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Is *Mycobacterium tuberculosis* stressed out? A critical assessment of the genetic evidence

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Abstract

Mycobacterium tuberculosis is an obligate human intracellular pathogen which remains a major killer worldwide. A remarkable feature of *M. tuberculosis* infection is the ability of the pathogen to persist within the host for decades despite an impressive onslaught of stresses. In this review we seek to outline the host inflicted stresses experienced by *Mycobacterium tuberculosis*, the bacterial strategies used to withstand these stresses, and how this information should guide our efforts to combat this global pathogen.

Keywords

Mycobacterium tuberculosis; Stress Responses; Pathogenesis

I. Introduction

Infection with *Mycobacterium tuberculosis* remains a major global health problem. Although active infection with this organism can be effectively treated with multidrug antimicrobial therapy, the shortest antibiotic regimens that will achieve reliable clinical cure are six months in duration. Regimens for drug resistant infections, or drug sensitive infections that arise in immunosuppressed hosts, are longer. A major goal of efforts to improve Tuberculosis (TB) treatment is to develop agents that will shorten therapy, thereby facilitating more widespread administration of curative antimicrobial regimens. One model of TB drug development holds that small molecule inhibitors of proteins necessary for *M. tuberculosis* to tolerate the harsh in vivo environment of the host would be promising candidates to shorten therapy. This rational path to antimicrobial development, while clearly not the only or most expeditious approach [1], posits that the important bacterial pathways that allow *M. tuberculosis* to persist within the host, and resist rapid elimination by available antimicrobials, have not been targeted. In part, this failure could be due to the traditional approach to antimicrobial discovery, which has identified compounds that inhibit processes important for cell growth. Development of antimicrobials that efficiently sterilize *M. tuberculosis* within the host, requires an understanding of the pathways in *M. tuberculosis*

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that are critical to withstand host-inflicted stresses. Our goal in this review is to summarize the current state of knowledge of:

1. The stresses experienced by *M. tuberculosis* during persistent infection
2. The defenses *M. tuberculosis* elaborates to withstand these stresses
3. How this information should guide further efforts in the research community to understand the *in vivo* environment of *M. tuberculosis*

II. Tuberculosis in Humans

M. tuberculosis infection begins when inhaled bacilli enter the airways and are immediately exposed to phagocytic cells of the innate immune system and antimicrobial peptide filled fluid covering the alveolar surface. Infection of naive alveolar macrophages and dendritic cells leads to a proinflammatory response and the recruitment of lymphocytes, monocytes, and fibroblasts to form a granuloma. Within the granuloma, T lymphocytes secrete cytokines such as gamma interferon (IFN- γ), which is critical for anti-mycobacterial defense. The specific antimicrobial effector functions stimulated by IFN- γ that kill *M. tuberculosis* in human cells are still being elucidated. Despite these attacks, primary infection with *M. tuberculosis* is highly successful and leads to widespread dissemination of *M. tuberculosis* to most organs in the body, but is usually asymptomatic in immunocompetent hosts and rarely leads to progressive Tuberculosis. Primary infection is subsequently controlled by antigen-specific cell mediated immunity, which reduces bacterial numbers to uncultivable levels, a clinical state that we refer to as latency. It is impossible to know with certainty whether immunity is completely sterilizing in some infected people, because the only residual evidence of prior infection is delayed type hypersensitivity reaction against mycobacterial antigens, detected either by a tuberculin skin test, or more recently IFN γ production from antigen stimulated lymphocytes. Definitive evidence for clinical latency in TB came from autopsy studies of asymptomatic people who died from non-Tuberculosis causes. These studies clearly revealed viable *M. tuberculosis* that could be recovered through inoculation of experimental animals or prolonged incubation [2]. Epidemiologic studies also revealed that a significant proportion (approximately 15-20%) of people who become tuberculin skin test positive after exposure to active Tuberculosis will reactivate the infection after a variable period of latent infection, most often less than 5 years, but sometime decades. These studies provided strong evidence for the existence of clinical latency. Thus, in a substantial fraction of primary exposures to *M. tuberculosis*, the bacterium is able to resist elimination by the host during a prolonged state of clinical and presumably microbiologic dormancy, a feat that must require novel strategies of pathogenesis.

In addition to its ability to resist elimination by host immunity, *M. tuberculosis* infection is also slowly sterilized by antimicrobial agents that are highly active against *M. tuberculosis* in vitro. Clinically, this drug tolerance is manifested by a biphasic kill curve in infected patients: a rapid early reduction in bacterial load followed by a more prolonged phase of slower sterilization. To reliably achieve clinical cure in greater than 90% of patients, multidrug antibiotic therapy for six months is required. Perhaps the most dramatic clinical example of drug tolerance in *M. tuberculosis* comes from the administration of preventative antimicrobial therapy for latent disease. Despite a low bacterial burden, the beneficial effect of Isoniazid monotherapy in preventing reactivation of latent disease continues to accrue through 9 months of therapy [3].

Thus, the clinical “persistence” of *M. tuberculosis* that we seek to understand as a research community is multifaceted. It includes the following bacterial phenotypes:

1. Resistance to elimination during the onset of antigen specific immunity during primary infection.
2. Resistance to sterilization by host immunity during prolonged latent infection
3. Persistence during preventative antimicrobial therapy for latent infection.
4. Persistence despite multidrug antimicrobial therapy during active disease, requiring prolonged administration to achieve cure.

Most efforts to reproduce these features of human Tuberculosis in controlled experimental settings have used animal models to understand *M. tuberculosis* persistence.

III. Animal Models of *M. tuberculosis* Infection and Persistence

No animal model replicates the multiple phases of *M. tuberculosis* infection in the human host as outlined above. Nevertheless, small animal models are necessary to understand *M. tuberculosis* pathogenesis because they allow analysis of bacterial burden, tissue pathology, and host survival of various host and pathogen mutants. The animal models that are used most extensively are discussed below.

The mouse is the most widely used animal model for *M. tuberculosis* infection because of the broad availability of immunological reagents, genetically engineered strains, and ease of housing. *M. tuberculosis* infection of the mouse by aerosol inhalation or intravenous injection follows a reproducible pattern characterized by bacterial growth over the first 2-3 weeks, followed by a plateau of the bacterial burden. The beginning of this plateau phase is coincident with onset of host adaptive immunity and can continue for over a year, until the death of the animal. This “persistence phase” of the murine infection clearly represents a balance between host immunity and the pathogen's ability to resist elimination. Host defects in cell mediated immunity such as lack of IFN γ , CD4 T cells, or TNF α cause progressive infection without a persistence phase [4].

However, several aspects of murine infection with *M. tuberculosis* do not replicate human infection, at least as assessed in standard laboratory mouse strains such as C57Bl/6. *M. tuberculosis* infected C57Bl/6 mice do not develop the architecturally complex cavitory granulomas that are characteristic of human Tuberculosis. Recently, alternative mouse strains have been generated by breeding which do develop cavitory lesions and the murine genetic determinant has been identified [5]. However, in all models, the persistence phase of the murine infection is characterized by stably high bacterial loads, indicating that the murine immune system is incapable of reducing *M. tuberculosis* to latency. In addition, recent evidence indicates that *M. tuberculosis* continues to replicate during the persistence phase [6-7], further reducing similarities to human latency.

In an attempt to better reconstitute the granulomatous environment of the murine model, the mouse hollow fiber model was developed [8]. The hollow fiber model uses encapsulation of bacilli in semidiffusible hollow fibers that are implanted subcutaneously into mice. Hypoxic granulomatous lesions develop around the hollow fibers, wherein the bacteria exhibit decreased metabolic activity and an antimicrobial susceptibility pattern similar to persistent bacilli [8].

Another variation of mouse infection is the Cornell model of persistent infection and reactivation, in which *M. tuberculosis* infected mice are treated with antimycobacterial agents until the bacterial titer becomes undetectable by microscopic inspection or culture. Following suppression of the host immune system by steroids, mice develop reactivated infection. However, the observed phenomenon of reactivation in infected mice is highly variable [9-10].

An alternative animal model to the mouse is the guinea pig. Following low dose aerosol infection, lung lesions in guinea pigs infected with *M. tuberculosis* have striking similarities to natural infections in humans, such as necrosis, mineralization, and hypoxia. However, this infection invariably results in progressive Tuberculosis characterized by marked weight loss, cachexia, and death occurring usually within 15-20 weeks after infection, and therefore natural latent infection does not occur.

The quest for an animal model that more closely replicates human infection has led some investigators to study infection in non-human primates. Infection of these animals does reproduce many aspects of human Tuberculosis, including latency, reactivation, and granuloma structure [11]. Although the proportion of infected animals that develop progressive primary infection (approximately 50%) is higher than adult human infection, latency is observed, making this model the closest to human TB [11]. The major limitation of this model is the cost and logistics of maintaining primate colonies, an obstacle that will likely limit its general use in the research community.

IV. Stresses Associated with *M. tuberculosis* Persistent Infection

In seeking to understand the coordinated bacterial response required to persist within the human host, we will attempt to parse this phenotype into individual component stresses applied by the host and relate this information to studies in the described animal models. In examining the experimental evidence for the relevance of a specific stress, we will apply the following criteria:

1. Is there evidence that such a stress exists in human TB or in animal models?
2. Have mutants of *M. tuberculosis* been identified that are deficient in response to given stress in vitro?
3. Do these mutants have phenotypes in animal models in which the particular stress is present?

Fulfillment of these criteria would provide strong evidence for the relevance of a particular stress during *M. tuberculosis* infection.

A. Attacks on the mycobacterial cell surface

The mycobacterial cell wall and cell membrane are the first lines of defense against most host-derived stresses. In response to in vitro stresses believed relevant to infection, *M. tuberculosis* modifies its cell wall architecture, including cell wall thickening and changes in surface lipid and protein composition [12-13]. Such alterations in the cell surface were linked to resisting host stress when Singh *et al.* showed that the *M. tuberculosis* WhiB3 transcription factor responds to fluctuations in the intracellular redox environment by directing the synthesis of virulence lipids that possibly act as a reductant sink [12].

The most directed and specific attack on the bacterial surface is believed to be imposed by antimicrobial peptides and proteins present in the airway surface fluid covering the respiratory epithelium and within the phagolysosome during infection. Several of these antimicrobials, including β -defensins, the cathelicidin LL-37, granulysin and ubiquitin-derived peptides have antimycobacterial activity, most likely by altering membrane permeability [14-18]. Using treatment with detergents or lysozyme, several groups have tested whether mutations in genes involved in cell wall biogenesis render mycobacteria more sensitive to cell surface stress. For example, an *M. marinum* strain lacking *kasB*, which encodes an enzyme responsible for elongating cell wall mycolic acid chains, is hypersusceptible to killing by defensins and is attenuated in macrophages, while the *M. tuberculosis* *DkasB* strain is highly attenuated in mice [19-20]. These studies suggest that

within the host *M. tuberculosis* may require proper cell wall composition to defend against cell surface stress imposed by antimicrobial peptides and similar molecules. Similarly, deletion of *M. tuberculosis lysX*, which is required for lysine modification of phosphatidylglycerol phospholipids, leads to increased sensitivity to cationic antimicrobial peptides and attenuation at later time points during infection of mice and guinea pigs, but the failure of genetic complementation of this mutant raises some question about whether the *in vivo* phenotype is due to *lysX* inactivation [21], especially given the inability of other investigators to find any attenuation of a *lysX* transposon mutant in mice [22].

Many other components of the *M. tuberculosis* cell wall and membrane have also been shown to be important for pathogenesis. These include mycolic acid biosynthesis and cyclopropane modification, PDIM biosynthesis, as well as signaling cascades that regulate cell wall and membrane biogenesis [23-28]. However, changes in the outer surface of *M. tuberculosis* influence the general fluidity, permeability, and antigenicity of the bacterial membrane and cell wall, thus affecting the susceptibility of *M. tuberculosis* to host immunity and the very character of the immune response [12, 29-31]. As a result, it is difficult to distinguish if attenuation of *M. tuberculosis* cell surface mutants results from the inability to resist cell surface attacks from the host, or from modulation of host responses.

B. Acidification of phagosomes

Once the macrophage becomes infected with *M. tuberculosis*, one of the host's key defenses is fusion of phagosomes containing *M. tuberculosis* with lysosomes to destroy the bacteria with an acid cocktail that damages DNA, proteins, lipids, and disrupts biochemical reactions. Within the unactivated macrophage, *M. tuberculosis* initially blocks phagosome maturation, thus resisting fusion with lysosomes and enabling *M. tuberculosis* to avoid a degradative fate [32-33]. Activation of the macrophages with IFN- γ overcomes the block in phagosome maturation, leading to acidification and exposure to an environment where hydrolases, reactive nitrogen and reactive oxygen operate most effectively [33].

Wild-type *M. tuberculosis* is quite resistant to acid stress in low pH media when certain detergents and albumin are eliminated [34] and some *M. tuberculosis* mutants that allow phagolysosome fusion and acidification are not attenuated in unactivated macrophages [35-36], indicating that *M. tuberculosis* encodes mechanisms to withstand killing by acid stress. To identify what mycobacterial proteins mediate acid resistance, Vandal *et al.* performed a transposon mutagenesis screen to isolate mutants unable to recover from acid exposure at pH 4.5, which is believed to be the pH of the macrophage lysosome. Among the genes identified in this screen, two predicted envelope associated proteases, Rv3671c and Rv2224c, were verified as being crucial for maintaining intrabacterial pH in culture and in IFN- γ activated macrophages [22, 34]. Inability of the mutants to maintain intrabacterial pH was directly associated with a decrease in viability in low pH media and in activated macrophages and was rescued by alkalinizing phagosomes [22, 37]. The *rv3671c* transposon mutant was severely attenuated in mice, especially at later time points, and the *rv2224c* transposon mutant yielded slightly lower bacterial loads in lungs of mice during chronic infection, indicating that these genes are essential for virulence [22, 34, 37].

Other transposon insertion mutants identified as decreasing the viability of *M. tuberculosis* during acid stress and in mice were in *rv2136c* and *ponA2* [22, 34]. In addition to the genes identified in the transposon screen, *mgtC*, a probable integral membrane protein, is required for *M. tuberculosis* survival in conditions of low pH and low Mg²⁺ concentration as well as growth within human macrophages and mice [38]. Similarly, an *M. tuberculosis* mutant lacking the pH responsive porin OmpATb is sensitive to low pH in vitro and attenuated in both macrophages and mice [39-40]. The mechanisms by which these proteins confer acid resistance are still unknown.

Collectively, these reports demonstrate that acid stress is clearly present in vivo during *M. tuberculosis* infection and specific bacterial mutants have been isolated which are deficient for acid resistance. In many cases, these mutants have strong animal phenotypes, confirming that *M. tuberculosis* actively resists the acid environment of the host. Lowering the pH of the phagosome not only imposes acid stress on *M. tuberculosis*, but also creates an environment where nitric oxide, hydrolases, and reactive oxygen species are all more potent. It is, therefore, not surprising that acid sensitive mutants of *M. tuberculosis* are also susceptible to killing by the cell wall damaging detergent SDS, reactive oxygen species, and nitric oxide [22, 34]. However, this broad stress sensitivity complicates conclusions about the host-inflicted stress that is responsible for the attenuation phenotypes of these mutants.

C. Reactive nitrogen intermediates

Mice lacking inducible nitric oxide synthase (iNOS), which are defective in the production of NO and NO derived reactive nitrogen intermediates (RNI), are highly susceptible to wide variety of infections, including *M. tuberculosis* [41-42]. These early studies demonstrated that iNOS is required to contain *M. tuberculosis* infection in the mouse and suggest that *M. tuberculosis* has mechanisms to resist killing by NO. To test this idea, Darwin and colleagues executed a screen for *M. tuberculosis* mutants that exhibited delayed outgrowth after exposure to acidified sodium nitrate, an NO donor. This screen identified multiple genetic loci required for NO resistance [43]. The *uvrB* mutant identified in this screen is discussed in the DNA damage section. A major finding of this screen was that two proteins (Mpa and PafA), functionally linked to the bacterial proteasome, were required for NO, but not hydrogen peroxide resistance. Mpa is an ATPase that forms a hexameric ring that most likely interacts with the proteasome core subunits. PafA attaches the small ubiquitin-like modifier Pup to target proteins [44].

Testing of these mutant strains in mice revealed that mutant titers were 1-2 logs lower than wild type. The *mpa* mutant is also attenuated for killing of infected mice [45]. Additional experiments demonstrated similar virulence defects in *M. tuberculosis* mutants lacking proteasome core subunits encoded by *prcBA* [46]. A prediction of the NO sensitive phenotype of proteasome mutants was that their virulence would be restored in mice lacking iNOS because the in vivo stress against which the proteasome defends is no longer present in these mice. The attenuation phenotypes of the *mpa* and *paf* mutants were partially reversed in iNOS deficient mice, although not to the level of wild type *M. tuberculosis* in iNOS deficient mice [43, 45]. These experiments indicated that one role of the proteasome is to defend against nitric oxide, but other in vivo stresses play a prominent role in limiting the growth of proteasome deficient *M. tuberculosis*. The mechanism by which loss of proteasome function sensitizes cells to killing by RNI remains to be fully elucidated, but is presumed to reflect a function of the proteasome in removing proteins damaged by RNI or another stress.

D. Reactive oxygen species

The production of superoxide ($O_2^{\cdot-}$) by phagocytic cells is a critical part of the human innate immune response to infection. The oxidative burst involves the production of $O_2^{\cdot-}$ by NADPH oxidase gp91^{phox} and gp47^{phox} proteins and can damage all components of the cell including proteins, lipids, and DNA. However, this innate immune response does little to prevent the growth of the bacteria in the mouse lungs, since during early infection bacterial titers increase rapidly. This suggests that *M. tuberculosis* possesses efficient mechanisms to evade the initial phagocytic oxidative burst. Activation with IFN- γ further induces infected murine macrophages to produce reactive oxygen species (ROS), along with RNI, to control and destroy the infecting bacteria [47-48].

The production of ROS appears to be transiently important in controlling infection as *M. tuberculosis* aerosol infection of mice lacking the $p47^{phox}$ gene resulted in a significant increase in bacterial growth over days 14-30 of infection [49]. Once antigen-specific IFN- γ producing lymphocytes were detected in the draining lymph nodes, however, bacterial growth in the lung stopped and bacterial titers resembled wild-type mice, indicating that other host defense mechanisms help control bacterial burden at this stage of infection [49].

To control damage caused by O_2^- , aerobic bacteria like mycobacteria degrade O_2^- to water and molecular oxygen by the action of superoxide dismutase (SOD) and catalase. *M. tuberculosis* expresses two SODs, SodA and SodC, and one catalase, KatG, which protect the bacteria against oxidative killing during the phagocyte oxidative burst. *sodA* is believed to be an essential gene for *M. tuberculosis* survival, indicating that the enzyme is critical for maintaining an optimal redox environment even in the absence of exogenous stress [50]. Depleting *M. tuberculosis* SodA by antisense silencing leads to increased sensitivity to H_2O_2 and severe attenuation during mouse infection [51]. Deletion of the nonessential *M. tuberculosis* *sodC* gene caused a growth defect in activated wild-type and iNOS deficient macrophages, but not in phagocyte oxidase deficient macrophages ($gp91^{phox-/-}$), indicating that the increased killing was due to the oxidative burst [52]. However, the $\Delta DsodC$ strain had no phenotype in guinea pig infection [50, 52].

A null mutant of *M. tuberculosis* *katG* is highly sensitive to H_2O_2 and attenuated during chronic infection of mice [53]. In contrast, during infection of $gp91^{phox-/-}$ mice, wild-type and $\Delta katG$ *M. tuberculosis* strains were indistinguishable throughout 22 weeks of infection, indicating that the attenuation in wild-type mice was due to increased sensitivity to ROS production [53]. Ng *et al.* also compared the growth of wild-type and $\Delta katG$ *M. tuberculosis* in iNOS deficient mice. iNOS deficient mice were able to contain the $\Delta katG$ mutant growth for at least 9 weeks, while the *katG* mutant was as virulent as wild-type in iNOS deficient/ $gp91^{phox-/-}$ mice emphasizing that *katG* specifically counters the oxidative burst [53].

In addition to the SOD and catalase genes, Cirillo *et al.* identified a *mel2* locus in mycobacteria that contains three luciferase-like genes involved in surviving oxidative stress [54]. Bacterial luciferases are thought to scavenge H_2O_2 in a catalase-like reaction where H_2O_2 is converted to water, light and an oxidized intermediate. Deletion of the *mel2* locus in *M. tuberculosis* led to increased sensitivity to H_2O_2 treatment in culture and mildly diminished bacterial loads during persistent infection [54]. The $\Delta mel2$ mutant behaved similar to wild-type in $gp91^{phox-/-}$ mice but not in iNOS deficient mice where it was still attenuated during persistent infection, suggesting that *mel2* is specifically important for resistance to ROS during persistence [54]. Taken together, these reports provide strong evidence that resisting oxidative stress is important for *M. tuberculosis* growth and persistence in macrophages and mice.

E. Hypoxia

The most common site of reactivation of latent *M. tuberculosis* infection is the lung, which is also the portal of entry for infection. As such, the gaseous environment which *M. tuberculosis* encounters during infection has been a longstanding area of interest for TB researchers. Most recently, much of this interest has centered on the notion that hypoxic microenvironments play an important role in *M. tuberculosis* infection, especially in the establishment or maintenance of latency. Much of this interest was stimulated by an in vitro model of *M. tuberculosis* hypoxia. *M. tuberculosis* grown in a stirred culture with a defined headspace ratio will progressively experience oxygen depletion from the closed environment, resulting in hypoxia and cessation of growth. This model, often called the "Wayne model" after its creator [55-56], results in physiologic changes in the bacterium that are reminiscent of what we assume to be true about latent *M. tuberculosis* in the human

lung. Specifically, Wayne model grown bacteria are in a state of nonreplicating persistence characterized by absence of DNA synthesis but minimal loss of bacterial viability [55] and are resistant to killing by Isoniazid, Rifampin, and Ciprofloxacin, antibiotics that are active against replicating bacteria [55-57]. Bacteria in the Wayne model also have higher enzyme activity for isocitrate lyase, an enzyme of the glyoxylate shunt which is required for utilization of fatty acids, a nutrient source that *M. tuberculosis* uses in vivo [58-59]. The relevance of the Wayne model to infection is supported by the observation of hypoxic areas in *M. tuberculosis* infected primate and guinea pig granulomas [60].

Many investigators have used the Wayne model to understand the physiologic adaptation to hypoxia. Transcriptional profiling of *M. tuberculosis* in hypoxia revealed a coordinated transcriptional response which depends upon a two-component system variously termed DosS/DosR or DevS/DevR. DosR actually has two upstream sensor kinases, DosS and DosT [61]. Although the DosR regulon was originally defined as a gene set induced in response to hypoxia, more recent data indicates that the extracellular domain of DosS/DosT can also sense carbon monoxide and nitric oxide [61-63]. Deletion of DosR abolishes early induction of the DosR regulon during hypoxia [64]. Multiple groups have tested *M. tuberculosis* lacking DosR in experimental animal infections. Most studies have shown little or no attenuation of the DosR mutant in mouse infection, including the mouse hollow fiber model [65-67]. The lack of an in vivo phenotype for *dosR* mutants in mice may reflect the lack of hypoxic areas in mouse granulomas [60]. In contrast, the detection of hypoxic areas of guinea pig and rabbit granulomas [60] would suggest that DosR would be required in these animal models, a prediction that has been confirmed by mild phenotypes of the DosR mutant in these animals [65, 68].

Recently, examination of *M. tuberculosis* gene expression in response to more extended hypoxia has revealed a gene expression program that is mostly DosR independent [66]. This “enduring hypoxic response” may explain the mild or absent phenotypes for DosR mutants, as loss of DosR alone may only cripple the earliest phase of the hypoxic response. This idea is supported by recent evidence indicating that a chemical inhibitor of DevR (DosR) binding to promoter DNA is highly effective at killing hypoxic *M. tuberculosis* if added at the beginning of the static culture phase, but has no effect if added once the hypoxic phase is established [69]. In summary, although *M. tuberculosis* clearly responds to hypoxia, the DosR system also responds to other gases such as NO and CO, making the specific role of hypoxia difficult to parse. The mild phenotypes of DosR mutants in vivo could either indicate that hypoxia is a minor stress in the animal models tested, or that DosR mutants are not null for the hypoxia response. Sorting out the role of hypoxia in *M. tuberculosis* will require more study, including genetic dissection of the extended hypoxic response and testing of these mutants (possibly in combination with DosR mutations) in appropriate animal models.

F. Nutrient starvation

When nutrients in their environment are limiting, bacteria must synthesize their own essential molecules. The existence of a nutrient limited environment during *M. tuberculosis* infection is evinced by the attenuation of numerous auxotrophic *M. tuberculosis* strains during mouse infection [70-73]. In many bacteria, amino acid biosynthesis genes are upregulated during nutrient deprivation via the stringent response. The stringent response is a global regulatory response which downregulates the cellular translational machinery through RelA catalyzed synthesis of hyperphosphorylated guanine nucleotides, (p)ppGpp [74].

M. tuberculosis lacking *relA* fails to upregulate (p)ppGpp during starvation and is defective for long term survival during starvation or hypoxic conditions [75]. The *M. tuberculosis*

DreIA mutant had similar kinetics and virulence as wild-type in mice early during infection, but failed to persist to the same extent as wild-type [74]. A *relA* transposon mutant was also attenuated in the mouse hollow fiber model [8]. The effects of inactivating *relA* on *M. tuberculosis* pathogenesis suggest a requirement for responding to nutrient deprivation for bacterial survival *in vivo* and demonstrated that the stringent response is necessary for persistent infection. However, the stringent response is also induced during phosphate depletion and oxidative stress, therefore, RelA is most likely mediating survival in response to a combination of stresses [7, 76].

G. Phosphate deprivation

Phosphate is an essential component of several biomolecules, such as membrane lipids, complex carbohydrates and nucleic acids. Therefore, assimilation and metabolism of phosphate from the environment is essential for bacterial survival. When phosphate is limiting, bacteria must acquire this essential nutrient through several transport systems. It became clear that phosphate may be limiting during *M. tuberculosis* infection of macrophages when transposon site hybridization experiments identified phosphate transport genes *pstA1*, *pstC1*, *pstS3* and *phoT* as important for survival during prolonged infections of primary murine macrophages [77]. By depleting phosphate in liquid cultures, Rengarajan *et al.* verified that the *pstA1* and *phoT* transposon mutants were more sensitive to phosphate limitation than wild-type *M. tuberculosis* [77]. The *phoT* transposon mutant was also attenuated during acute and persistent infection of mice [77]. Similar results were seen in an *M. bovis* BCG *phoT* deletion mutant, which was less virulent in guinea pigs and possums, and had a slight defect during chronic infection of mice [78]. *pstA1*, *pstC1*, *pstS3* and *phoT* are part of the Pho regulon, which is upregulated during Pi starvation by the sensor histidine kinase and response regulator pair of the two component regulatory system *senX3/regX3* [76, 79]. An *M. tuberculosis* *regX3* transposon mutant or deletions of either *senX3*, *regX3* or both were reported to be attenuated for persistent infection in mice [76, 80-81]. These results support the notion that the *M. tuberculosis* experiences a phosphate starved environment *in vivo*.

H. DNA damage

Many of the conditions imposed on *M. tuberculosis* by the host are capable of damaging pathogen DNA, including oxidative stress, acid stress, and starvation. In the screen for nitric oxide sensitive mutants mentioned above, Darwin and colleagues identified a strain with a transposon insertion in *uvrB* (Rv1633) [43]. UvrB is a component of the nucleotide excision repair (NER) machinery, which repairs damage to nucleotides by incising the DNA phosphodiester backbone on both sides of the lesion, dissociating the incised oligonucleotide containing the damaged segment, and filling the gap with DNA polymerases. Further testing of the *uvrB::Tn* strain revealed that it was not sensitive to organic peroxides or H₂O₂ [82]. Testing of this strain in the mouse model showed a relatively small difference in lung and spleen bacterial loads between wild type and *uvrB::Tn* strains, but a prolonged time to death in *uvrB::Tn* infected mice. In contrast to expectations, the difference between WT and *uvrB::Tn* strains was more dramatic in iNOS knockout mice, but the two strains behaved similarly in iNOS/phox doubly deficient animals [82]. This study supports that idea that host generated reactive nitrogen and oxygen intermediates damage the bacterial chromosome and that NER contributes to repair of this damage.

RecA is a widely conserved bacterial strand exchange protein that forms filaments on 3' tailed single stranded DNA and mediates homology search to initiate homologous recombination (HR). Several studies have examined mycobacterial mutants lacking RecA. Consistent with findings in other organisms, *M. smegmatis* [83] and *M. bovis* BCG [84] lacking RecA are hypersusceptible to diverse DNA damaging agents. The extreme *in vitro*

susceptibility of *recA* mutants suggests that this mutant might be hypersusceptible to host derived DNA damage, as is the case during Salmonella infection [85]. However, only one publication has examined the role of *recA* in mycobacterial pathogenesis. *M. bovis* BCG $\Delta recA$ was not attenuated in either wild type BalbC mice or SCID mice, suggesting either that double-strand breaks (DSBs) are not induced in the mycobacterial chromosome during murine infection, or that additional DSB repair pathways can compensate for loss of HR. The recent discovery of a non homologous end-joining pathway in *M. tuberculosis* [86] and *M. smegmatis* [87-88], and the demonstration of redundancy for DSB repair during stationary phase [83] indicate that NHEJ is able to repair DSBs when *recA* is inactivated. Further study will be required to know whether DSBs are a significant source of genotoxic stress for *M. tuberculosis* in vivo and whether HR and/or NHEJ repair such damage.

A wide variety of clastogens have been used to interrogate the DNA repair pathways of *M. tuberculosis*. The most commonly used agents include UV light, Ionizing radiation, alkylating agents, and homing endonucleases. A screen for *M. tuberculosis* genes transcriptionally upregulated after UV exposure identified *dnaE2*, encoding a nonessential homolog of the replicative DNA polymerase subunit DnaE1 [89]. Deletion of *dnaE2* abolished UV induced mutagenesis in vitro, as measured by rifampin resistance due to missense mutations in *rpoB* [89]. In a very important finding, *M. tuberculosis DdnaE2* was attenuated in mice, measured by time to death, and did not evolve rifampin resistance in vivo [89]. These experiments indicated DnaE2 contributes to mutagenic DNA repair in vivo and that this process is important for rifampin resistance. This study provides the clearest example to date of mutagenic repair of the *M. tuberculosis* chromosome in response to host derived clastogenic stress. Thus, DNA repair pathways not only protect mycobacteria from genotoxicity but also allow for survival and persistence during chemotherapy by the acquisition of drug resistance. In contrast, more recent examination of two Y-family polymerases, predicted to be error prone, revealed no role in virulence or mutagenesis [90].

A functional genomic screen investigating genes upregulated in *M. smegmatis* following a homing endonuclease induced DSB identified the essential mycobacterial CarD protein as responsive to genotoxic stress and necessary for survival during DNA damage [7]. Further investigations showed that *carD* is transcriptionally induced by a wide range of stresses relevant to *M. tuberculosis* infection and is also required for resistance to starvation and oxidative stress. CarD binds the bacterial RNA polymerase to control rRNA transcription both at steady state and during these stresses. Experiments that depleted CarD during *M. tuberculosis* infection of mice demonstrated that CarD is required for not only bacterial replication in vivo, but also during the persistent phase of infection, when bacterial titers are stable [7]. The pleiotropic role of CarD in stress responses highlights the importance of a coordinated response to a combination of host derived stresses experienced by *M. tuberculosis* in vivo.

In summary, *M. tuberculosis* clearly experiences DNA damage during infection and repair of this damage is responsible for antimicrobial resistance. Further definition of the specific DNA repair pathways that repair host inflicted DNA damage is an important area for further study.

V. Conclusions

The studies discussed above provide strong experimental evidence that *M. tuberculosis* experiences multiple host derived stresses in vivo and requires pathways to respond and resist eradication in order to persist in the host. We have summarized many of these studies in Table 1, which lists each stress, genes implicated in resisting that stress, and the in vivo phenotypes of mutants in those genes.

What is also clear is that our ability to replicate the multiple phases of the human infection in experimental systems is extremely limited. Using available model systems, we simply cannot examine what stresses are relevant to certain phases of infection such as induction of latency, maintenance of latency, reactivation from latency, survival of the bacterium within a liquefied cavity, and survival in an aerosolized droplet during transmission. As such, although we can clearly define genetic determinants of resistance to in vitro stress, the in vivo correlates of that resistance are difficult to determine with certainty. For example, it might be the case that repair of UV induced DNA damage is critical during aerosol transmission, when the bacterium may be exposed to UV light and may desiccate, but this function may be dispensable during latency. Resistance to RNI may be important during growth within activated macrophages, but it may be irrelevant for extracellular bacteria within a cavity. None of our animal models can query these phenotypes despite our ability to define specific responses of the pathogen to a wide variety of stresses in vitro.

An additional limitation of the studies to date (although understandable from a reductionist experimental point of view) is the application of monolithic stresses to the bacterium. In the context of infection, the bacterium is not experiencing a single stress at a time, but is experiencing multiple stresses simultaneously. From the perspective of the infecting *M. tuberculosis* cell, the response to all of these stresses must be coordinated. Consistent with this idea, it is notable that many mutants discussed in this review, although isolated in screens using monolithic stress conditions, are sensitive to a range of stresses. Table 2, which presents the phenotypes of individual mutant strains across stresses, illustrates that rarely is a single mutation responsible of sensitizing *M. tuberculosis* to a single stress. In addition, although beyond the scope of this review, there is growing evidence that networks of alternative sigma factors in *M. tuberculosis* integrate some of the stress responses discussed above [please see the following recent review⁹¹]. Although we know very little about how such responses are coordinated, they clearly involve adaptation of multiple basic cellular processes such as transcription, cell membrane/wall biogenesis, and metabolism.

There is growing interest in using in vitro conditions that mimic the in vivo environment to screen for antimycobacterial agents that might be active within the host. We believe that the idea of integrated stress responses could be incorporated into these efforts to allow isolation of candidate antimicrobials that sensitize *M. tuberculosis* to multiple stresses, hopefully enhancing their in vivo efficacy. It seems likely that paralysis of multiple stress responses required for in vivo persistence by a chemical inhibitor would be more effective than paralysis of any single stress response. We hope that testing of this hypothesis will be possible with the ongoing efforts of our research community to identify and test such inhibitors in experimental animals and eventually in patients.

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TABLE 1

Stress response pathways of *M. tuberculosis*. For each stress, we list the presence of the stress in relevant animal models, genes implicated in the stress response, and our assessment of the phenotypes of *M. tuberculosis* mutants in animals. We emphasize that our designation of “yes” or “no” to describe the in vivo phenotypes of the mutants reflects a diversity of in vivo phenotypes which can be found in the accompanying references.

Stress	Stress exists in vivo?	Genes involved in stress response in vitro	Defined mutants impaired during in vivo growth	References
Hypoxia	Mice-no, Guinea pigs-yes, Nonhuman primates- Yes	<i>dosS/dosT/dosR</i>	<i>dosR</i> - mice: no, guinea pigs: yes	[65-68]
Phosphate Deprivation	Mice-yes	<i>phoT, regX3, senX3, pstA1</i>	<i>senX3/regX3</i> : yes [*] , <i>phoT</i> :yes	[76-77, 80-81]
ROS	Mice and humans-yes	<i>katG, sodA, sodC, mel2</i>	<i>sodA</i> : yes <i>sodC</i> : no <i>katG</i> : yes <i>mel2</i> :yes	[50-54]
RNI	Mice-yes	<i>uvrB</i> , proteasome (<i>mpa</i> , <i>pafA, prcAB</i>)	UvrB: yes Proteasome: yes	[43, 46, 82]
Acid	Mice-yes	<i>Rv3671c</i> <i>Rv2224c, Rv2136c</i> , <i>ponA2, mgtC, ompA</i>	<i>Rv2224c</i> :yes, <i>ponA2</i> : yes, <i>Rv3671c</i> and <i>Rv2136c</i> : yes <i>mgtC</i> :yes <i>ompAT</i> :yes	[22, 34, 37-38, 40]
DNA damage	Yes	<i>dnaE2, recA, carD</i>	<i>dnaE2</i> :Yes <i>recA</i> still unknown <i>carD</i> : yes	[7, 84, 89]
Nutrient starvation	Yes	AA biosynthesis; <i>relA</i>	<i>relA</i> :Yes AA biosynthesis: yes	[70-74]
Cell membrane stress	Yes	<i>whiB3, kasB</i>	<i>whiB3</i> : yes <i>kasB</i> : yes,	[12, 19, 92-93]

* Designation of “yes” based on deletion mutants. One study shows no attenuation of a *regX3::Tn* strain in mice [94]

TABLE 2

Interrelated stress responses of *M. tuberculosis*

Genes presented in this review as involved in *M. tuberculosis* stress responses are listed and designated as being transcriptionally upregulated in response to a given stress (“responds”), required for survival during exposure to that particular stress (“required”), or neither responsive nor required (“no”).

GENE	Cell Surface *	Acid	RNI	ROS	Hypoxia	Nutrient Starvation	Phosphate Starvation	DNA Damage
<i>whiB3</i>	ND [12]	ND	Responds, not required [92]	ND	Responds, not required [92]	Required [92]	Responds [76]	ND
<i>kasB</i>	Required [20]	ND	ND	ND	ND	ND	ND	ND
<i>lysX</i>	Required [21]	Required [34]	No-[22]	No-[22]	ND	ND	ND	ND
<i>Rv3671c</i>	ND	Required [34]	ND	ND	ND	ND	ND	ND
<i>Rv2224c</i>	Required [22] [37]	Required [34]	Required [22]	Required [22]	ND	ND	ND	ND
<i>Rv2136c</i>	Required [22]	Required [34]	Required [22]	Required [22]	ND	ND	ND	ND
<i>ponA2</i>	Required [22]	Required [34]	No- [22]	Required [22]	ND	ND	ND	ND
<i>mgfC</i>	ND	Required [38]	ND	ND	ND	ND	ND	ND
<i>ompATb</i>	ND	Required and responds [40]	ND	ND	ND	ND	ND	ND
<i>mpa</i>	ND	ND	Required [43]	Not required (actually resistant)-[43]	ND	ND	ND	ND
<i>pafA</i>	ND	ND	Required [43]	Not required (actually resistant)-[43]	ND	ND	ND	ND
<i>prcAB</i>		Not required [46]	Required [46]	Not required [46]	ND	ND	ND	ND
<i>sodA</i>	ND	ND	ND	Required [51]	ND	ND	ND	ND

GENE	Cell Surface *	Acid	RNI	ROS	Hypoxia	Nutrient Starvation	Phosphate Starvation	DNA Damage
<i>sodC</i>	ND	ND	Slightly sensitive, but dramatic reduction in survival with combo of RNI and ROS [52]	Required [50, 52]	ND	ND	ND	ND
<i>katG</i>	ND	ND	ND	Required [53]	ND	ND	ND	ND
<i>mel2</i>	ND	ND	ND	Required [54]	ND	ND	ND	ND
<i>dosS/R regulon</i>	Responds [95]	Responds [95]	Responds [95]	ND	Responsive, Required	ND	ND	ND
<i>relA</i>	ND	ND	ND	No-[75]	Required [75]	Responds and required [75]	Responds [76]	ND
<i>phoT</i>	ND	ND	ND	ND	ND	ND	Required [77]	ND
<i>senX3/re gX3</i>	ND	ND	ND	ND	ND	ND	Responds and Required [76]	ND
<i>uvrB</i>	ND	ND	Required [43]	Not required in vitro [43] required in mice [82]	ND	ND	ND	Required UV [82]
<i>recA</i>	ND	ND	ND	Responds [89]	ND	ND	ND	Responds and required [89, 96]
<i>DnaE2</i>	ND	ND	ND	Responds and required for mutation [89]	ND	ND	ND	Responds and required [89]
<i>carD</i>	ND	ND	ND	Required and responds [7]	ND	Required and responds [7]	ND	Required and responds [7]

ND=not determined.

* As described in the text, cell surface stress is often induced by exposure to SDS, lysozyme, or antimicrobial peptides. If a mutant in question is sensitive to any one of these stresses, we list it as "required," even if not sensitive to the other two cell surface stresses.