StuI/NotI fragments respectively. The upstream region (991 bp) was amplified by RP1968 and RP1969 while the downstream region (999 bp) was amplified by RP1970 and RP1971. A *lacZ-sacB-hyg* cassette from pGOAL19 was then introduced as a PacI fragment in the resulting vector to obtain the final suicide plasmid that was electroporated into *M. tuberculosis*. Transformants were selected on plates containing both kanamycin and hygromycin. The occurrence of single crossover (SCO) was confirmed by PCR. One mutant with the correct integration of suicide plasmid was grown in the absence of any drug to allow a second homologous recombination (DCO). Recombinants were isolated as white colonies on plates containing sucrose and X-gal and the occurrence of a double crossover leading to *clgR* deletion was confirmed by PCR screening. These primer couples amplify two DNA regions whose length was 1559 bp (RP2110 – RP2111) and 1547 bp (RP2112-RP2113) respectively in the wild-type strain, and 1219 bp and 1207 bp respectively in the correct mutant. One strain with the proper chromosomal structure was named TB522.

PRIMER	**SEQUENZA 5'-3'**	**AIM**
RP1968 *Fw*	TTTAAAGGTCATGGCCGGGTCGACAGC	Amplification of upstream region of *clgR,* upper primer
RP1969 *Rv*	AGGCCTACGCACCAAAGCCGCCATCAA	Amplification of upstream region of *clgR,* lower primer

RP1970 *Fw*	AGGCCTACGCACCAAAGCCGCCATCAA	Amplification of downstream region of *clgR*, upper primer
RP1971 *Rv*	GCGGCCGCGCCGAACGTCTGCCCAACT	Amplification of downstream region of *clgR*, lower primer
RP2110 *Fw*	GAACACCTCGGCGGTGACCG	Screening SCO, upper primer
RP2111 *Rv*	ATTTATCGGGTCAGCGCGCA	Screening SCO, lower primer
RP2112 *Fw*	CACTTTCGGGTCCGCTGCAC	Screening DCO, upper primer
RP2113 *Rv*	AGCAACGAACGCCACGGCCG	Screening DCO, lower primer

Table 3: List of primers used for the construction and check of the mutant TB522.

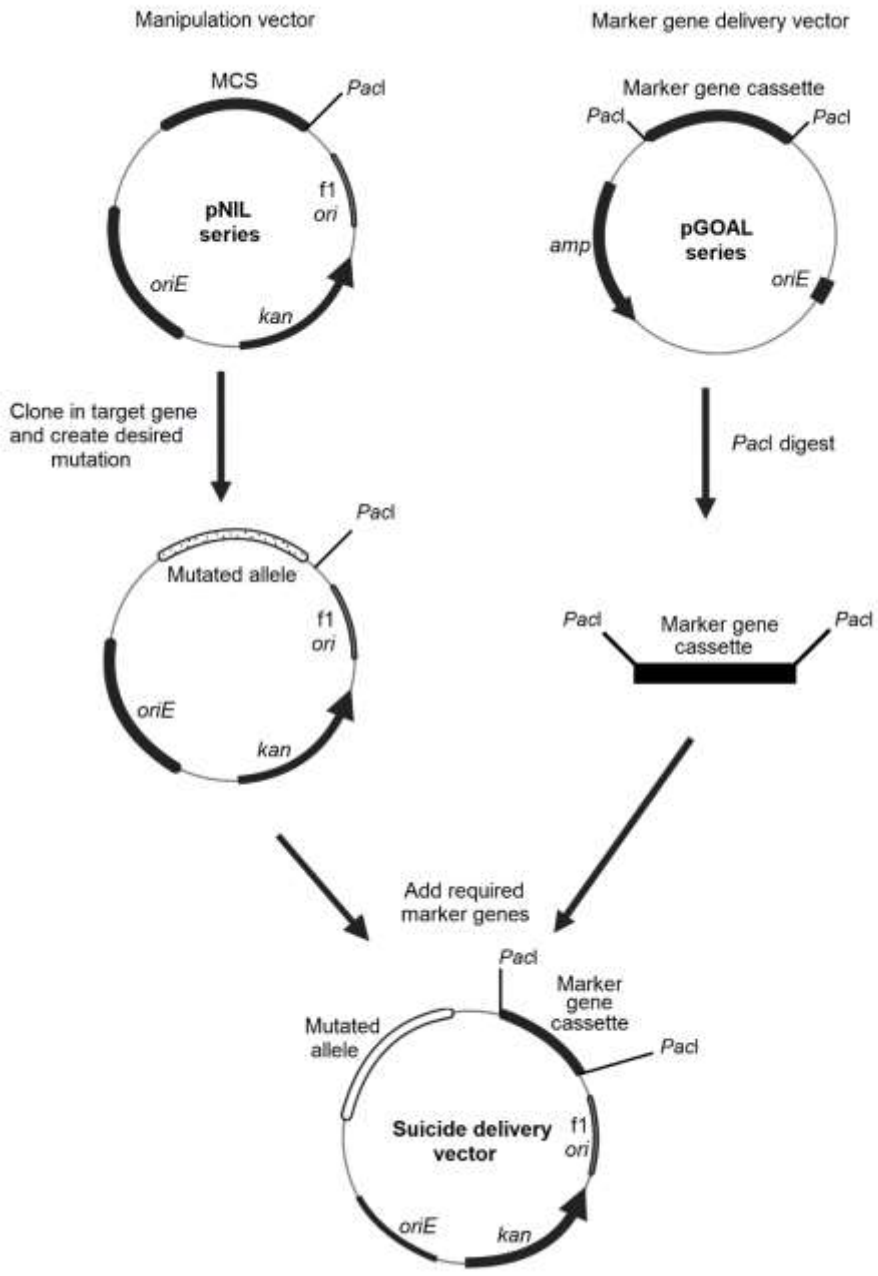


Fig. 11: Cloning strategy for generating suicide delivery vectors. (a) The regions upstream and downstream the target gene were cloned into the pNIL vector. (b) The PacI cassette containing the desired marker genes was then excised from the pGOAL vector and cloned into the unique PacI site of the pNIL/mutated gene vector, resulting in the final suicide delivery vector. (c) The final vector thus contains oriE, the kanamycin resistance gene (kan) and the f1 origin (f1 ori) (modified from ref. 57).

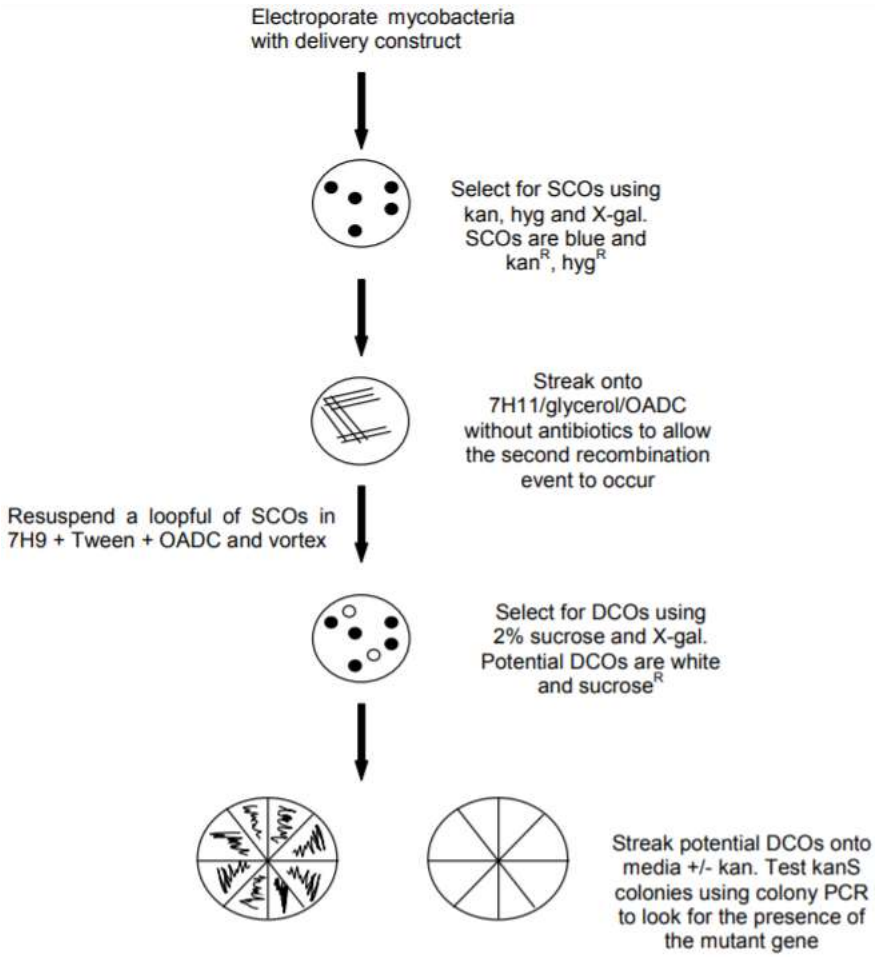


Fig. 12: Steps in the selection strategy for gene replacement using the pNIL/pGOAL system. The suicide delivery vector was electroporated into competent mycobacteria. The plasmid cannot survive autonomously so selecting for the presence of the plasmid marker genes (kan, hyg, lacZ) selects for those colonies that have undergone a single recombination event. SCOs (blue, kan R, hyg R) were plated onto media without selection while the second recombination event occurs. DCOs were selected for by plating onto media containing X-gal and sucrose. DCOs have lost the vector and were white and sucrose resistant. Finally, colonies were patch tested for sensitivity to kanamycin and were screened for the wild-type or mutant version of the gene by colony PCR (57).

3.2.2 Construction of a *ΔmprAB* mutant in *Mycobacterium tuberculosis*

All plasmids and primers used to obtain and check the strain were listed in table 4 and 5.

To obtain a mutant in which the double component system MprAB was deleted, a *new* method for genetic engineering of mycobacterial chromosomes, called ORBIT (Oligonucleotide-mediated Recombineering followed by Bxb1 Integrase Targeting) was used (58). The target specific oligonucleotide was designed considering the 60 bases across the start and stop codons of the gene of interest, the Bxb1 *attP* site sequence was inserted in the middle of it. The wild-type strain of *M. tuberculosis* wastransformed with the plasmid pKM461, to obtain the acceptor strain TB545. TB545 was grown up to OD_{540} = 0.8, treated with ATc for 8 h and with 2 M glycine for 16 h. Then the culture was pelleted at 4000 rpm for 10', washed twice in 10% glycerol and finally resuspended in 2 mL of glycerol. 380 µL aliquots of freshly made competent cells were then electroporated with 1 µg of RP2151 and 200 ng of payload plasmid pKM464. After a 24 h recovery period at 37 °C in 2 mL 7H9 ADC, cells were plated in 7H10 ADC Hyg plates and incubated at 37 °C for at least 3 weeks. The resulting colonies were then transferred in plates of 7H10 enriched with sucrose allowing the expulsion of pKM461. Genetic analysis was performed and a hygromycin resistant colony was selected, checked by PCR and sequenced. As a final control, the region was Sanger sequenced (BMR Genomics) to verify replacement of the target gene by correct insertion of pKM464. The final strain was named TB552.

Plasmid	**Description**	**Reswastence**
pKM461	PTet-Che9c RecT-Bxb1-Int, SacRB, TerR	Kanamycin
pKM464	Bxb1 attB,	Hygromycin

Table 4: List of plasmids used to obtain the mutant TB552.

PRIMER	SEQUENZA 5'-3'	AIM
RP2168 *Fw*	CGACTGAACGGGTGCGTTGATC	Amplification of plasmid pKM461, upper primer
RP2169 *Rv*	AACGCCAGTCGAACTGCTGG	Amplification of plasmid pKM461, lower primer

Table 5: List of primers used to check the mutant TB552.

Oligo RP2151:

TGGGTGTGGCTTTCAACAGTAACTGCACAACTAGGTTGCGCGCGTGGACTGAGATTCCACGGTTTGT
ACCGTACACCACTGAGACCGCGGTGGTTGACCAGACAAACCACGATCGTCGTCAACGACAAGAATTC
GCACGGACACCAGTGTCGTCGCAGCGCCTGAGAC

3.3 Cell Viability

In order to test the viability under surface stress condition mediated by sodium dodecyl sulfate (SDS), *M. smegmatis* and *M. tuberculosis* strains were cultured in 30 ml of 7H9 until OD_{600}/OD_{560} 0.4. Aliquots of serial dilutions of untreated samples was plated. Afterwards, SDS was added at final concentration of 0.05% to the culture and aliquots of serial dilutions of samples were plated 30, 90 minutes and 24 hours after the incubation at 37°C with mild shaking.

In order to test the viability in acidic pH condition, *M. smegmatis* and *M. tuberculosis* strains were cultured in 30 ml of Sauton medium until OD_{600}/OD_{560}.

Subsequently, bacteria were centrifuged (3000 rpm, 5 min) and resuspended in the same medium at pH 4.5. The number of viable cells was tested by spreading aliquots of serial dilutions at different time points: 30, 90 minutes and 24 hours after the incubation at 37°C with mild shaking.

3.4 RNA extraction and retro-transcription

Strains were inoculated overnight in the standard medium. The day after, cultures were refreshed in 7H9 or centrifuged and resuspended in 30 ml of Sauton medium at pH 6.8 and 4.5. Starting from early-log phase (OD600= 0.4) samples were collected at determined time points. Each sample was centrifuged at 13000 rpm for 5 min at room temperature, and frozen. The frozen cell pellets were suspended in 1 ml of TRIzol reagent and transferred to 2-ml screw cap tubes containing 0.8 ml of 0.1-mm-diameter zirconia/silica beads (BioSpec Products). Cells were disrupted with three 45-s pulses in a Mini-Bead-Beater (BioSpec Products). After 10 min of incubation at room temperature, chloroform and isoamilic alcool (24:1 ratio) was added. The aqueous phase was added to 500 µl of isopropanol and 2 µl of glycogen to allow the precipitation of nucleic acid. Samples were incubated overnight at -20°C. This step was repeated twice. The RNA pellets were washed with 300 µl of 75% ethanol, centrifuged twice for 15 min, and air dried (59). RNA pellets were resuspended in 0.02 ml of DEPC water and the concentration was quantified with Nanodrop. RNA samples were then retro-transcribed to first strand cDNA with M-MLV Reverse Transcriptase (Invitrogen™) following manufacturer instructions, and conserved at -20 °C.

3.5 Real time PCR

Quantitative reverse transcription real-time PCR (RT-PCR) was performed on a 7000 Sequence Detection System (Applied Biosystems) using PowerUP SYBR Green Master Mix (Applied Biosystems). Results were normalized to the amount of *mysA*/*sigA* mRNA (60). RNA samples that had not been reverse transcribed were included in all experiments to exclude significant DNA contamination. For each sample, melting curves were used to confirm the purity of the amplification products. Experiments were performed at least twice, starting from independent biological samples. Primers used for quantitative real-time PCR in *M. smegmatis* were described in table 6 and those used for *M. tuberculosis* in table 7.

Gene	Forward primer	Reverse primer
mysA	5′- accaagggctacaagttctcg -3′	3′- ccttctcgggcgtgatgtc-5′
sigB	5′- tcgtgcgcgtgtatctgaa -3′	3′- caggttggcctcgagcag-5′
sigE	5′- gcaggtcaggtcccagcc -3′	3′- cgaaggctggctacaccgca -5′
rseA	5′- gacggtgagctgcggatgtc -3′	3′- gtcgggtcatcggcgaactt -5′
clgR	5′ - cgaggtgattggcgacgt-3′	3′ 3′- catgctctcaccggcgtc- 5′
clp	5′- cccgctacatcctgccgtcc -3′	3′- cgggatccagcgactccagc -5′

Table 6: Oligonucleotides used for quantitative RT-PCR assays. *mysA = MSMEG_2758; sigB = MSMEG_2752; sigE = MSMEG_5072; rseA = MSMEG_5071; clgR = MSMEG_2694; clp = MSMEG_4672.*

Gene	Forward primer	Reverse primer
sigA	5′- ccatcccgaaaaggaagacc-3′	3′- aggtctggttcagcgtcgag-5′
sigB	5′- gtctatctgaacggcatcgg -3′	3′- ccgcctcgccatcacgcac-5′
sigE	5′- cgaaggctggctacaccgca -3′	3′- gcaggtcaggtcccagcc -5′
rseA	5′- cagttccgttccaccgagca -3′	3′- ggtggacaacgcgggatct-5′
clgR	5′ - ctttggtgcgtgaggtcgttg-3′	3′- atcgatgagcaccaccgacaa - 5′
clp	5′- cctgggctcggaggtgaacg -3′	3′- ttgcccttggtacctgccgc-5′
a	5′- gcggacctgttggggatgag -3′	3′ - cggtacgcgacggtaattcc - 5′
b	5′ - tttgcgttgccgacggtgac - 3′	3′ - cggtacgcgacggtaattcc - 5′
c	5′- acgacttgccaacttattgcag -3′	3′ - tcagacggctccacccact - 5′

Table 7: Oligonucleotides used for quantitative RT-PCR assays. *sigA = Rv2703; sigB = Rv2710; sigE = Rv1221; rseA = Rv1222; clgR = Rv2745c; clp = Rv2461c;* couples *a,b,c* (Fig. 13) amplified three different *sigE* promoter region.

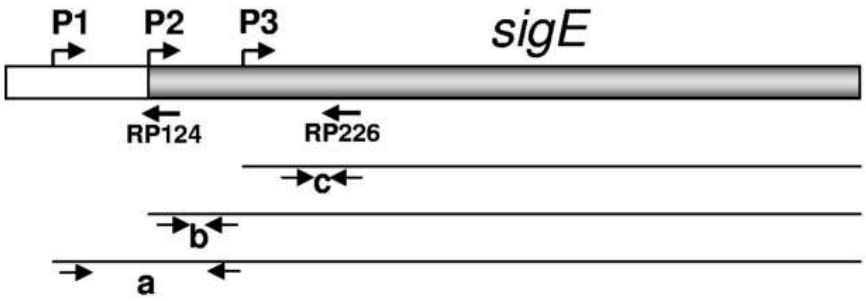


Fig. 13: Schematic representation of the *sigE* promoter region. The three thin lines represent the transcripts originating from each of the three promoters. Arrow pairs below each transcript indicate the primer couples used in quantitative RT-PCR experiments (a, b, c).

4. RESULTS

4.1 Characterization of *sigE* regulatory network in *M. smegmatis* under surface stress conditions

4.1.1 Basal level of different genes involved in *sigE* regulatory network in mutant strains

Before to study the dynamics of expression of the genes involved in the *sigE* network (*sigE, sigB, rseA, clp*), we determined their basal level of expression in the different mutants used in the study and found that their basal level of expression was comparable in all the strains (data not shown).

4.1.2 Killing curves after addition of SDS 0.05% in *M. smegmatis*

To test the capability of the various *M. smegmatis* strains used in this work to survive under surface stress condition mediated by SDS, killing curves were performed. In figure 14 the survival rate of *M. smegmatis* mc^2 155 wild-type strain, MS266 (Δ*mprAB*), MS133 (Δ*clgR*) and MS265 (RseA T36A = RseA*) were indicated. As clear from the figure 14, there is not significant differences among them, even if survival of all the strains was strongly decreased after 30 and 90 minutes of exposure.

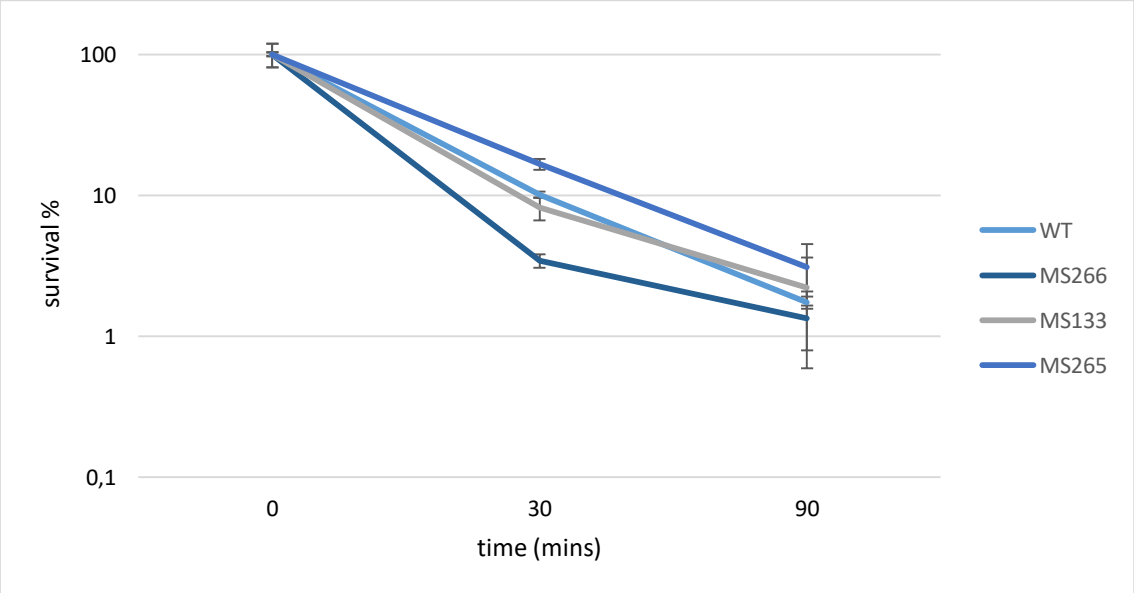


Fig. 14. Killing assay of *M. smegmatis* strains exposed to SDS 0.05%. The samples were collected before treatment (time 0) and 30 and 90 minutes after the addition of the detergent. wild-type = mc^2 155; MS266 = *ΔmprAB*; MS133 = *ΔclgR; MS265 = RseA*.*

4.1.3 Gene expression studies in *M. smegmatis* wild-type after SDS 0.05% exposure

The change in the expression level of *sigE, rseA, sigB* and *clp* after exposure to stress was first studied in wild-type strain mc^{2}155. *sigE* induction was visible since the beginning of the experiment (5 minutes post-exposure) and was stable during the whole experiment. Consequently, also *sigB* induction was stable and high. In addition, also *rseA* and *clp* were induced, even if their induction was not always statistically significant (Fig. 15). Curiously, we could not detect the induction of *clgR*, which at least in *M. tuberculosis* was shown to be responsible of *clp* induction. These data suggest that either in *M. smegmatis*, *clp* is not regulated by ClgR, or that the ClgR molecules normally present in the cell, become able to activate *clp* expression in response to stress.

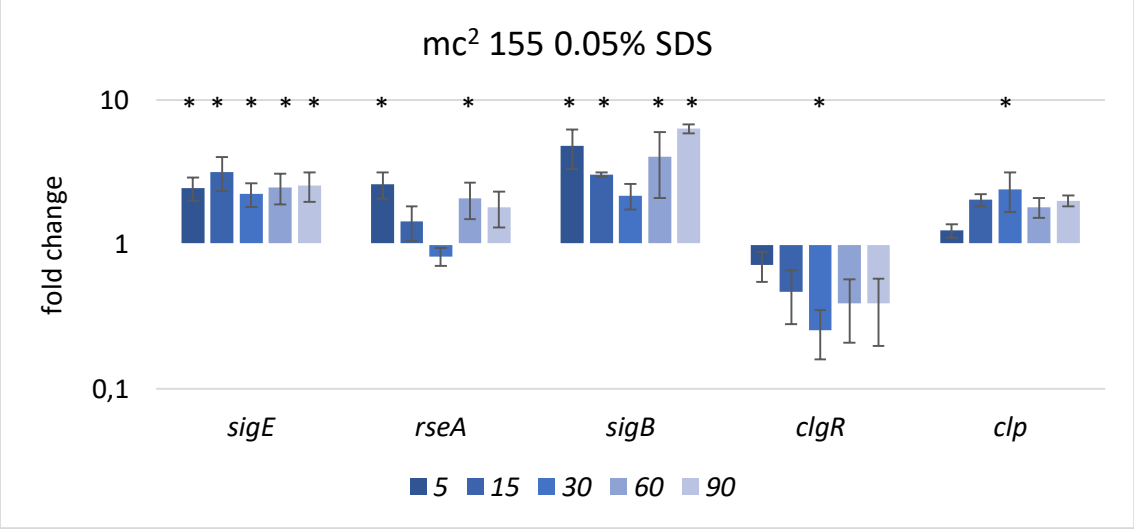


Fig. 15: Fold-changes of mRNA level of genes belonging to the *sigE* network in wild-type strain upon exposure to 0.05% SDS. The values were expressed as the ratio between the numbers of cDNA copies detected by quantitative RT–PCR in samples obtained from exponentially growing strains and were normalized to the level of *mysA* cDNA, which represents the internal invariant control. The values were measured 5, 15, 30, 60, 90 minutes after SDS 0.05% addition

and normalized to untreated sample. The reported values derive from at least two independent experiments. * pValue < 0.05 versus untreated samples (Student's *t*-test).

4.1.4 Gene expression levels in *M. smegmatis ΔmprAB* mutant after SDS 0.05% exposure

The experiment was then repeated using the *mprAB* mutant MS266. In this strain the genes encoding the anti-sigma factor RseA and *clgR* were severely repressed, while both *sigE* and *sigB* expression remained at the basal level (Fig. 16). These data show that MprAB has a fundamental role in starting the SigE-mediated stress response under these conditions.

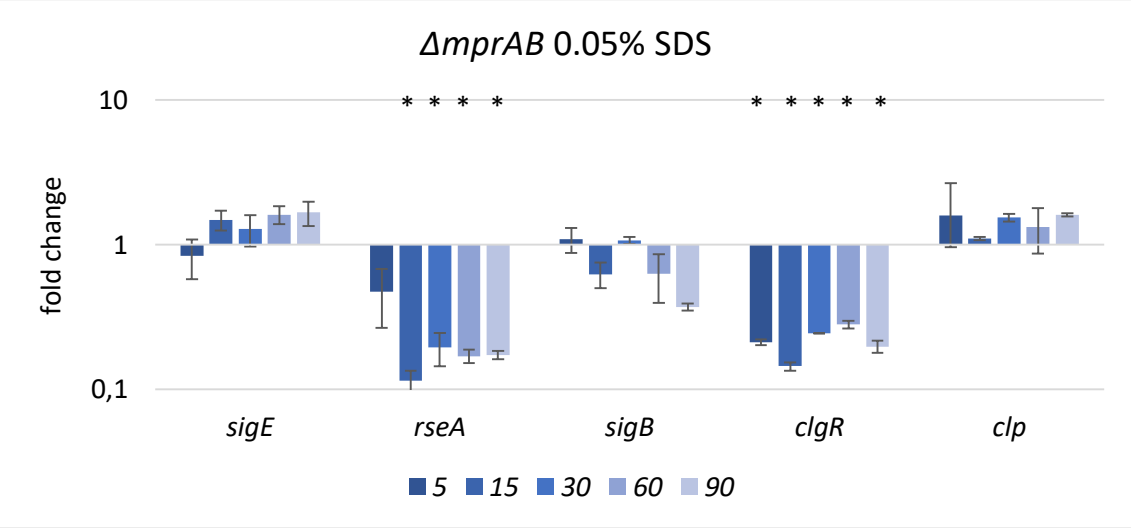


Fig. 16. Fold-changes of mRNA level of genes belonging to the *sigE* network in MS266 upon exposure to 0.05% SDS. The values were expressed as the ratio between the numbers of cDNA copies detected by quantitative RT–PCR in samples obtained from exponentially growing strains and were normalized to the level of *mysA* cDNA, which represents the internal invariant control. The values were measured 5, 15, 30, 60, 90 minutes after SDS 0.05% addition and normalized to untreated sample. The reported values derive from at least two independent experiments. * pValue < 0.05 versus untreated samples (Student's *t*-test).

4.1.5 Gene expression levels in *M. smegmatis ΔclgR* mutant after SDS 0.05% exposure

In the *clgR* mutant MS133, after exposure to surface stress, *sigE* expression level was significantly induced, even if only starting after 30 minutes from exposure to SDS. *sigB* induction was present, but unstable during the experiment (Fig. 17) suggesting that, while MprAB is essential to start the SigE-mediated stress response, ClgR is essential to sustain the response during time. In fact, while in MprAB mutant the stress response was absent, in the *clgR* mutant, the response was present, but its strength was lower, and its intensity decreases after 30 minutes. As expected for the absence of ClgR, the level of *clp* expression was very low.

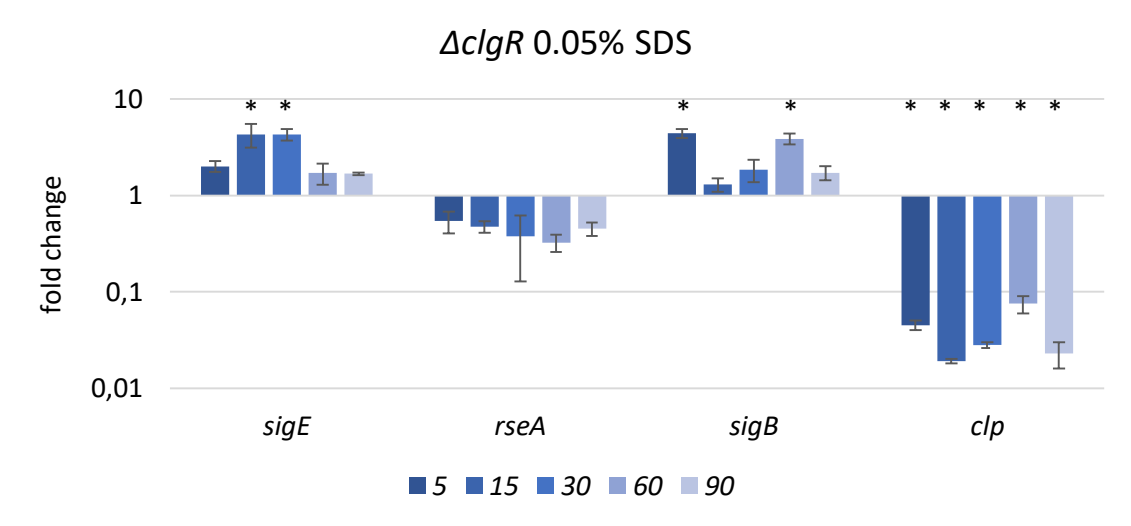


Fig. 17: Fold-changes of mRNA level of genes belonging to the *sigE* network in MS133 upon exposure to 0.05% SDS. The values were expressed as the ratio between the numbers of cDNA copies detected by quantitative RT–PCR in samples obtained from exponentially growing strains and were normalized to the level of *mysA* cDNA, which represented the internal invariant control. The values were measured 5, 15, 30, 60, 90 minutes after SDS 0.05% addition and normalized to untreated sample. The reported values derive from at least two independent experiments.* pValue < 0.05 versus untreated samples (Student's *t*-test).

4.1.6 Gene expression levels in *M. smegmatis* RseA* mutant after SDS 0.05% addition

Finally, we analysed the mutant MS265 in which the anti-sigma factor contained a mutation preventing its phosphorylation by PknB and thus its degradation following surface stress. In this strain *sigE* and *sigB* expression levels were significantly upregulated. *clgR* was induced despite the gene encoding the protease *clp* remained at basal levels (Fig. 18). These results were interesting, since the impossibility to phosphorylate RseA should result in a lower activation of SigE that we did not observe. These data suggest that at least in *M. smegmatis* the relation among ClgR-Clp and RseA degradation could be different from that suggested in literature.

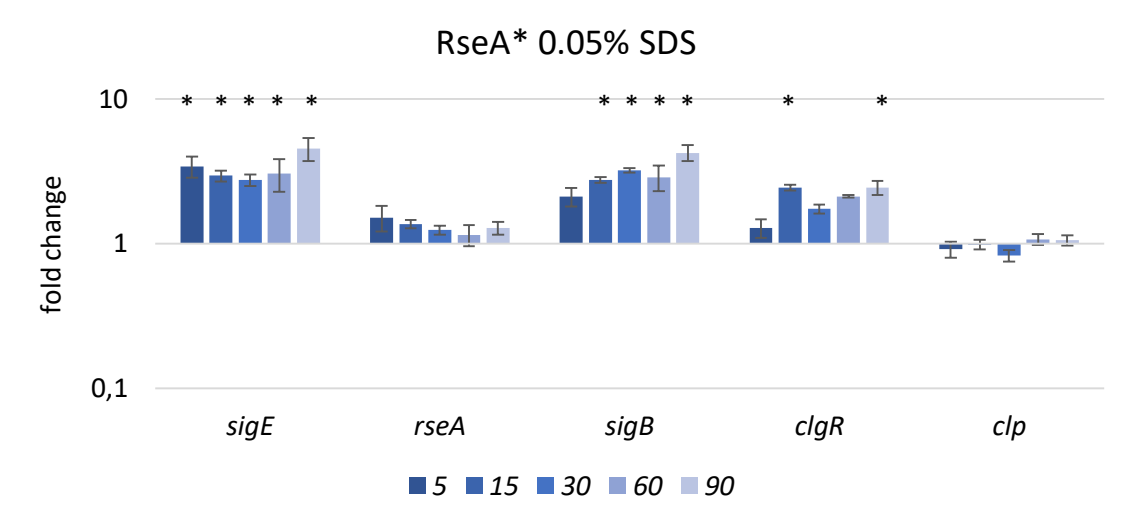


Fig. 18: Fold-changes of mRNA level of genes belonging to the *sigE* network in MS265 upon exposure to 0.05% SDS. The values were expressed as the ratio between the numbers of cDNA copies detected by quantitative RT–PCR in samples obtained from exponentially growing strains and were normalized to the level of *mysA* cDNA, which represented the internal invariant control. The values were measured 5, 15, 30, 60, 90 minutes after SDS 0.05% addition and normalized to untreated sample. The reported values derive from at least two independent experiments.* pValue < 0.05 versus untreated samples (Student's *t*-test).

4.2 Characterization of the *sigE* regulatory network in *M. smegmatis* in acidic pH conditions

4.2.1 Killing curves at pH 4.5 in *M. smegmatis*

To test the capability of the different *M. smegmatis* strains to survive in acidic pH, killing curves were performed. In figure 19, it was shown that the cells viability was comparable for all strains.

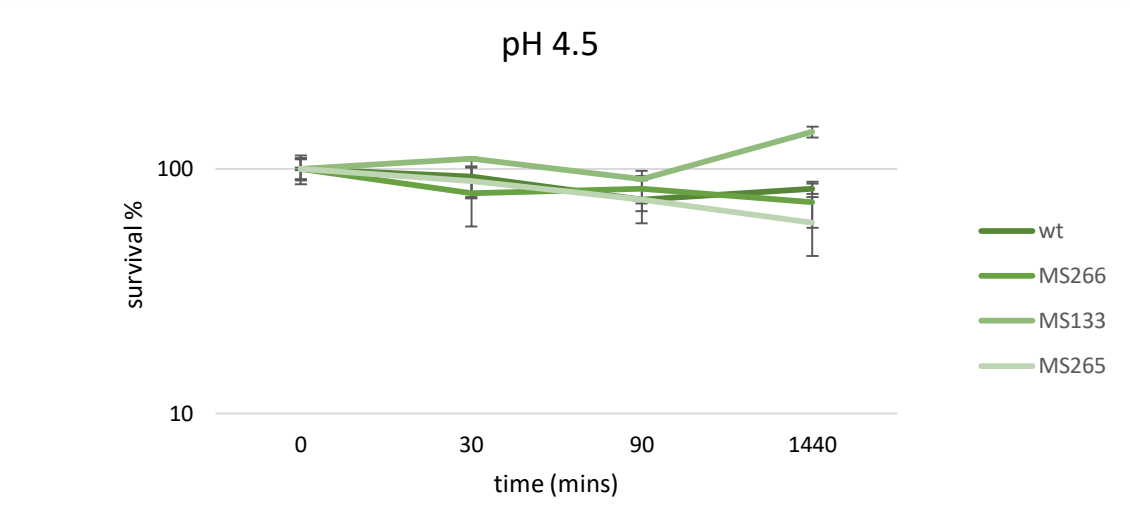


Fig. 19: Killing assay of *M. smegmatis* strains at pH 4.5. The samples were collected immediately after the resuspension in minimal medium at pH 4.5 (time 0), and 30, 90 minutes and 24 hours after the refreshing of the cultures. wt = mc^2 155; MS266 = *ΔmprAB*; MS133 = *ΔclgR; MS265* = RseA*

4.2.2 Gene expression studies in *M. smegmatis* wild-type strain after exposure to pH 4.5

In order to investigate if acidic pH influences expression of the *sigE* regulon, we performed real time PCR experiments comparing the expression of representative *sigE* regulated genes in bacteria grown under acid stress versus normal conditions. Specifically, we decided to analyse the dynamic of transcription of these genes at pH 4.5 compared to pH 6.8.

In the wild-type strain (fig. 20), *sigE* was always induced, in particular after 30 and 60 minutes when fold change values were close to five. In parallel, *sigB* showed comparable values of induction in the first two time points, with a peak after 60 minutes when upregulation was

about 8 times. In addition, *clgR* followed the same pattern with very high induction. Surprisingly, transcription of *clp*, known to be regulated by ClgR was not induced. A possible explanation is that *clgR* induction alone was not sufficient to activate *clp* transcription, but another signal, not activated in acidic pH, was required.

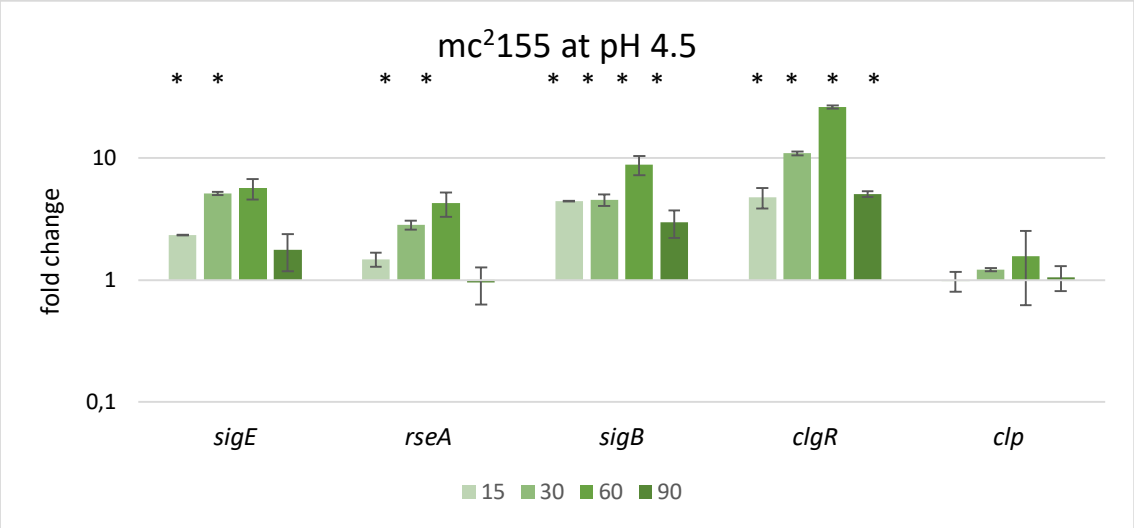


Fig. 20: Fold-changes of mRNA level of genes belonging to the *sigE* network in wild-type strain at pH 4.5. The samples were collected at 15, 30, 60, 90 mins after the refreshing in Sauton medium at pH 6.8 and 4.5. Results were given as means ± SD of at least two biological replicates. Significant differences from the transcriptional level of mutant strain grown at pH 4.5 versus pH 6.8 (Student's *t*-test pValue<0.05) were indicated by the asterisk.

4.2.3 Gene expression studies in *M. smegmatis ΔmprAB* mutant after exposure to pH 4.5

In the *ΔmprAB* strain MS266 (fig. 21), a strong upregulation of *sigE* was observed: indeed its induction after 15 minutes of acidic pH exposure was very high (around 10 times more than culture grown at pH 6.8). *rseA* keeps the same fold change values during the experiment while *clgR* expression had a sharp increase at 60 minutes. On the contrary, *clp* was never induced. Even if the values were not the same, the dynamic of gene expression follows the same pattern obtained in wild-type strain. The data obtained demonstrates that the two-component system *mprAB* does not work as a pH sensor as for surface stress, where its deletion almost totally abrogated *sigE* induction after stress. In this case, the induction of *sigE* was clearly dependent on a different regulator that will be interesting to identify. A good

candidate was SigH, which might be induced by the oxidative stress induced by the growth arrest caused by acidic pH and was known to recognize *sigE* P3. However, the slower induction dynamics of *clgR* and *sigB* in the *ΔmprAB* mutant suggest the possibility of a minor role of MprAB also in these conditions. In fact, at least for the *sigB* gene it was known that its SigE-dependant promoter requires the binding of phosphorylated MprA for its full activity (49).

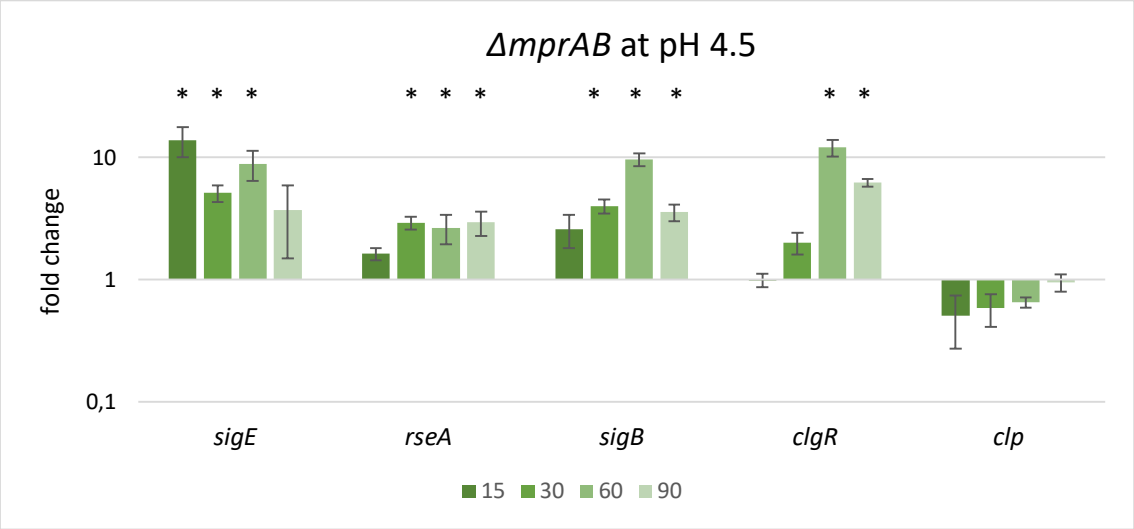


Fig. 21: Fold-changes of mRNA level of genes belonging to the *sigE* network in *ΔmprAB* strain MS266 at pH 4.5. The samples were collected at 15, 30, 60, 90 mins after the refreshing in Sauton medium at pH 6.8 and 4.5. Results were given as means ± SD of at least two biological replicates. Significant differences from the transcriptional level of mutant strain grown at pH 4.5 versus pH 6.8 (Student's *t*-test pValue<0.05) were indicated by the asterisk.

4.2.4 Gene expression studies in *M. smegmatis ΔclgR* mutant after exposure to pH 4.5

In the *ΔclgR* strain MS133 (fig. 22), *sigB* induction was sligthly delayed. In fact, no *sigB* induction was visible after 15 minutes of low pH exposure, suggesting a role of ClgR in *sigB* promoter activation. However, after the first 15 minutes, all genes were well induced. In addition, in this case, it was clear that in acidic conditions ClgR has not the same role seen in response to surface stress where it was essential to sustain the response initiated by MprAB.

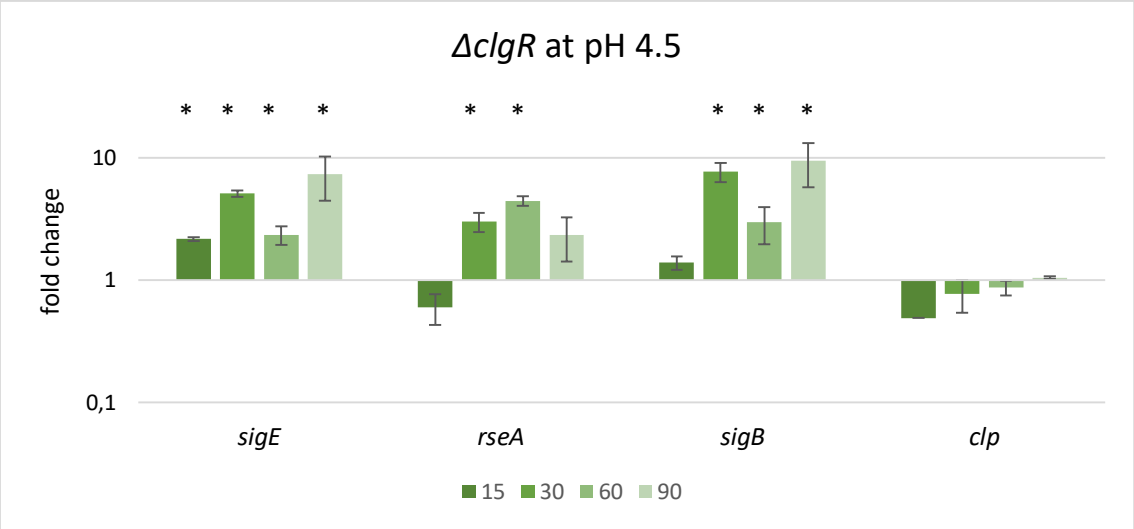


Fig. 22: Fold-changes of mRNA level of genes belonging to the *sigE* network in *ΔclgR* strain at pH 4.5. The samples were collected at 15, 30, 60, 90 mins after the refreshing in Sauton medium at pH 6.8 and 4.5. Results were given as means ± SD of at least two biological replicates. Significant differences from the transcriptional level of mutant strain grown at pH 4.5 versus pH 6.8 (Student's *t*-test pValue<0.05) were indicated by the asterisk.

4.2.5 Gene expression studies in *M. smegmatis* RseA* mutant after exposure to pH 4.5

When the SigE–network dynamics were analysed in a strain encoding RseA T36A (fig. 23), the only clear difference compared to the wild-type after 15 minutes of exposure to low pH was the weaker induction of all genes but we detected a strong upregulation of *sigB* and *clgR* at 15, 60, 90 minutes after the refreshing in low pH.

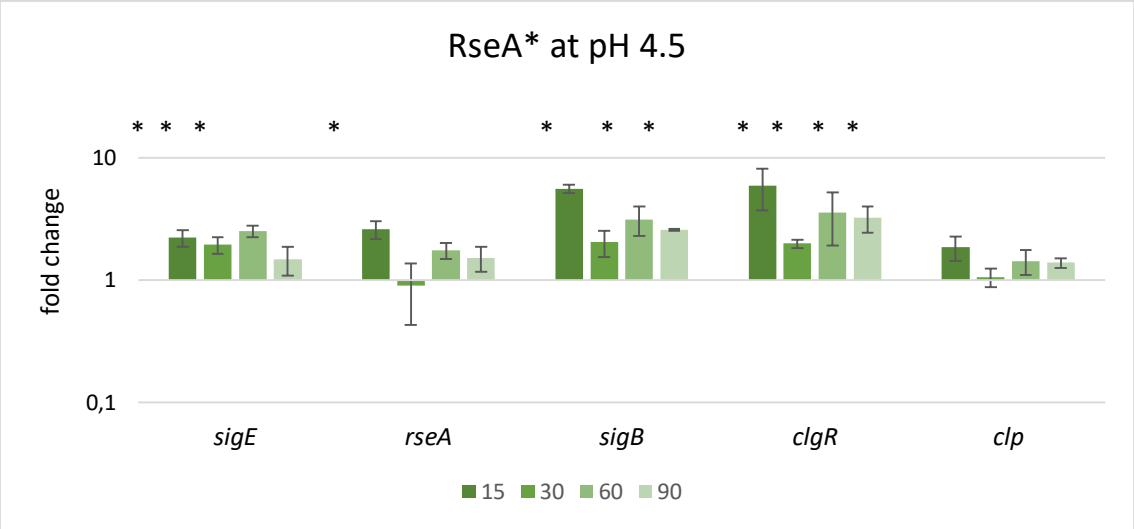


Fig. 23: Fold-changes of mRNA level of genes belonging to the *sigE* network in RseA* strain at pH 4.5. The samples were collected at 15, 30, 60, 90 mins after the refreshing in Sauton medium at pH 6.8 and 4.5. Results were given as means ± SD of at least two biological replicates. Significant differences from the transcriptional level of mutant strain grown at pH 4.5 versus pH 6.8 (Student's *t*-test pValue<0.05) were indicated by the asterisk.

4.3 Characterization of *sigE* regulatory network in *M. tuberculosis* under surface stress conditions

4.3.1 Basal level of different genes involved in *sigE* regulatory network in mutant strains

Before to study the dynamics of expression of the genes involved in the *sigE* network (*sigE, sigB, rseA, clp*), we determined their basal level of expression in the different mutants used in the study and found that their basal level of expression was comparable in all the strains (data not shown).

4.3.2 Killing curves after addition of SDS 0.05% in *M. tuberculosis*

To test the capability to survive of *M. tuberculosis* strains under surface stress condition mediated by SDS, killing curves were performed (fig. 24). The growth pattern was similar in all strains. After 24 hours, the viability of the strain in which RseA has a point mutation was about 69%, that of the strain in which *clgR* was deleted was around 50% while in wild-type strain only 30% of bacteria were resistant.

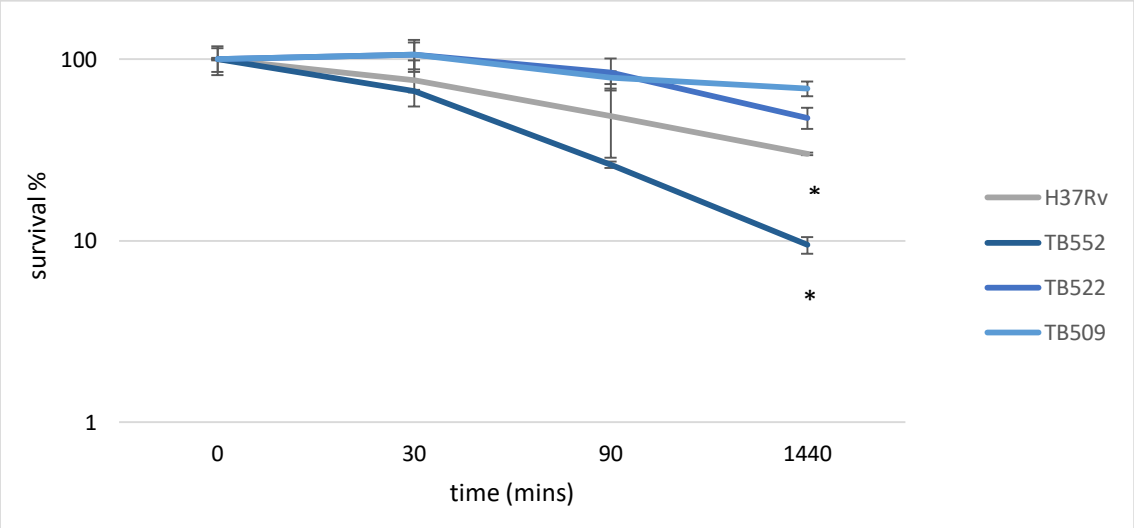


Fig. 24: Killing assay of *M. tuberculosis* strains exposed to SDS 0.05%. The samples were collected before treatment (time 0) and 30 and 90 minutes after the addition of the detergent.

H37Rv = wild-type; TB552 = ΔmprAB; TB522 = ΔclgR; TB509 = RseA*. pValue * < 0.05 Student's *t*-test viability at 1440 minutes versus wild-type strain.

4.3.3 Gene expression studies in *M. tuberculosis* wild-type after SDS 0.05% exposure

The change in expression level of *sigE, rseA, sigB* and *clp* after exposure to stress was studied in the wild-type strain H37Rv (fig. 25). *sigE* induction was immediate and stable during the experiment. Consequently, also *sigB* induction was stable. *clgR* showed two peaks of induction at 30 and 90 minutes, even if not statistically significant, while *clp* showed a mild and not statistically significant induction after 30 minutes of exposure. *rseA* was repressed.

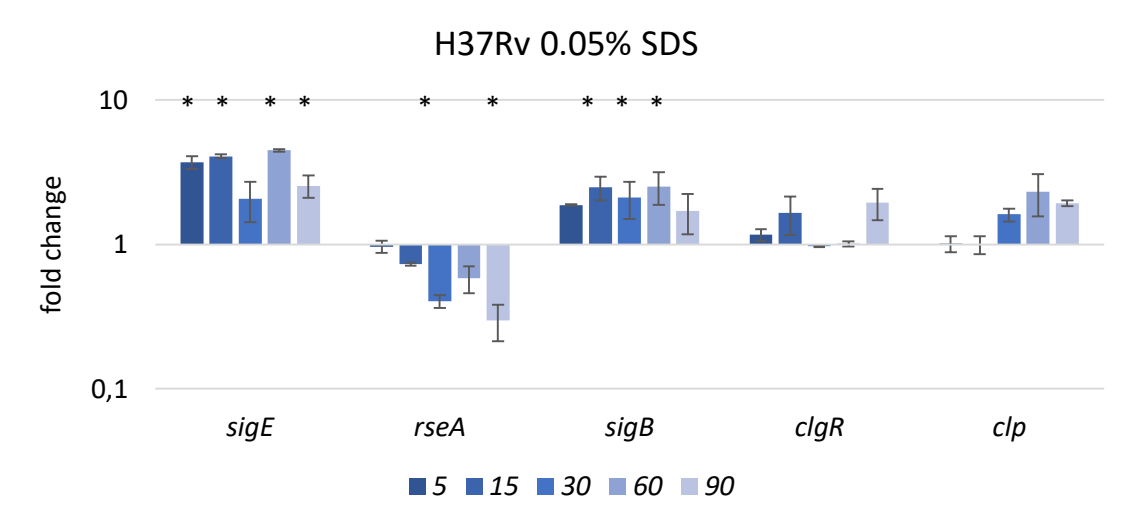


Fig. 25: Fold-changes of mRNA level of genes belonging to the *sigE* network in wild-type strain upon exposure to 0.05% SDS. The values were expressed as the ratio between the numbers of cDNA copies detected by quantitative RT–PCR in samples obtained from exponentially growing strains and were normalized to the level of *sigA* cDNA, which represented the internal invariant control. The values were measured 5, 15, 30, 60, 90 minutes after SDS 0.05% addition and normalized to untreated sample. The reported values derive from at least two independent experiments. pValue * <0.05 Student's *t*-test versus untreated samples.

4.3.4 Gene expression studies in *M. tuberculosis ΔmprAB* mutant after SDS 0.05% exposure

In this *ΔmprAB* mutant, TB552, all the analysed gene were severely repressed except for *sigE,* which showed a mild induction at 5 minutes after the addition of the detergent.

These data show that MprAB has a fundamental role in starting the SigE-mediated stress response in these conditions, since in its absence the system was not able to sense the stress and start the response. *sigE* expression in this mutant can only work from the constitutive promoter P1 since the strong inducible promoter P2 cannot be activated due to the absence of MprAB (54).

SigE was known to be required for most of the SDS induction of *sigB*. MprAB is another component involved in *sigB* regulation activated in response to macrophage infection, surface-damaging agents, nutrient limitation, alkaline pH, and other stresses (44). In mutant strain missing MprAB indeed, *sigB* was not induced after SDS addition.

As assessed in *M. smegmatis*, also in the pathogenic species this two-component system was essential for an efficient stress response (fig. 26).

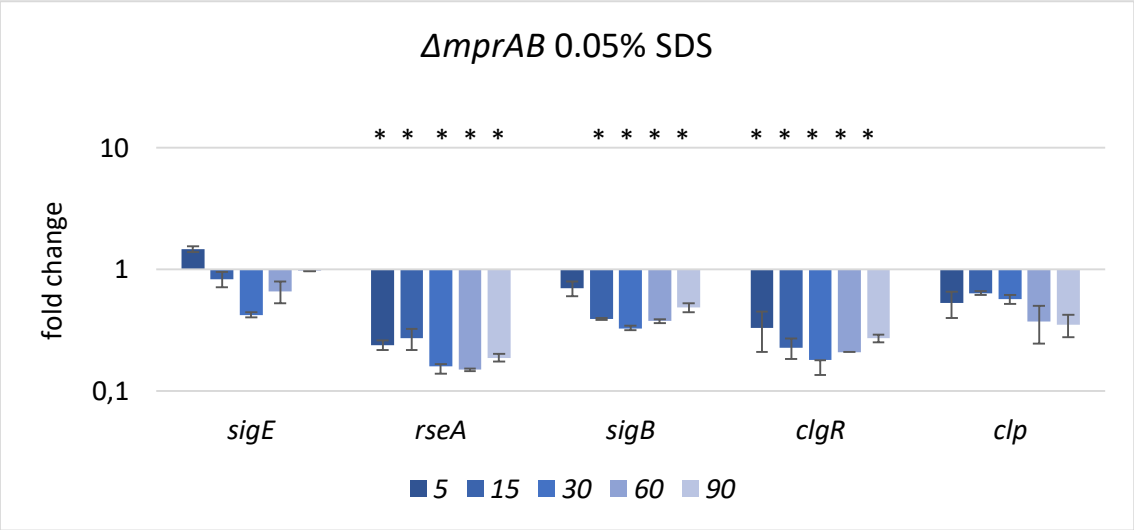


Fig. 26: Fold-changes of mRNA level of genes belonging to the *sigE* network in TB552 upon exposure to 0.05% SDS. The values were expressed as the ratio between the numbers of cDNA copies detected by quantitative RT–PCR in samples obtained from exponentially growing strains and were normalized to the level of *sigA* cDNA, which represented the internal

invariant control. The values were measured 5, 15, 30, 60, 90 minutes after SDS 0.05% addition and normalized to untreated sample. The reported values derive from at least two independent experiments. pValue * <0.05 Student's *t*-test versus untreated samples.

4.3.5 Gene expression studies in *M. tuberculosis ΔclgR* mutant after SDS 0.05% exposure

Also in this mutant, TB522, all the analysed gene were severely repressed except for *sigE* whose decrease of mRNA level started after 30 minutes of exposure to stress (fig. 27). From these data it was clear that in *M. tuberculosis* also ClgR was required to activate *sigE*-mediated stress response.

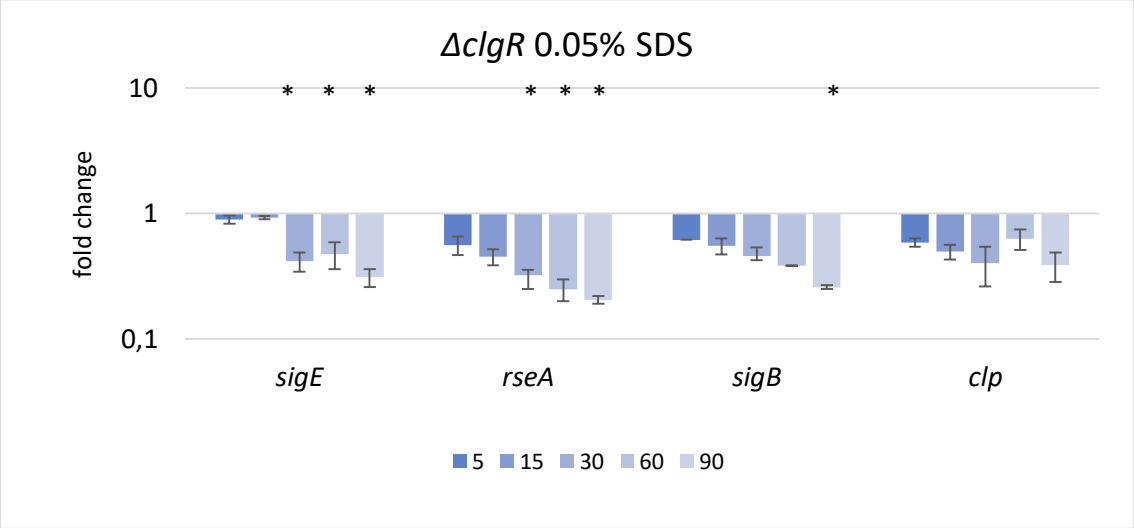


Fig. 27: Fold-changes of mRNA level of genes belonging to the *sigE* network in TB522 upon exposure to 0.05% SDS. The values were expressed as the ratio between the number of cDNA copies detected by quantitative RT–PCR in samples obtained from exponentially growing strains and were normalized to the level of *sigA* cDNA, which represented the internal invariant control. The values were measured 5, 15, 30, 60, 90 minutes after SDS 0.05% addition and normalized to untreated sample. The reported values derive from at least two independent experiments. pValue * <0.05 Student's *t*-test versus untreated samples.

4.3.6 Gene expression studies in *M. tuberculosis RseA** mutant after SDS 0.05% exposure

Also in this strain, TB509, in which the mutation on RseA, should prevent its degradation operated by the protease ClpC1P2, all genes were either not induced or repressed except for *clp*, which was induced. This support the hypothesis that RseA degradation was essential to release an active form of SigE in these conditions. The upregulation of *clp* in the absence of *clgR* induction, suggests that ClgR already present in the cytoplasm in these conditions can be activated inducing *clp* expression (fig. 28).

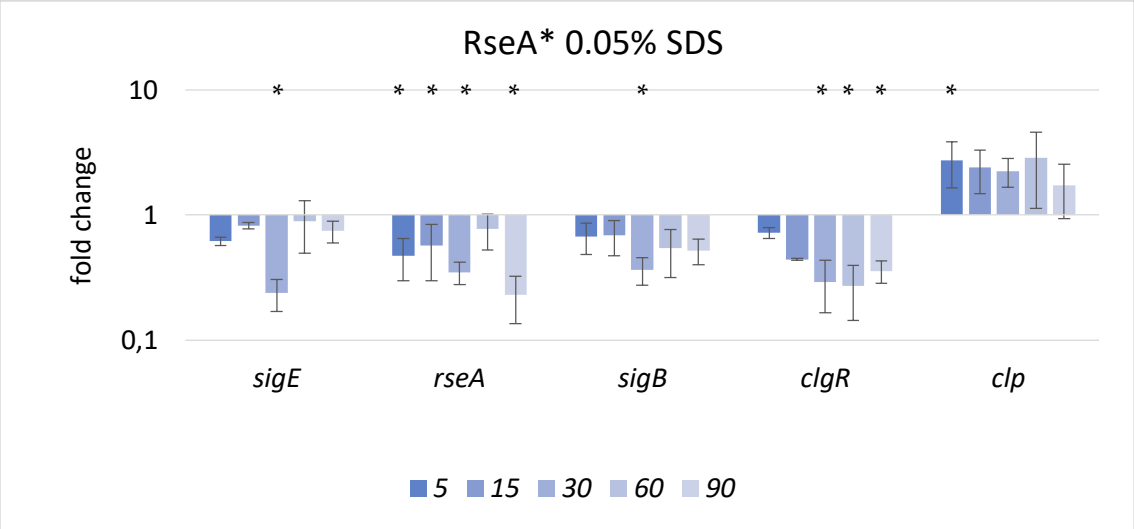


Fig. 28: Fold-changes of mRNA level of genes belonging to the *sigE* network in TB509 upon exposure to 0.05% SDS. The values were expressed as the ratio between the numbers of cDNA copies detected by quantitative RT–PCR in samples obtained from exponentially growing strains and were normalized to the level of *sigA* cDNA, which represented the internal invariant control. The values were measured 5, 15, 30, 60, 90 minutes after SDS 0.05% addition and normalized to untreated sample. The reported values derive from at least two independent experiments. pValue * <0.05 Student's *t*-test versus untreated samples.

4.4 Characterization of *sigE* regulatory network in *M. tuberculosis* in acidic pH conditions

4.4.1 Killing curve at pH 4.5 in *M. tuberculosis*

To test the capability to survive of *M. tuberculosis* strains in acidic pH, killing curves were performed. The number of viable cells was tested by spreading aliquots of serial dilutions at different time points: 0, 30, 90 minutes and 24 hours (1440 minutes) after the resuspension in minimal medium at pH 4.5. In figure 29, it was shown that the cells viability was comparable for all strains except for TB522 in which, after 24 hours, around 75% of bacteria were killed by the effect of acidic pH. For the other strains, significant mortality was not detected.

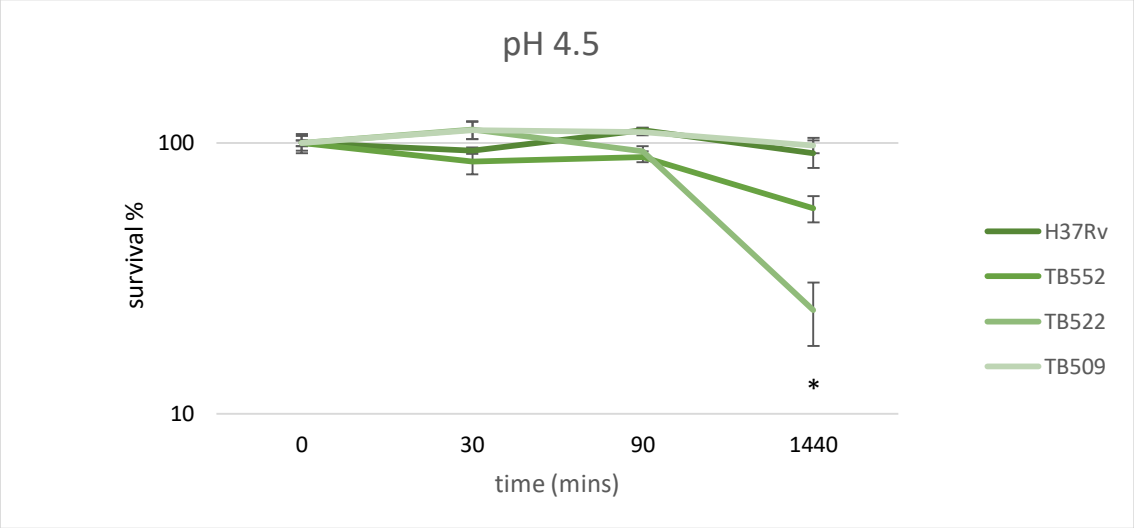


Fig. 29: Killing assay of *M. tuberculosis* strains. The samples were collected immediately after the resuspension in minimal medium at pH 4.5 (time 0), and 30, 90 minutes and 24 hours after the refreshing of the cultures. H37Rv = wild-type; TB552 = *ΔmprAB*; TB522 = *ΔclgR*; TB509 = RseA*. pValue * < 0.05 Student's *t*-test viability at 1440 minutes versus wild-type strain.

4.4.2 Gene expression studies in *M. tuberculosis* wild-type strain after exposure to pH 4.5

In order to investigate if acidic pH influences expressions of the *sigE* regulon, we performed real time PCR experiments comparing the expression of representative *sigE* regulated genes in bacteria grown under acid stress versus normal conditions. Specifically, we decided to analyse the dynamic of transcription of these genes at pH 4.5 compared to pH 6.8.

We evaluated the expression levels of *sigE*-related genes after 15, 30, 60, 90 minutes at pH 4.5. In the wild-type strain (fig. 30) we could see that *sigE* was induced during the experiment and, in parallel, *sigB* showed similar values of induction. Also, *clgR* followed the same pattern at 60 minutes. Surprisingly, transcription of *clp*, known to be regulated by ClgR was not induced These results suggest that *clgR* induction alone was not enough to activate protease transcription, and another signal not present under acidic pH conditions, was required.

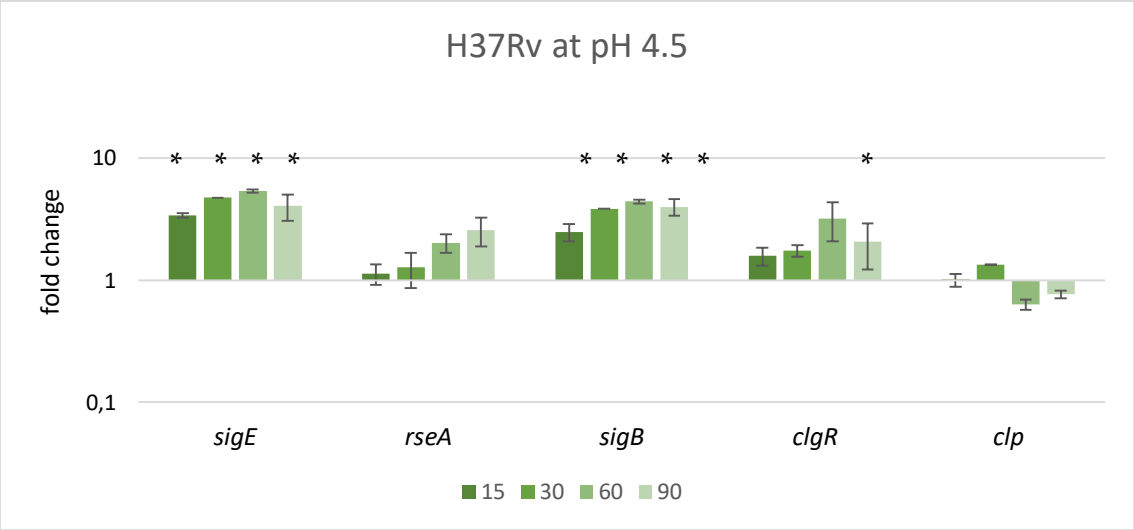


Fig. 30: Fold-changes of mRNA level of genes belonging to the *sigE* network in wild-type strain at pH 4.5. The samples were collected at 15, 30, 60, 90 mins after the refreshing in Sauton medium at pH 6.8 and 4.5. Results were given as means ± SD of at least two biological replicates. Significant differences from the transcriptional level of mutant strain grown at pH 4.5 versus pH 6.8 (Student's *t*-test pValue<0.05) were indicated by the asterisk.

4.4.3 Gene expression studies in *M. tuberculosis ΔmprAB strain* after exposure to pH 4.5

In *ΔmprAB* strain, TB552, a strong upregulation of *sigE* was observed after 30 minutes of acidic pH exposure physiological pH (fig. 31). *sigB* was more induced after 60 minutes when it has the same induction of *sigE* and *rseA* follows the same dynamic. *clgR* was strongly upregulated especially after 30 minutes. The ClgR dependent gene encoding the Clp protease was also induced. The dynamic of stress response was very close to that showed for the wild-type strain even though in *ΔmprAB* mutant it was delayed. The data obtained suggest that the two-component system MprAB doesn't work a strong role as a pH sensor as it had for surface

stress. The induction of *sigE* was clearly dependent on a different regulator that will be interesting to identify. A good candidate was SigH, which might be induced by the oxidative stress induced by the growth arrest caused by acidic pH and was known to recognize *sigE* P3.

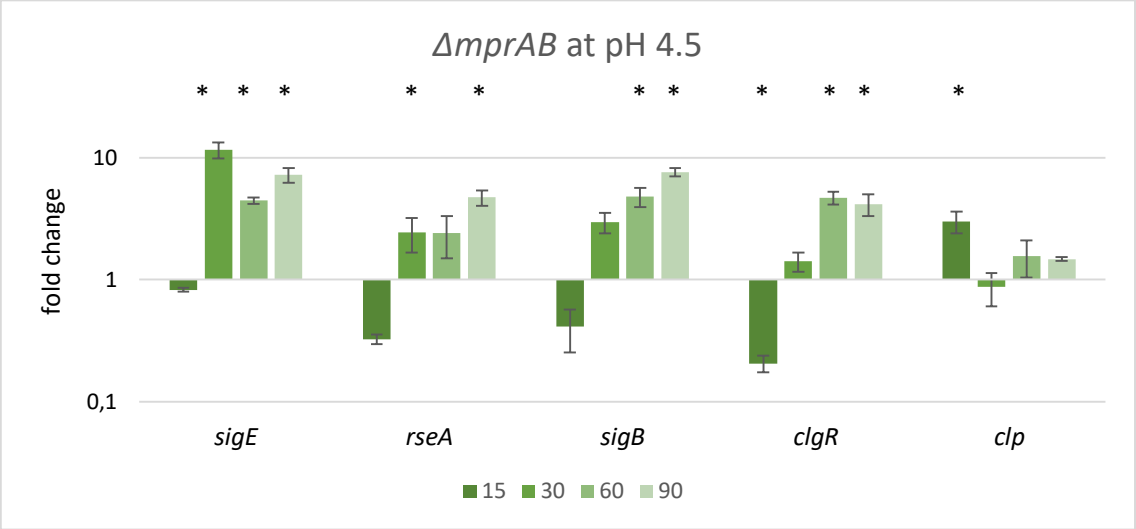


Fig. 31: Fold-changes of mRNA level of genes belonging to the *sigE* network in *ΔmprAB* strain at pH 4.5. The samples were collected at 15, 30, 60, 90 mins after the refreshing in Sauton medium at pH 6.8 and 4.5. Results were given as means ± SD of at least two biological replicates. Significant differences from the transcriptional level of mutant strain grown at pH 4.5 versus pH 6.8 (Student's *t*-test pValue < 0.05) were indicated by the asterisk.

4.4.4 Gene expression studies in *M. tuberculosis ΔclgR strain* after exposure to pH 4.5

In *ΔclgR* strain, TB522 (fig. 32), the induction of *sigE* was delayed, similarly to that of *ΔmprAB* strain, even if the peak of induction was at 60 minutes. Also in this case, the data suggest that ClgR was not essential for *sigE*-mediated low pH response, even if the delay in the activation of the system suggest at least a minor role of ClgR also in these conditions.

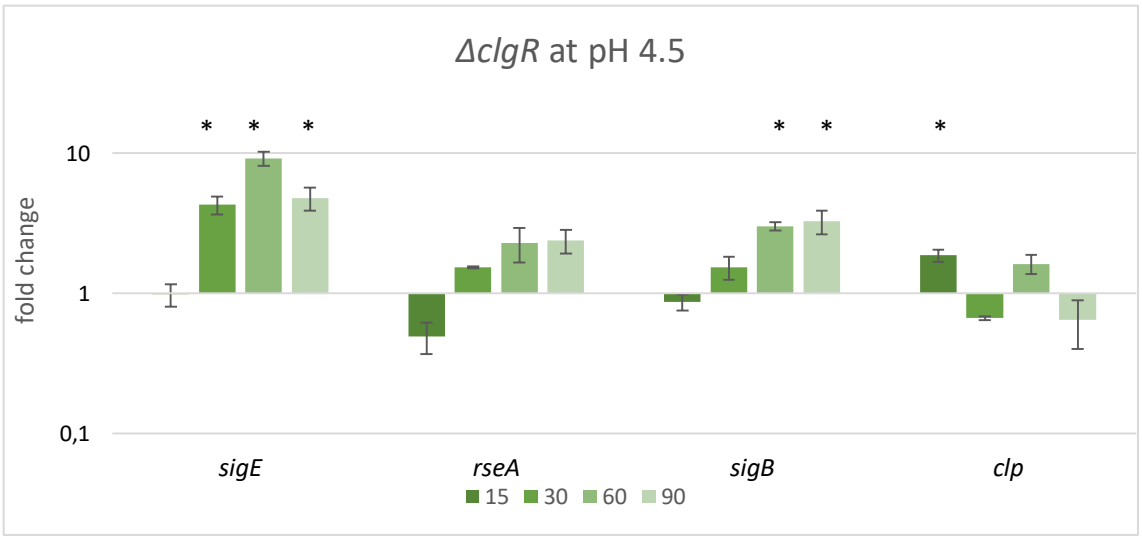


Fig. 32*:* Fold-changes of mRNA level of genes belonging to the *sigE* network in *ΔclgR* strain at pH 4.5. The samples were collected at 15, 30, 60, 90 mins after the refreshing in Sauton medium at pH 6.8 and 4.5. Results were given as means ± SD of at least two biological replicates. Significant differences from the transcriptional level of mutant strain grown at pH 4.5 versus pH 6.8 (Student's *t*-test pValue < 0. 05) were indicated by the asterisk.

4.4.5 Gene expression studies in *M. tuberculosis* RseA* *strain* after exposure to pH 4.5

In RseA mutant strain (fig. 33), TB509, the only clear difference compared to wild-type was a lower induction of all genes, but the system was still active, suggesting a minor role of RseA degradation in the activation of SigE activity in conditions of low pH.

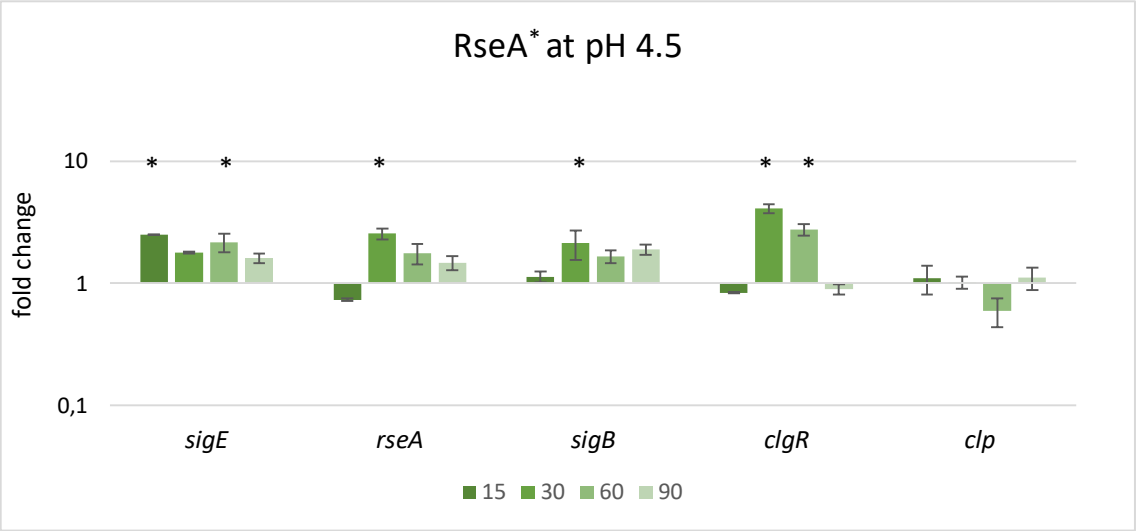


Fig. 33: Fold-changes of mRNA level of genes belonging to the *sigE* network in RseA* strain at pH 4.5. The samples were collected at 15, 30, 60, 90 mins after the refreshing in Sauton medium at pH 6.8 a 4.5. Results were given as means ± SD of at least two biological replicates. Significant differences from the transcriptional level of mutant strain grown at pH 4.5 versus pH 6.8 (Student's *t*-test pValue < 0.05) were indicated by the asterisk.

4.5 Gene expression studies in *sigE* promoter region in *M. tuberculosis* wild-type and *ΔmprAB strain* after exposure to pH 4.5

In order to determine the contribution of each of the three *sigE* promoters under acidic pH condition, real time PCR experiments were performed using three couples of primers (Table 7) Couple "a" detects only transcripts originating from P1, couple "b" detects transcripts from P1 and P2, and couple "c" detects transcripts originating from all promoters. Fig. 34 shows the variation of the mRNA levels detected with these three couples of primers and normalized to the levels of *sigA*-specific mRNA, used as an internal invariant control. RNA was obtained from cells grown in Sauton at pH 4.5 and 6.8 and collected 60 minutes after the refreshing in the appropriate medium in H37Rv and in TB552 (Δ*mprAB*).

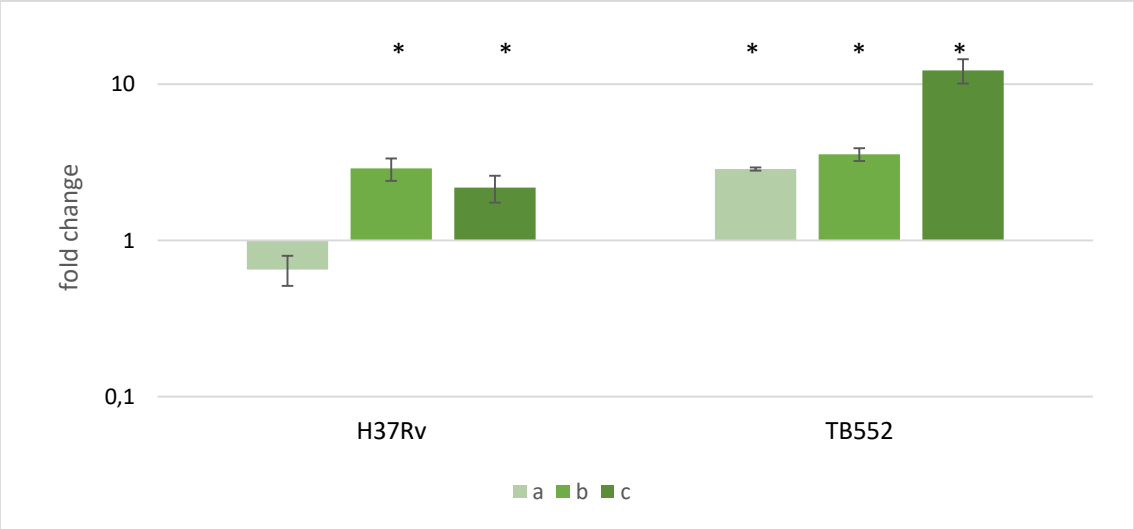


Fig. 34: Changes in mRNA levels after exposure to low pH in *M. tuberculosis* H37Rv and TB552. Three different primer couples were used to evaluate the variation of transcripts initiated at P1, P1 and P2 or from all three promoters (P1+P2+P3). Values were expressed as the ratio between the number of cDNA copies detected in samples obtained from the cultures grown in acidic medium and the number of cDNA copies detected in samples obtained from bacteria grown in standard medium. The values were normalized to the level of *sigA* cDNA, which represents the internal invariant control. Results were given as means±SD of at least two biological replicates. Significant differences from the transcriptional level of mutant strain grown at pH 4.5 versus pH 6.8 (Student's *t*-test pValue< 0.05) were indicated by the asterisk.

As expected, the total amount of transcripts due to the cumulative transcription from the three promoters increased under stress conditions. In wild-type strain, P2 was involved in *sigE* transcription under acidic pH conditions. Since this promoter was activated by MprAB, this suggest that this two-component system was responsible of the activation of the SigE system in the wild-type strain. In order to better characterize the induction of *sigE*, we also performed a preliminary experiment in *ΔmprAB.* In this case, P2 was not involved in *sigE* induction, however most of the induction was due to P3 suggesting that, in the absence of MprAB, *sigE* induction at low pH was due to SigH that was known to be activated under different stress conditions such as oxidative stress. It was possible that in the absence of the activation of the stress response due to the absence of MprAB, the cells experience oxidative stress that activate SigH, which in this case works as a backup system. Experiments were ongoing to evaluate if also in the absence of ClgR a similar mechanism can be activated.

5. CONCLUSIONS

This project contributes to characterize the *sigE* regulatory network in *M. smegmatis* and in *M. tuberculosis* in conditions of surface stress and low pH in order to clarify the hierarchy of the different regulators involved.

Surface stress:

First, we could clarify that in this condition the network works in slightly different manner in *M. tuberculosis* and in *M. smegmatis.* In the absence of MprAB, both species were unable to respond to SDS, even in the first few minutes of exposure, when viability of the strains was not affected at all. However, in the absence of ClgR, while the expression profile of the *M. tuberculosis* mutant was similar to that of the MprAB mutant, in *M. smegmatis* the system was able to be activated, even if at lower intensity and stability, suggesting a role for ClgR less important in this species than in *M. tuberculosis*. Similarly, while the mutation of RseA preventing its degradation had a major role in *M. tuberculosis*, totally abrogating the SDS response, it did not have almost any effect in *M. smegmatis,* suggesting a minor role of SigE post-translational regulation in *M. smegmatis* in these conditions or a minor involvement of RseA phosphorylation in its degradation in this species.

Low pH:

In these conditions, both species behaved mostly in the same way, as neither the deletion of *mprAB* or the deletion of *clgR* had a major role in the activation of the *sigE* network, if we exclude the clear 15 minutes delay only visible in *M. tuberculosis* and in the *M. smegmatis clgR* mutant. This suggests that neither of the two regulators are involed in low pH response or that in their absence, a back up system is activated. To explore these possibilities, we characterized the activity of the three promoters of *sigE* in *M. tuberculosis* comparing their activation in the wild-type strain and in the *mprAB* mutant. We discovered that, while in the wild-type strain the main promoter responsible of *sigE* transcription after exposure to SDS was the MprAB-dependant promoter P2, in the *mprAB* mutant the main promoter responsible of *sigE* transcription was the SigH-dependant promoter P3. These data suggest that in the wild-type strain, MprAB is the main sensor of stress (probably due to surface damages due to low pH). In its absence, the cell cannot react to this damage and, during time, the low pH can

induce the formation in the cytoplasm of reactive oxygen intermediates that activate SigH. Experiments are ongoing to evaluate if this mechanism is also present in the *clgR* mutant. The fact that in both strains with a mutation in RseA that preclude its phosphorylation and thus its degradation, the response is active, but clearly at lower level, is still difficult to understand in the absence of data indicating the *sigE* promoters active in this strain and in the *clgR* mutant. A possibility is that in this strain the absence of degradation of RseA decreases the concentration of free SigE (induced from P2 or P3) maintaining its activity at lower levels. Further experiments are needed to clarify the meaning of these finding.

In conclusion, the data presented in this thesis help to better understand the effective implication of the different key players of the *sigE* regulatory network under critical conditions and contribute to clarify the mechanisms by which the tubercular pathogen manages to adapt and successfully survive into the host.

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