All Stressed Out: Mycobacterial Responses to Stress

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Infection of a host by a pathogenic microorganism requires the resistance of the colonizing microbe to a variety of stressors. These stressors can be produced by the host's immune system in an effort to thwart the invading microbe's attempt to grow in a host environment. Stresses can be in the form of acidic, oxidative, nitrositive, and low oxygen tension (Fig 1b). Additional stresses can come from the environment as the invading pathogen endures varying periods of time externally before being taken up by a host. External environmental stresses include desiccation and UV light exposure (Fig 1a). Researchers studying these stressors in vitro can gain a more complete understanding of pathogenic processes that occur in vivo.

1. Introduction

Mycobacteria are naturally resistant to a variety of stresses due to their thick waxy cell wall. They resist many environmental insults including exposure to antibiotics. Trehalose dimycolate, or cord factor, confers on mycobacteria resistance to multiple drugs [1]. It is also necessary for the proper structure of the cell wall of the organism and for mycobacterial ultrastructures such as colony morphology. Trehalose dimycolate is responsible for the phenomenon of cording where *Mycobacterium tuberculosis* self associates into structures that appear as cords. This self association likely provides a physical barrier to stresses and protects from cell damage.

In addition to innate resistance to stress due to a robust cell wall, mycobacteria also respond to the environmental stressors via specific gene inductions. Thus, in the presence of a variety of stresses including heat shock, acidity, and low oxygen tension, bacteria will upregulate various genes needed to withstand environmental stressors. Genes are induced, proteins produced, and mycobacterial cells are rendered more resistant to harsh environmental conditions. This system allows for the optimum use of mycobacterial resources. Bacteria ramp up certain stress induced pathways that are not needed or perhaps are detrimental during growth under non stress conditions. Needed resources are only consumed at the point where mycobacterial survival is in jeopardy.

2. Desiccation

During the course of infection, *M. tuberculosis* is expelled into the airway in an individual with active cavitary disease. As a sick individual coughs to expel mycobacteria*, M. tuberculosis* after many generations inside the human body finally arrives to the outside environment within microdroplets which then evaporate to produce droplet nuclei (Fig 1a). These droplet nuclei remain suspended in the air for hours to efficiently infect the next individual. Thus is it of vital importance for the infectivity of *M. tuberculosis* that it can resist desiccation, albeit for several hours. The ability of *M. tuberculosis* to persist in the environment is of great relevance to its overall pathogenesis. Mycobacteria capable of persisting for longer periods of time may, in the end, infect a greater number of hosts.

Trehalose dimycolate previously found to be important for intrinsic resistance to antibiotics also appears to give model mycobacterial membranes resistance to desiccation [2]. This resistance could potentially aid the microorganism in a transition from a relatively wet environment to an aerosol where desiccation predominates. In fact synthetic trehalose glycolipids also impart desiccation resistance to lipid membranes [3]. This compound can stabilize membranes which often do not fare well under conditions where drying is a concern. Thus production of trehalose dimycolate could give mycobacteria a competitive advantage by allowing their membranes to resist desiccation and thus survive to establish a productive infection in another host inhaling the droplet nuclei.

3. Exposure to Ultraviolet (UV) Light

Once in the external environment *M. tuberculosis* can be exposed to UV light from solar radiation or artificial sources (Fig 1b). This UV light can readily inactivate mycobacteria. In fact treatment of tuberculosis in the first part of the 20th century often entailed exposure to fresh air and sunlight. This not only provided vitamin D to the infected patient, but also discouraged transmission to other individuals. In fact a sanatorium that was established in a cave in the United States resulted in all the patients becoming markedly worse, as well as the infection with *M. tuberculosis* of non ill companions of the patients [4]. Due to the absence of UV light, infection of the healthy companions was probably

encouraged by the persistence of droplet nuclei with viable *M. tuberculosis* bacilli in the air of the cave. In a clinical setting UV light is often provided in rooms of tuberculosis patients to inactivate bacteria expelled due to cavitary disease and coughing.

Genes involved in excision repair in *M. tuberculosis* seem to be important in resisting DNA damage due to exposure to UV light [5]. Interestingly a mutated version of the *uvrB* gene, that normally functions is to excise damaged nucleotides, confers on mycobacteria more susceptibility to reactive oxygen and reactive nitrogen intermediates within a mouse host. In fact *uvrB* was also upregulated during *M. tuberculosis* infection of human macrophages [5]. Thus DNA repair mechanisms are important to resist damage due to exposure to UV light as well as in vivo stresses.

4. Oxidative stress

Once inside a host, mycobacteria can experience a variety of stresses from the host immune system. An individual exposed to a patient with active tuberculosis may inhale droplet nuclei. The droplet nuclei travel to the lung alveoli where *M. tuberculosis* encounters alveolar macrophages. These macrophages interact with the mycobacteria and phagocytose them. Mycobacteria then traffic to the phagocytic compartment. As the immune system senses the presence of the invading mycobacteria it may stimulate macrophages to become more efficient at killing foreign invaders. These activated macrophages produce increased quantities of reactive oxygen intermediates (Fig 1b). In one study a significantly greater numbers of genes are upregulated in *M. tuberculosis* than *Mycobacterium avium subsp paratuberculosis* indicating oxidative stress is a major stressor of *M. tuberculosis* in vivo [6].

Bacterial stationary phase growth seems to increase intracellular oxidative stress. When mycobacteria experience nutrient limitation, as they might in host macrophages, they may enter stationary phase. Thus within macrophages, mycobacteria may experience oxidative damage due to external exposure of reactive oxygen intermediates as well as intra-bacterial cytoplasmic oxidative damage. *M. tuberculosis* may combat this oxidative stress with the *whiB1* gene which becomes more active during stationary phase and functions to reduce cellular disulphide brides that may predominate during stationary phase [7].

Mycobacteria produce mycothiol which acts to combat the toxic effects of oxidative damage. Cysteine residues in mycothiol become oxidized under oxidative conditions to form disulphide bond containing mycothione, thus preventing the oxidation of other bacterial molecules. Mycothione can then be recycled to mycothiol. Many other bacterial species as well as human cells produce glutathione to combat oxidative damage. Glutathione, however, is toxic to mycobacteria when provided in growth media or produced by human macrophages. This toxic effect may result from glutathione disrupting the redox balance within mycobacterial cells [8,9].

In addition to mycothiol, it has been shown that superoxide dimutases (Sods) and catalase (KatG) can detoxify superoxide [10]. Interestingly these genes are upregulated in *M. tuberculosis* early in infection and then down regulated during chronic infection indicating that early in infection oxidative damage may be important for controlling mycobacteria growth and less so later in infection. *M. tuberculosis* possesses one heat labile catalase (KatG) and is more sensitive to oxidative stress than *Mycobacterium fortuitum* which possesses a heat labile catalase (KatG) as well as a heat-stable catalase (KatE). This supports the idea that a greater quantity of catalase can increase resistance to oxidative damage [11].

Oxidative damage may act to damage DNA. A histone like protein Lsr2 can confer protection on mycobacteria to reactive oxygen intermediates [12]. This histone like protein is thought to act by binding DNA, compacting it, and acting as a physical barrier to damage. As mentioned previously, *uvrB* when mutated results in mycobacteria which are more sensitive to oxidative damage as well as more sensitive to UV damage showing that diverse systems can confer resistance to multiple stresses [5]. In support of this, multiple mutations that result in sensitivity to acidic stress also result in sensitivity to oxidative stress [13].

5. Acid Responses

Within immunologically activated macrophages, phagosomal pH initially drops to 5.5 and can rebound to 6.5. Low pH can inhibit growth of mycobacteria or is bactericidal and can also be encountered within granulomas or liquefied lesions within the host (Fig 1b) [14,15]. Additionally, low pH may be a signal to increase virulence indicating that infection has taken place and additional genetic pathways need to be activated for mycobacteria to resist novel environmental insults.

Many genes which are upregulated during exposure to acidic stress relate to the cell wall. Transposon mutants selected for increased sensitivity to acidic stress mapped to genes important for cell wall formation. These mutants proved to be susceptible to oxidative stress as well [13]. One study found that the *mymA* operon is upregulated after 15-30 min of exposure to acidic pH 5.5 [16]. Deletion of genes in this operon resulted in defects within the mycobacterial cell wall and the deletion strain was more sensitive to acidic pH [17,18]. The *lipF* gene encodes an esterase that may modify the mycobacterial cell wall in response to acidic stress to increase resistance. In fact LipF localizes to the mycobacterial cell wall and its gene's promoter is upregulated at a minimum of 1.5 hours of exposure to acidic stress

[19]. When mutated, a putative magnesium transporter in *M. tuberculosis* resulted in attenuated growth at mildly acidic pH and low mg^{2+} concentrations [20]. Mg^{2+} may be important to maintain cell envelope structure which is altered at acidic pH. OmpA, a protein predicted to form pores is induced at pH 5.5 and mutation of this gene showed attenuation in a mice model of tuberculosis [21,22]. The mechanism by which OmpA helps mycobacteria resist acidic stress is unknown.

Many genes upregulated by acidic stress fall within the PhoP/PhoR regulon. PhoP is part of a two component system consisting of a transcriptional regulator and a sensor kinase. PhoR is the sensor kinase which responds to an unknown signal. PhoP is presumably activated by PhoR and regulates a variety of genes. A deletion mutant of *phoP* results in the loss of upregulation of a variety of genes, a subset of which have been shown previously to be upregulated by acidic stress [23].

Analysis of genes upregulated by acidic stress in *M. avium subsp paratuberculosis* revealed 613 genes upregulated by this stress. This constituted a greater number of genes upregulated than was found in *M. tuberculosis* in response to acidic stress. During infection, *M. avium subsp paratuberculosis* must traverse the host intestinal tract including the acidic environment of the stomach. *M. tuberculosis* in contrast infects via the lung and encounters acidity within macrophages, granulomas, and liquefied lesions [14,15]. The greater response of *M. avium subsp paratuberculosis* may result from adaptation to an increased exposure to acidic stress. Nonetheless, acidity seems to be an important stressor in vivo for *M. tuberculosis*.

6. Heat Stress

As infection progresses, the host immune system responds. A consequence of *M. tuberculosis* infection is fever which can lead to heat stress in mycobacterial species (Fig 1b). Heat stress can cause unfolding and degredation of proteins within the bacterial cytoplasm as well as the cell wall. Bacterial responses include production of factors such as heat shock proteins which act as chaparonins to refold proteins. The α–crystalline protein Acr-2 is activated by heat shock and SDS treatment. *acr2* is regulated by the two component system *mtrAB* and *sigE*. Acr2 also exhibits chaperone activity in vitro [24]. While heat shock proteins have chaperone activity to aid with refolding heat damaged proteins, these same heat shock proteins can elicit an immune response. The 65 Kd heat shock protein is found in cerebral spinal fluid of patients with tuberculosis meningitis [25]. Hsp70 from *M. tuberculosis* is released in exosomes by heat shock and stress [26]. This protein can cause an inflammatory response in individuals infected with *M. tuberculosis*. *Mycobacterium leprae* is defective in heat shock, lacking Hsp70, Hsp60, and *sigE* which is a pseudogene in this species [27]. *M. leprae* tends to colonize and grow within tissues in a host which have a mean lower temperature such as the extremities.

7. Hypoxic Growth

M. tuberculosis experiences low oxygen tension in the human host. As the immune system responds to the presence of tubercle bacilli, macrophages and T-cells accumulate at sites of infection. Granulomas form and the center of granulomas can have decreased oxygen tension (Fig 1b). In fact studies have shown that tuberculous granulomas from guinea pigs, rabbits, and nonhuman primates are hypoxic [28]. There have been many in vitro models of hypoxia that potentially can lead to dormancy of *M. tuberculosis*. Low oxygen tension may be a signal for mycobacteria to enter a latent, dormant, or persistent state. These low oxygen in vitro models include slowly depleting oxygen, rapidly depleting oxygen, and addition of nitric oxide.

Early in exposure an initial hypoxic response predominates. This early response is controlled by the two component system DosS/DosT-DosR and serves to upregulate genes in what has come to be called the "dormancy regulon". DosR is a transcriptional regulator, whereas DosT and DosS are sensor kinases which respond to low oxygen as well as nitric oxide (NO) [29,30]. DosR is itself controlled by another two component system, the PhoP-PhoR regulon which responds to unknown environmental signals [23]. One gene which is upregulated by DosR and low oxygen tension is *hspX* (*acr*, *Rv2031c)* which produces a protein thought to aid in refolding proteins [31,32]. This protein is induced in vivo, as T-cells from latently infected individuals with *M. tuberculosis* show reactivity to the HspX protein [33].

One half the genes in the DosR regulon of this initial hypoxic response return to baseline within 24 hours. After DosR upregulation other regulatory networks such as sigE and sigC replace DosR. An enduring hypoxic response occurs after the initial hypoxic response and may be important for the mycobacteria to enter or maintain stasis [34]. The PhoP/PhoR two component system has been shown to be important for the upregulation of genes within this enduring hypoxic response [23].

In order to survive the dormant/ latent phase, *M. tuberculosis* needs to maintain cell viability, and proton motive force as well as ATP homeostasis [35]. After a period of time in stasis perhaps initiated due to the lack of oxygen, mycobacteria may need to exit the dormant/latent phase to produce an active infection. The transition may occur with waning immunity and constitutes reactivation. *M. tuberculosis* is known to accumulate triacylglycerol during stress such as exposure to an acidic environment, treatment with NO, hypoxic growth, and stasis [36,37]. Triacylglycerol

utilization has also been shown to be important to transition from dormancy to active growth [38]. In fact certain pathogenic strains of *M. tuberculosis* such as w/Beijing lineage overproduce triacylglycerols which may aid in their pathogenesis [39].

8. Toxin-Antitoxin Systems

Toxin-antitoxin systems are common in bacteria. These systems contain a "toxin" which can be inhibitory to critical self-cellular processes and is a relatively stable protein. The antitoxin often acts as transcriptional repressor of the toxin gene and is relatively unstable. Environmental stresses can act to inactivate the unstable antitoxin gene resulting in increased transcription of the toxin gene and increased toxin concentrations. Toxins may function to cleave mRNA and thus inhibit cellular protein production [40]. The toxin can down regulate cellular processes and help bacterial cells adapt to stress conditions. There are 88 putative TA systems which are present in *M. tuberculosis.* Four of these toxin-antitoxin systems have been shown to be activated by hypoxia and phagocytosis.

9. Two Component systems

Two component systems are ubiquitous among bacteria. They function via the transfer of phosphate between a sensor kinase and a response regulator. The sensor kinases are often present in the cellular membrane, sense a signal, and phosphorylate the response regulator which can be a transcriptional activator or repressor. Thus two component systems constitute a mechanism by which to transmit signals from the external environment, such as stress conditions, to upregulate key genes needed to modulate bacterial functions. *M. tuberculosis* contains within its genome 11 two component systems [41]. Some of these systems have already been shown to be important to respond to specific stresses. DosS/DosT-DosR responds to hypoxia and upregulates many genes needed for an initial hypoxic response [29]. Others such as PhoP/PhoR are important to upregulate genes involved in several stress responses such as acidity and low oxygen tension, yet the environmental signal remains unknown [23].

10. Sigma Factors

In order to activate transcription, bacterial RNA polymerases bind to promoter regions. RNA polymerase is composed of a core enzyme with two α subunits, a β, a β', and a ω subunit. This core enzyme binds to promoters in a non specific manner. Sigma factors determine promoter specificity by associating with the RNA polymerase holoenzyme and recognizing and binding to -10 and -35 promoter sequences. Thus in addition to specific transcription factors, bacteria can use sigma factors to activate transcription of broad categories of genes, some of which may be responsive to environmental stressors.

Bacterial sigma factors belong to differing categories. The *M. tuberculosis* σ^A is essential and regulates transcription of housekeeping genes. σ^B is nonessential but highly similar to σ^A and is induced by heat shock, cold shock, low aeration, oxidative stress, and stationary phase [42]. The extracellular function σ factors (ECF) are environmentally responsive and are often involved in envelope synthesis. ECF sigma factors include SigC, sigD, SigE, SigG, SigH, SigI, SigJ, SigK, SigL, and SigM. SigF is another alternate sigma factor with homology to sporulation sigma factors in other bacterial species and responds to heat shock, cold shock and nutrient starvation. A *sigC* mutation resulted in *M. tuberculosis* more susceptible to hydrogen peroxide [43]. SigB, SigE, sidD, and SigF respond to nutrient starvation while sigE also responds to heat shock and SDS exposure [43]. SigJ and SigF are induced by antibiotic exposure [43]. SigH responds to heat shock and oxidative stress [43]. Thus sigma factors function as master regulators. In *M. tuberculosis* the ECF sigma factors serve to respond to environmental stressors and to activate many genes requires for each stress condition.

11. Summary

It is apparent that mycobacteria contain a plethora of mechanisms that respond to environmental stressors. These systems likely evolved under selective pressure from the host immune system. While mycobacteria possess an innate ability to withstand stress due to their robust cell wall, they also possess systems which are upregulated only upon exposure to specific stresses. Thus they possess systems which are inducible and render the bacteria more resistant to their environment. By understanding how mycobacteria sense and respond to environmental stresses we can gain insight as to how these microbes are such successful pathogens, specifically of the human body. Understanding stress response systems will hopefully lead to the development of treatments which will thwart mycobacterial attempts to resist host insults and which will promote elimination of mycobacteria from the host. In addition, understanding the mechanism by which stress induces *M. tuberculosis* to enter a dormant/ latent state within the host may lead the way to treatments that prevent *M. tuberculosis* from entering or from exiting this state.

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