Microreview

Mycobacterial survival strategies in the phagosome: defence against host stresses

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Summary

Infections with Mycobacterium tuberculosis remain a major cause of disease and death in humans. Among the factors that contribute to M. tuberculosis’s success as a pathogen is its ability to withstand potentially bactericidal host defences and to resist elimination by an activated immune system. This resistance to killing by the host is in part due to the low permeability of the mycobacterial cell envelope for many toxic molecules. In addition, it depends upon the detoxication of reactive oxygen and reactive nitrogen molecules produced by the host, the repair of the damage these molecules cause and maintenance of a neutral intrabacterial pH within acidic environments. The latter three mechanisms are the focus of this review.

Introduction

Bacterial pathogens have evolved diverse strategies to ensure growth and survival within the host niche. Mycobacterium tuberculosis (Mtb) is one of the most prevalent human pathogens that has evolved to prevent the maturation of phagosomes into phagolysosomes (Armstrong and Hart, 1971; Sturgill-Koszycki et al., 1994; MacMicking et al., 2003). For Mtb, the ability to arrest phagosome maturation is an important survival strategy because it allows replication within macrophages, the cell type most capable of killing intracellular microbes. Several Mtb mutants unable to prevent phagosome–lysosome fusion showed impaired growth in macrophages, but many of them were still able to survive in phagolysosomes (Pethe et al., 2004; MacGurn and Cox, 2007). A few even replicated intracellularly like wild-type (wt) Mtb despite their impaired ability to stall phagosome–lysosome fusion. Maturation of Mtb containing phagosomes into phagolysosomes after activation of macrophages with IFNγ (Schaible et al., 1998; Via et al., 1998; MacMicking et al., 2003) similarly elicits several potentially mycobactericidal chemistries, which Mtb has adapted to (Nathan and Shiloh, 2000; Rhee, 2005). Survival of Mtb is, thus, not only facilitated by the manipulation of its primary host cell, but also due to resistance against stresses that the bacteria encounter in immunologically activated macrophages. Here, we discuss the genes and mechanisms that help Mtb to specifically withstand the acidic, nitrooxidative stresses of the host niche and review their importance for pathogenicity. Additional aspects of the Mtb host interaction, including the role of mycobacterial proteins and lipids in manipulating phagosome maturation and mechanisms by which Mtb interferes with host signalling pathways, have been reviewed elsewhere (Koul et al., 2004; Philips, 2008).

Oxidative and nitrosative stress

Activated macrophages express two enzymes, phagocyte oxidase (NOX2/gp91phox) and inducible nitric oxide synthase (iNOS), which generate reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) respectively (Fig. 1). Upon phagocytosis, the preformed NOX2 subunits assemble into an enzymatically active enzyme complex that transfers electrons across the membrane from cytosolic NADPH to molecular oxygen. This produces superoxide anions (O2-), which dismutate into hydrogen peroxide (H2O2) and generate toxic hydroxyl radicals (Bedard and Krause, 2007). iNOS is induced by IFNγ and produces nitrite and nitrate via nitric oxide. Under acidic conditions, such as in the phagosomes of IFNγ-activated macrophages, nitrite forms nitrous acid, which dismutates to nitric oxide (NO) and another toxic radical, nitrogen dioxide (Nathan and Shiloh, 2000). NO can synergize with superoxide (produced by the macrophage or generated as byproduct of respiratory metabolism by the pathogen) to form poisonous peroxynitrite (Beckman et al., 1990; Nathan and Ehrt, 2004). These
ROI and RNI can be bactericidal as they react with a wide range of molecules, including nucleic acids, proteins, lipids and carbohydrates. To counteract such stress, Mtb uses a variety of molecules to either detoxify ROI and RNI before they can cause harm or to repair the damage they caused. In the following we review mechanisms involved in Mtb’s ability to withstand ROI and RNI stress. For some mechanisms the evidence that they are important during an infection is incomplete and often effects of inactivating a single resistance mechanism may be masked by redundancy. Notwithstanding, the importance of ROI and RNI in control of Mtb and the importance of Mtb’s ability to resist nitro-oxidative stress are evident.

Detoxification of ROI and RNI

Increased rates of mycobacterial diseases, including tuberculosis in people with chronic granulomatous disease, indicated a potential role for ROI in the control of Mtb infection in humans (Bustamante et al., 2007). Mice deficient in NOX2, however, were found to be relatively resistant to tuberculosis (Adams et al., 1997; Cooper et al., 2000; Jung et al., 2002). It has been suggested that Mtb possesses resistance mechanisms to evade or counter the phagocyte respiratory burst, thus masking the initial impact of oxidative burst might have been able to express alternative ROI-resistance mechanisms or have acquired compensatory mutations. Notwithstanding, this work demonstrated that Mtb’s catalase contributes to virulence in a host that is capable of generating ROI.

Superoxide dismutases catalyse the conversion of superoxide anions to hydrogen peroxide and are important for virulence in several bacterial pathogens, including Helicobacter pylori (Seyler et al., 2001), Salmonella typhimurium (Fang et al., 1999) and Yersinia enterocolitica (Igwe et al., 1999). Mtb contains two genes encoding superoxide dismutases, sodA and sodC. SodC is a Cu,Zn superoxide dismutase localized to the mycobacterial cell envelope (Wu et al., 1998). Lack of sodC increases susceptibility of Mtb to superoxide, to the combination of superoxide and nitric oxide and to killing by IFNγ activated murine peritoneal macrophages (Piddington et al., 2001). In contrast, resting wt macrophages and IFNγ-activated NOX2-deficient macrophages did not kill MtbΔsodC, demonstrating that the Cu,Zn superoxide dismutase contributes to Mtb’s resistance against oxidative burst products generated by activated macrophages. SodC transposon mutants were, however, not attenuated in mice up to 60 days post infection (Piddington et al., 2001; Sassetti and Rubin, 2003) and MtbΔsodC was virulent in guinea pigs (Dussurget et al., 2001). The importance of the Cu,Zn superoxide dismutase during long-term chronic infections has not been determined.

SodA, which uses iron instead of Cu,Zn, may compensate for SodC to protect against the respiratory burst in naïve macrophages and during mouse infections. SodA appears to be essential for in vitro growth (Dussurget...
et al., 2001). Mtb mutants with reduced sodA expression displayed increased susceptibility to H2O2 and were markedly attenuated in mice (Edwards et al., 2001). That sodA and katG are required for growth of Mtb in macrophages and to cause disease in mice is also supported by phenotypes of an Mtb secA2 mutant. SecA2 is part of a specialized secretion system that is important for mycobacterial virulence (Braunstein et al., 2003; Kurtz et al., 2006; Rigel and Braunstein, 2008). MtbΔsecA2 is defective in the export of SodA and KatG, unable to grow in nonactivated macrophages and showed reduced growth in mice (Braunstein et al., 2003; Kurtz et al., 2006).

However, the intracellular growth defect was not reversed in macrophages deficient in NOX2 pointing towards additional functions of SecA2 during intracellular growth. Indeed, macrophages infected with the MtbΔsecA2 mutant released elevated levels of proinflammatory cytokines, suggesting a role for SecA2-dependent export in restricting host cell responses (Kurtz et al., 2006).

Low-molecular-weight thiols are important cellular antioxidants. Glutathione is one of the most abundant thiols in cyanobacteria and proteobacteria and protects against oxidative and other forms of stress, including low pH and osmotic stress (Masip et al., 2006). Actinobacteria contain mycothiol (MSH) instead of glutathione. In Mtb MSH serves as an antioxidant and helps to maintain the reducing environment within the cytoplasm (Newton et al., 2008). Mutants with reduced cellular levels of MSH showed increased sensitivity to oxidative stress (Buchmeier and Fahey, 2006; Buchmeier et al., 2006). Deletion of mshA, encoding the first enzyme in mycothiol biosynthesis, resulted in an Mtb strain with undetectable levels of mycothiol. This mutant required catalase for in vitro growth, but had no significant growth defect in mice and killed immunocompetent and immunodeficient mice with similar kinetics as wt Mtb (Vilcheze et al., 2008). While intracellular thiol levels were significantly reduced in vitro, it is plausible that alternative mechanisms of redox defence compensate for the lack of mycothiol during mouse infections.

The production of reduced-sulfur-containing molecules such as mycothiol depends on the sulfur assimilation pathway whose first step, the conversion of adenosine 5′-phosphosulfate to sulfite, is catalysed by the 5′-adenosinephosphosulfate reductase CysH (Carroll et al., 2005). Deletion of cysH results in methionine and cysteine auxotrophy and attenuation of Mtb in immunocompetent mice (Senaratne et al., 2006). Growth of the cysH mutant during the active phase of infection was not impaired, but with the onset of adaptive immunity the mutant was killed and subsequently persisted at 50–100-fold-reduced titers during chronic infection. Survival of mice infected with MtbΔcysH was significantly prolonged compared with mice infected with wt Mtb. Consistent with the unimpaired in vivo growth prior to the onset of adaptive immunity, the mutant was almost fully virulent in Rag2−/− mice, which lack functional T and B cells. Thus, in mice Mtb appears to have access to cysteine and methionine and might be able to scavenge reduced-sulfur-containing metabolites from the host. CysH is, however, important during survival in the face of adaptive immunity. This might be due to the antioxidant activity of CysH as MtbΔcysH displayed increased susceptibility to H2O2 and peroxynitrite. Decreased levels of downstream metabolic products including mycothiol could be the cause for this (Senaratne et al., 2006). MtbΔcysH was partially attenuated in iNOS−/− mice but displayed full virulence in NOX2-deficient mice treated with the iNOS inhibitor aminoguanidine and in iNOS/NOX2 double deficient mice, supporting the hypothesis that the impaired antioxidant activity of Mtb lacking CysH is responsible for its attenuation in vivo.

To detoxify both RNI and ROI, Mtb expresses an NADH-dependent peroxidase and peroxynitrite reductase that consists of four proteins: aldehyd:peroxidase reductase subunit C (AhpC), a thioredoxin-related oxidoreductase (AhpD), dihydrolipoamide acyltransferase (DiaT) and lipoamide dehydrogenase (Lpd) (Bryk et al., 2000; Bryk et al., 2002; Tian et al., 2005). DiaT and Lpd also serve as E2 and E3 components of pyruvate dehydrogenase, which produces acetyl coenzyme A (Tian et al., 2005). The loss of pyruvate dehydrogenase activity in MtbΔdlaT deprives the bacteria of carbohydrate-derived acetyl coenzyme A. Accordingly, growth of MtbΔdlaT was severely reduced in medium with glucose and glycerol (Shi and Ehrt, 2006) but normal in medium with the fatty acid butyrate as carbon source (S. Ehrt, unpublished). The mutant was hypersusceptible to RNI in vitro and died in wt and iNOS−/− mouse macrophages (Shi and Ehrt, 2006). The mechanism by which the mutant was killed in iNOS−/− macrophages remains to be identified. Susceptibility to the respiratory burst does not appear to be responsible as the mutant is hyperresistant to H2O2 (S. Ehrt, unpublished). The dlaT mutant was attenuated for growth in mouse lungs but persisted at reduced titers during the chronic phase of infection. Attenuation of virulence was even more pronounced in guinea pigs as MtbΔdlaT failed to grow in lungs and spleens and did not cause disease (Bryk et al., 2008), suggesting that Mtb encounters different metabolic environments and/or different degrees of oxidative stress in mice and guinea pigs.

Besides the enzymes and antioxidants described above Mtb most likely contains additional RNI and ROI detoxification mechanisms. For example, the enzymatic specificity of and high efficiency for RNI detoxification by truncated haemoglobins has been well characterized biochemically (Ascenzi et al., 2007; Lama et al., 2009). However, the physiological role of truncated haemoglobins for virulence...
of Mtb remains to be determined. Similarly, there is biochemical evidence that coenzyme F-420 converts NO to NO and thereby might protect Mtb from nitrosative damage (Purwantini and Mukhopadhyay, 2009). Accordingly, Mtb with an interrupted $fbIC$, which is required for F-420 biosynthesis, was hypersusceptible to RNI in vitro (Darwin et al., 2003), but was reported not to be attenuated in mice (Darwin and Nathan, 2005).

**Protein repair and degradation**

The sulfur of cysteine and methionine residues in proteins is highly susceptible to oxidation (Weissbach et al., 2002) and exposure of methionine to ROI or RNI leads to the generation of methionine sulfoxide, which can interfere with protein function. Methionine sulfoxide can be converted back to methionine by methionine sulfoxide reductase (Msr) (Weissbach et al., 2002; Boschi-Muller et al., 2005). In addition to this repair function, the reversible oxidation of methionine by Msr might serve as sink for oxidants before they damage cellular macromolecules (Levine et al., 1996). Accordingly, MsrA deficiency confers hypersusceptibility to killing by ROI and RNI in several bacterial species (St John et al., 2001; Singh and Moskovitz, 2003; Alamuri and Maier, 2004; Douglas et al., 2004). Mtb expresses two MsrA enzymes, each stereospecific for one of the epimers of methionine sulfoxide. Mtb lacking both msrA and msrB was more readily killed by acidified nitrite or hypochlorite, but the mutant was not hypersusceptible to H$_2$O$_2$ or the organic peroxide cumene hydroperoxide (Lee et al., 2009). Growth of the double mutant was slightly attenuated in mouse lungs; however, this growth defect was not reversed in the complemented mutant. Thus, it is not clear if Msr play a role in the pathogenesis of Mtb in the mouse. Nonetheless, the data suggest that while MsrA and MsrB contribute to resistance of Mtb against RNI and ROI, other antioxidant defences compensate for Msr deficiency during infection.

A genetic screen of Mtb transposon mutants identified insertions in the proteasome-associated genes, $mpa$ (Mycobacterium proteasome ATPase) and $pafA$ (proteasome-accessory factor) as sensitive to RNI stress (Darwin et al., 2003). Chemical inhibition of proteasome activity and genetic silencing of the core-proteasome encoding genes $prcBA$ also sensitized Mtb to RNI in vitro (Darwin et al., 2003; Gandotra et al., 2007). By what mechanism the proteasome protects Mtb against RNI remains to be elucidated. Proteasome activity might be required to turn over irreversibly damaged proteins or to control the stability of regulatory factors that are important for RNI resistance (Butler et al., 2006). Interestingly, mutants with defects in proteasome function are more resistant to H$_2$O$_2$ than wt Mtb. This phenotype is shared with other RNI-susceptible mutants, such as Mtb$\Delta dlaT$

(Darwin et al., 2003; Gandotra et al., 2007; S. Ehr, unpublished). The underlying mechanism is undefined, but may involve compensatory upregulation of peroxidases that protect against oxidant-induced damage (De Mot et al., 2007). Mutants with defects in proteasome-associated genes were attenuated for growth in mice; this attenuation was partially reversed in iNOS$^{-/-}$ mice, indicating that Mtb requires these genes to resist RNI in vivo. However, that the mutants were still partially attenuated in mice lacking iNOS suggests a role of proteasome-associated genes for protection against host defences other than RNI. Similarly, proteasome depletion attenuated Mtb in IFN-$\gamma$-deficient mice, pointing to a function of the proteasome beyond resistance against IFN-$\gamma$-dependent host defence mechanisms (Gandotra et al., 2007).

**DNA repair, protection and mutagenesis**

Screening for RNI-hypersusceptible Mtb transposon mutants also revealed that mutations in the nucleotide excision repair gene $uvrB$ result in severe sensitivity to RNI (Darwin et al., 2003). Mtb lacking $uvrB$ was extremely sensitive to UV light but not more susceptible to several sources of reactive oxygen species in vitro. Wt mice infected with the mutant survived significantly longer than those infected with wt Mtb. While the mutant was still attenuated in iNOS$^{-/-}$ mice, attenuation was almost completely reversed in mice lacking both iNOS and NOX2. Thus, UvrB, an enzyme important for DNA repair, is required for resistance of Mtb to both RNI and ROI in mice, implicating DNA as a critical target for RNI and ROI derived from iNOS and NOX2.

A role for DNA repair mechanisms in resisting host defences has separately emerged from the failure of an Mtb $dnaE2$ mutant to persist during chronic mouse infection (Boshoff et al., 2003). DnaE2 encodes a replicative DNA polymerase dispensable for in vitro growth but required for resistance to UV light and for the increase in mutation rate that is observed in Mtb following DNA damage. Mtb$\Delta dnaE2$ also failed to develop drug-resistant mutants during mouse infections. Induction of dnaE2 transcription in vivo and the persistence defect of the dnaE2 mutant may indicate that DNA damage during infection is countered by a DnaE2-mediated increase in mutation frequency, potentially generating mutants with a survival advantage in the face of an active host immune system. Thus, DnaE2 appears to fulfill two functions important for Mtb’s pathogenicity: DNA damage repair and generation of mutations that increase Mtb’s fitness. To prevent mutation, Mtb chromosomal DNA also seems to be physically shielded from ROI-mediated damage by Lsr2, a histone-like protein (Colangeli et al., 2009). Accordingly, an Mycobacterium smegmatis $lsr2$ deletion mutant was
hypersusceptible to H$_2$O$_2$ while overexpression of Isr2 increased ROI resistance in Mtb. The apparent essentiality of Isr2 in Mtb prevented the construction of a deletion mutant (Sassetti and Rubin, 2003; Colangeli et al., 2007) and whether Isr2 contributes to virulence of Mtb remains to be determined.

**Phagosome acidification and its impact on Mtb**

Mtb inhibits phagosome maturation and prevents phagosome–lysosome fusion in resting macrophages (Fig. 1). As a consequence, the phagosomal environment is only mildly acidic (pH 6.2–6.4) (Sturgill-Koszycki et al., 1994; MacMicking et al., 2003). The lack of acidification is most likely due to the exclusion of the vacuolar proton-ATPase (Sturgill-Koszycki et al., 1994). After immunologic activation of the macrophage, such as by exposure to IFN$_\gamma$, the phagosome maturation arrest is overcome, and the phagosomal compartment acidifies to pH 4.5–5.4 (Schaible et al., 1998; Via et al., 1998; MacMicking et al., 2003). Evidence that Mtb resides in acidified phagosomes in vivo comes from the observation that pyrazinamide, which kills Mtb in vitro exclusively at low pH, is effective during infections (Zhang and Mitchison, 2003). In addition IFN$_\gamma$, which is crucial for control of mycobacterial infections (Casanova and Abel, 2002), causes macrophage activation and phagosome acidification in isolated macrophages, and likely has the same activities in vivo. Finally, transcription of pH-responsive genes is induced in activated macrophages and acid-susceptible mutants are attenuated in vivo, suggesting that Mtb encounters an acidic environment during infections (Buchmeier et al., 2000; Raynaud et al., 2002; Rohde et al., 2007; Vandal et al., 2008).

Susceptibility of many bacteria to acid varies according to the composition of the medium and the density of the bacterial population during acid challenge (Foster, 1999; Bodmer et al., 2000; Sung and Collins, 2003; Vandal et al., 2009a,b). However, in a simple phosphate citrate buffer at pH 4.5, Mtb’s viability did not decline over a period of 6 days at densities ranging from $5 \times 10^6$ to $5 \times 10^8$ cfu ml$^{-1}$ (Jackett et al., 1978; Vandal et al., 2008; Vandal et al., 2009b). Thus, at least in this medium, Mtb is resistant to phagolysosomal concentrations of acid and the intrabacterial pH of Mtb remained near neutral even when the external pH was at 4.5. The intrabacterial pH of Mtb residing in IFN$_{\gamma}$-activated wt macrophages also remained close to neutral, indicating that Mtb expresses mechanisms of pH homeostasis that function both in vitro and within macrophages (Vandal et al., 2008). It has been demonstrated that viability of Mtb increased in IFN$_{\gamma}$-activated macrophages that were unable to fully acidify their phagosomes because they lacked the GTPase LRG47 (MacMicking et al., 2003). While this supports a role for phagosome acidification in the intracellular control of Mtb, it is important to consider that other antibacterial factors of macrophages may depend on acidification. For example, free fatty acids (Akaki et al., 2000; Vandal et al., 2006) or ubiquitin-derived peptides (Alonso et al., 2007) are not delivered to the mycobacterial phagosome in LRG-47-deficient macrophages. The effect of acid per se on intracellular survival of Mtb is therefore difficult to evaluate. It is, however, evident that acid facilitates and in some cases synergizes with other antibacterial defence mechanisms.

**Mechanisms of acid resistance**

Acid resistance and pH homeostasis in bacteria are mediated by a variety of mechanisms, including proton extrusion, production of ammonia, amino acid decarboxylation, cell envelope modification and macromolecule protection (Booth, 1985; Foster, 2004). The mechanisms that protect Mtb against acid have been less extensively studied even though it was suggested over 100 years ago that Mtb’s lipid-rich cell envelope could act as an effective barrier against the entry of protons (Metchnikoff, 1905). In agreement with this hypothesis, several acid-sensitive mutants were found to be hypersusceptible to cell wall-perturbing agents, such as lipophilic antibiotics and SDS (Vandal et al., 2008; 2009a). Moreover, of 21 acid-susceptible mutants, isolated in a screen of 10 100 Mtb transposon mutants, 15 mutants were affected in genes predicted to be involved in cell wall functions. Several of these likely participate in the biosynthesis of peptidoglycan or the cell wall lipid lipoarabinomannan (Cole et al., 1998; Vandal et al., 2008; 2009a). Another cell envelope component that contributes to acid resistance and virulence of Mtb is the outer membrane protein OmpATb (Raynaud et al., 2002; Molle et al., 2006). An ompATb mutant showed delayed growth at pH 5.5 in vitro, and reduced growth in macrophages and in mice. The mechanism by which OmpATb confers acid resistance is unknown. In lipid bilayers this protein exhibits pore-forming activity, which is pH-sensitive in that the channel has a propensity to close at low pH (Molle et al., 2006). Decreasing cell envelope permeability at low pH may represent an adaptive mechanism used by Mtb to survive in the low pH of the phagosome.

Two acid-sensitive transposon mutants isolated in the screen mentioned above were impaired in their ability to maintain a neutral intrabacterial pH during acid challenge and inside IFN$_{\gamma}$-activated mouse macrophages (Vandal et al., 2008; 2009a). Both mutants were severely attenuated for growth in mice and cleared from mouse lungs and spleens during the chronic phase of infection. This suggests that acid resistance and intrabacterial pH homeostasis are important for virulence of Mtb. As dis-
cussed above and elsewhere (Vandal et al., 2009b), the activities of antibacterial host defences, including RNI and ROI and lysosomal hydrolases, are enhanced at low pH and thus the severe attenuation of these mutants may be caused by the synergistic action of acid with other macrophage factors. The two mutants described here have transposon insertions in Rv3671c and Rv2136c. Rv3671c encodes a membrane-associated serine protease and might protect Mtb against acid by modifying the bacterial cell envelope, regulating protein or lipid quality control and/or serving in signalling pathways that help the bacterium resist extracellular stress. Rv2136c encodes a putative homologue of Escherichia coli’s UppP, an undecaprenol pyrophosphate phosphatase involved in peptidoglycan biosynthesis (Cole et al., 1998; El Ghachi et al., 2004). The exact mechanisms by which Rv3671c and Rv2136c protect against acid and support virulence remain to be identified. The Rv2136c mutant could not be complemented with a single copy of the gene or the putative operon (Rv2133 to Rv2137) and it is, thus, possible that a secondary mutation on the chromosome may be causing the observed phenotypes.

The potential synergy of acid with other host defences has also pointed to Mg\(^{2+}\) transport as potential stress-resistance factor because an Mtb\(\Delta\)mgtC mutant was attenuated for growth in macrophages and mice. MgtC is a putative Mg\(^{2+}\) transporter and Mg\(^{2+}\) might be required at low pH for the maintenance of cell envelope integrity, as a cofactor for enzymes that become important during acid stress or for the function of a Mg\(^{2+}\)-dependent proton ATPase involved in extruding cytosolic protons (Cotter and Hill, 2003). Mtb\(\Delta\)mgtC was attenuated for growth in vitro at a mildly acidic pH of 6.25, but only at low Mg\(^{2+}\) concentrations (Buchmeier et al., 2000). Raising either the pH or the Mg\(^{2+}\) concentration allowed the MgtC mutant to grow like wt Mtb. The growth defect in isolated macrophages and in mice therefore suggests that Mtb depends on Mg\(^{2+}\) acquisition when it faces the low pH of the phagosome. Analyses of Salmonella MgtC suggest that it might be involved in regulating membrane potential, perhaps by activating a cation-translocating P-type ATPase (Moncrief and Maguire, 1998; Gunzel et al., 2006).

Concluding remarks

Among the survival strategies discussed here, resistance to RNI has been examined most extensively. This includes several studies, which reported attenuation of Mtb in mice due to inactivation of genes that participate in the pathogen’s defence against RNI. Evidence for a role in defence against ROI during infections mainly comes from Mtb\(\Delta\)katG. While a number of genes have been identified whose products seem to primarily protect Mtb from RNI or ROI in vitro, few bacterial genes appear to exclusively participate in resistance to RNI or ROI in vivo. Except for the katG mutant, which was fully virulent in NOX2-deficient mice, none of the mutants lacking genes involved in RNI/ROI resistance returned to full virulence in mice lacking either iNOS or NOX2. This might be because many antioxidants are multifunctional and required to protect against ROI and RNI and in some cases additional forms of stress. Few of the above-mentioned mutants have been tested for virulence in mice lacking both iNOS and NOX2, but those that were analysed showed normal growth. The interdependence of stress resistance observed for RNI and ROI defence probably also applies to acid resistance because oxidative and nitrosative stress is likely amplified in mutants incapable of intracellular pH homeostasis. While genes required for pH homeostasis have only recently been identified, it is equally likely that Mtb’s acid defence mechanism have coevolved with resistance mechanisms against the compound stresses presented by the macrophage. Nonetheless, while little is known about their mechanism of action, their requirement for virulence in mice has been clearly demonstrated.

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