Mycobacterial catalases, peroxidases, and superoxide dismutases and their effects on virulence and isoniazid-susceptibility in mycobacteria – a review

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ABSTRACT: Mycobacteria are intracellular bacterial parasites which survive and proliferate inside of macrophages for long periods of time. Because mycobacterial survival in macrophages is required for virulence, a great deal of effort has been focused on identifying the genetic and physiologic determinants of intracellular survival and growth. A number of factors, among them catalases, peroxidases, and superoxide dismutase have been suggested as agents permitting mycobacteria to overcome the intracellular defences of macrophages. The characteristic features of mycobacterial catalase/peroxidases and superoxide dismutase, their distribution within the genus *Mycobacterium*, and their mutual interactions in the inactivation of toxic oxygen products are reviewed. Focus is placed on evidence of the role of mycobacterial catalase-peroxidase and superoxide dismutase in virulence and on the role of catalase-peroxidase in susceptibility to isonicotinic acid hydrazide.

Keywords: tuberculosis; antituberculotic drugs; *Mycobacterium tuberculosis*; *Mycobacterium avium*; therapy

Contents

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1. Introduction

The molecular mechanisms associated with the pathogenesis of tuberculosis are just now being identified and characterized. Pathogenic mycobacteria are facultative intracellular bacteria with the ability to survive and proliferate inside the phagolysosomes of macrophages. Intracellular survival and proliferation of mycobacteria is required for their virulence (Rhoades and Ullrich, 2000) and consequently, there has been a focus on discovery of mechanisms of intracellular survival. Inhibition of phagosome-lysosome fusion (Via et al., 1997) and pH reduction in phagosomes (Crowle et al., 1991) are two of the ways *Mycobacterium tuberculosis* and *Mycobacterium avium* overcome the bactericidal activities of human macrophages. The interaction of mycobacterial cells with its host leads to their replication, yet the macrophages must remain viable (Grange, 1996). In addition, oxidative response gene products, including catalase, peroxidase and superoxide dismutase, have also been shown to be agents of survival of mycobacteria in macrophages (Manca et al., 1999; Firmani and Riley, 2002; Master et al., 2002).

One of the bactericidal mechanisms of macrophages is the production of reactive oxidative intermediates (ROI) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), and single oxygen ($^1O_2$). These oxygen species are extremely toxic to microorganisms (Edwards et al., 2001). Therefore, in order to survive within a macrophage it is necessary that any microbial cell is intrinsically resistant to these agents (Edwards et al., 2001). Mycobacteria have the capacity to inhibit production of ROI and produce enzymes that degrade these microbicidal host metabolites (Banerjee et al., 1994; Miller and Britigan, 1997). This review focuses on the roles of mycobacterial catalase-peroxidases and superoxide dismutases in providing defence against ROI and consequently affects virulence. In addition, the role of catalases, peroxidases, and superoxide dismutases in susceptibility to the antibiotic isonicotinic acid hydrazide (INH or isoniazid) is covered because of the long known interrelationship between virulence and susceptibility to isoniazid (Middlebrook and Cohn, 1953; Jackett et al., 1978).

For information on the utility of the presence and characteristics of catalases in identification of members of the genus *Mycobacterium*, the reader is referred to the article by (Dvorska et al., 2001). Catalases have also proven to be quite useful in identifying taxonomic relationships between members of the genus *Mycobacterium* (Wayne and Diaz, 1982).

2. Mycobacterial catalases and peroxidases

2.1. Mycobacterial catalases

Catalases (hydroxyperoxidases) degrade H_2O_2 to water and oxygen in a single reaction. Peroxidases also degrade H_2O_2, but use H_2O_2 to oxidize a variety of substrates. Cofactors for peroxidases include the red and green hemes. Mycobacteria produce a catalase-peroxidase, so-called because the enzyme has both these activities. A total of three genes affecting catalases have been described in *Escherichia coli*: *katG* encoding a hyperoxidase I-type catalase (HPI), *katE* encoding a hyperoxidase II-type catalase (HPII), and *katF*, which does not encode another catalase, but a regulatory gene for the *katE*-encoded HPII (Milano et al., 1996). Catalase HPI is found in the periplasmic space and the cytoplasmic membrane, while catalase HPII is found in the cytoplasm (Heimberger and Eisenstark, 1988).

2.2. Mycobacterial catalase-peroxidases

Within the genus *Mycobacterium* three different types of catalase-peroxidases have been described (Table 1). The heat-labile, H_2O_2-inducible Kat*G* catalase-peroxidase (T-catalase) is a member of the HPI group of catalases. The heat-stable, non-inducible Kat*E* catalase-peroxidase (M-catalase), belongs to the HPII group. The HPI Kat*G* catalase-peroxidase is resistant to aminotriazole, while the HPII Kat*E* catalase-peroxidase is sensitive. The third type of catalase (A-catalase) was identified and described in strains of *M. avium* and *M. intracellulare*. It is similar to the mycobacterial Kat*E* HPII catalase, but has greater resistance to high temperature, has a different charge, is more hydrophobic, and fails to react with antibody to Kat*E* (Wayne and Diaz, 1988).

M. tuberculosis expresses only a single catalase, the Kat*G*, heat-labile, H_2O_2-inducible, HPI type catalase-peroxidase (Wayne and Diaz, 1982). The first cloning of *M. tuberculosis* catalase-peroxidase (Kat*G*) and demonstration of its role in INH resistance published by Zhang et al. (1992). The enzyme consists of two similar domains, like cytochrome-c-peroxidase

Table 1: Presence of catalase-peroxidases and superoxide dismutase (SOD) in mycobacteria

Mycobacterial species	Catalase-peroxidase		SOD	Others	Authors
	HPI-type	HPII-type			
M. asiaticum	Kat*G*	Kat*E*	Nd	N	Wayne and Diaz (1986)
M. aurum	Nd	Kat*E*	Nd	Nd	Quemard et al. (1991)
M. avium	Kat*G*	Kat*E*	SOD	Nd	Wayne and Diaz (1986), Mayer and Falkinham (1986), Milano et al. (1996)
M. bovis	Kat*G*	Nd	Nd	Nd	Wilson et al. (1995)
M. intracellulare	Kat*G*	Kat*E*	SOD	AhpC	Wayne and Diaz (1982), Wayne and Diaz (1986), Mayer and Falkinham (1986), Wayne and Diaz (1988), Sherman et al. (1996)
M. gordonae	Kat*G*	Kat*E*	Nd	Nd	Wayne and Diaz (1986)
M. kansasii	Kat*G*	Kat*E*	Nd	Nd	Wayne and Diaz (1986)
M. leprae	Nd	Nd	SOD	Nd	Wheeler and Gregory (1980)
M. lepraemurium	Nd	Nd	SOD	Nd	Ichihara et al. (1977)
M. phlei	Nd	Nd	SOD	Nd	Chikata et al. (1975)
M. scrofulaceum	Kat*G*	Kat*E*	SOD	Nd	Wayne and Diaz (1986), Mayer and Falkinham (1986)
M. smegmatis	Kat*G*	Nd	SOD	Nd	Kusunose et al. (1976b), Marcinkeviciene et al. (1995)
M. terrae	Nd	Kat*E*	Nd	Nd	Wayne and Diaz (1986)
M. tuberculosis	Kat*G*	Nd	SOD	Nd	Middlebrook and Cohn (1953), Kusunose et al. (1976b), Wayne and Diaz (1986), Zhang et al. (1993)
M. xenopi	Kat*G*	Nd	Nd	Nd	Wayne and Diaz (1982)

Nd = not detected – identification of the enzyme in organism was not described so far

of yeast. The catalase-peroxidase domain and activity is located in the NH_2-terminus. The function of C-terminal end has not been described so far, but could be involved in the binding of the enzyme to substrate (Wayne and Diaz, 1982).

The *katE* gene of *M. avium*, encoding an HPII-type, heat-stable, M-type catalase-peroxidase has been cloned (Milano et al., 1996) as has the *katG* gene of *M. intracellulare*, encoding a HPI-type, heat-labile and T-type catalase (Morris et al., 1992).

2.3. Alkylhydroperoxide reductase

Another antioxidant protein is alkylhydroperoxide reductase (AhpC). This H_2O_2-inducible enzyme may act to protect mycobacteria from peroxides, especially in the absence of Kat*G* (Sherman et al., 1996; Heym et al., 1997).

3. Superoxide dismutases

Superoxide dismutases (SOD) catalyse the dismutation of the superoxide radical to H_2O_2 and molecular oxygen. Superoxide dismutases have been distinguished on the basis of their associated metals, namely iron, copper-zinc or manganese (Dussurget et al., 2001).

3.1. Mycobacterial superoxide dismutases

SOD activity has been detected in a variety of mycobacteria (Table 1). The first report about cloning of *M. tuberculosis* SOD and demonstration of a secreted feature of the SOD was described in Zhang et al. (1991). *M. tuberculosis* produces two SODs: one (Sod*A*) that employs an iron-cofactor (Kusunose et al., 1976a) and a second (Sod*C*) that

contains a copper-zinc co-factor (Dussurget et al., 2001). *M. leprae* (Wheeler and Gregory, 1980) and *M. smegmatis* strain TAKEO (Kusunose et al., 1976b) have been reported to produce a manganese-containing SOD. The pattern of inhibition of SOD activity of strains of *M. avium*, *M. intracellulare*, and *M. scrofulaceum*, suggested that these species produced SODs containing both iron and manganese (Mayer and Falkinham, 1986).

However, those studies were performed on crude extracts, not purified enzymes. The *sodA* gene of *M. avium* was cloned and shown to encode a 23 kDa protein consisting of 207 amino acids that shares a high degree of similarity with SodA superoxide dismutases of *M. tuberculosis* and *M. leprae* (Thangaraj et al., 1990; Escuyer et al., 1996). Unlike the Sod*A* of *M. tuberculosis*, the *M. avium* enzyme was described as a Mn SOD enzyme (Escuyer et al., 1996). Both the iron-SOD of *M. tuberculosis* and the Mn SOD of *M. avium* are exported from cells and large amounts are found in the extracellular medium (Zhang et al., 1991; Escuyer et al., 1996; Edwards et al., 2001). The iron-SOD of *M. tuberculosis* is secreted via a *secA2*-catalyzed export pathway (Braunstein et al., 2003).

3.2. Reactive oxidative intermediates

In addition to having a role in macrophage survival, the environmental opportunistic mycobacteria (e.g. *M. avium* and *M. intracellulare*) may be exposed to photochemically generated ROI in surface waters exposed to sunlight (Chikata et al., 1975) and after phagocytosis in free living phagocytic protozoa and amoebae (Strahl et al., 2001) and amoebae, where SOD activity may aid in their survival.

4. Mycobacterial catalase-peroxidases and superoxide dismutases as a virulence factors

Mycobacteria are considered the archetypical intracellular pathogens due to their capacity to invade and multiply within macrophages. Mycobacteria are readily phagocytised by macrophages due, in part, to their very high cell surface hydrophobicity (Crowle et al., 1991). Following phagocytosis, mycobacterial cells are exposed to toxic forms of oxygen including H_2O_2, superoxide, HOCl, •OH, and singlet oxygen produced as a consequence of the respiratory burst (Miller and Britigan, 1997). In addition to ROI, toxic reactive nitrogen intermediates (RNI) may also be produced in infected macrophages (Noronha-Dutra et al., 1993). Therefore, it has been proposed that mycobacterial survival in macrophages depends upon their ability to withstand the inhibitory and bactericidal activities of those compounds (Crowle et al., 1986).

M. tuberculosis virulence has been linked to resistance to H_2O_2. Low virulence isolates were shown to be more susceptible to H_2O_2 compared to isolates with high virulence (Jackett et al., 1978). Because catalase levels were not strictly associated with measures of virulence in guinea pigs, factors other than catalase are involved (Jackett et al., 1978; O'Brien et al., 1991). Factors (e.g. permeability) other than catalase-peroxidase activity have been proposed to contribute to H_2O_2 resistance in *M. intracellulare* (Gangadharam and Pratt, 1984). The catalase-peroxidase activities *of M. tuberculosis, M. bovis* BCG, and *M. avium* were the only agents resulting in the destruction of H_2O_2 (Laochumroonvorapong et al., 1997). Therefore, it is likely that protection against other reactive oxygen (ROI) and nitrogen (RNI) intermediates is mediated by additional enzymes.

4.1. Catalase-peroxidases as mycobacterial virulence factors

The Kat*G* catalase-peroxidase of *M. tuberculosis* appears to promote the persistence in infected tissue. KatG^-deletion mutants of *M. tuberculosis* had lower levels of survival in spleens of mice and guinea pigs (Wilson and Collins, 1996; Li et al., 1998). Introduction of a cloned *katG* catalase-peroxidase gene into the *katG*$^-$deletion strain led to persistence in spleens of mice and guinea pigs (Li et al., 1998). The same appears not to be the case for *M. intracellulare*. An isogenic *katG*$^+$ and *katG*$^-$ pair of *M. intracellulare* strains had the same growth kinetics in mouse tissue (Marklund et al., 1998).

Catalases and peroxidases may also have a role in mycobacterial resistance to reactive nitrogen intermediates (RNI). RNI, which include nitric oxide (NO•), are bactericidal. It has been shown that RNI are responsible for killing mycobacteria in alveolar macrophages (Noronha-Dutra et al., 1993). Recently it has been shown, that the *M. tuberculosis* Kat*G* catalase-peroxidase has per-

oxynitratase activity (Wengenack et al., 1999), suggesting that it could be involved in resistance to reactive nitrogen intermediates. Possibly, RNI interact with peroxide, generated by mycobacterial cell metabolism, to produce other bactericidal products such as singlet oxygen (Noronha-Dutra et al., 1993). Alternatively, the iron within catalase (i.e., Kat*G* and Kat*E*) may interact with, and thus detoxify, NO•.

Results of studies using different, non-isogenic strains of *M. tuberculosis* have provided conflicting data. *M. tuberculosis* strains that lacked catalase activity (i.e., strains B1453 and H37RaHR) were the most sensitive to RNI, but other *M. tuberculosis* strains that were catalase-positive (i.e. strains 79112 and H37a) were also sensitive to RNI (O'Brien et al., 1994). Those results show the weakness of comparing non-isogenic strains. It is quite likely that other RNI detoxification systems exist. A similar result was seen when hydrogen peroxide susceptibility of *M. tuberculosis* was investigated. H_2O_2 susceptibility was correlated with the presence of low levels of catalase activity in INH-resistant isolates, but not in low-virulence, INH-sensitive, catalase-positive isolates (O'Brien et al., 1994).

4.2. Superoxide dismutase as mycobacterial virulence factor

Like catalase-peroxidase, mycobacterial superoxide dismutase may be involved in macrophage survival and consequently, the virulence of mycobacteria. However, like the data on catalase-peroxidase there are conflicting reports. In one report, a null mutation in the *M. tuberculosis* copper-zinc superoxide dismutase gene (*sodC*) did not reduce survival in activated and inactivated murine bone marrow-derived macrophages. There was also no difference in the survival of the two isogenic strains in guinea pig tissues (Dussurget et al., 2001). In contrast, a second report demonstrated that an *sodC*-null mutant was more susceptible to killing by gamma interferon-activated murine peritoneal macrophages (Piddington et al., 2001). In both reports, the *sodC*-null mutants were more susceptible to superoxides compared to their *sodC*-wild type parents (Dussurget et al., 2001; Piddington et al., 2001). *M. tuberculosis* mutants with reduced levels of the exported iron-SOD were more susceptible to H_2O_2 and were almost avirulent (Edwards et al., 2001).

5. Role of mycobacterial catalase-peroxidase in isoniazid susceptibility

5.1. Mechanism of action of isoniazid

The association between virulence, H_2O_2-susceptibility, and catalase activity in *M. tuberculosis* has been known since the 1950's (Middlebrook and Cohn, 1953). Further, isoniazid-resistant isolates were shown to have low catalase activity (Michison et al., 1963). Later it was shown that isoniazid is a prodrug that requires activation by the mycobacterial catalase-peroxidase (Shoeb et al., 1985; Zhang et al., 1992). Isoniazid is activated to an unstable electrophilic state by the Kat*G* catalase-peroxidase with H_2O_2 serving as an electron acceptor (Shoeb et al., 1985). Activated isoniazid inhibits the biosynthesis of mycolic acid synthesis (Banerjee et al., 1994; Mdluli et al., 1998b), components of cell wall that protect mycobacterial cells against oxidising agents and other agents in the environment (Mdluli et al., 1998b). The targets for activated isoniazid include the enoyl acyl carrier protein (ACP) reductase encoded by the *inhA* gene (Banerjee et al., 1994) and the β-ketoacyl ACP synthase encoded by the *kasA* gene (Mdluli et al., 1998a). Only the Kat*G* catalase-peroxidase activates isoniazid in *M. tuberculosis* (Zhang et al., 1992; Li et al., 1998). It is likely that endogenous superoxide levels also contribute to isoniazid's antibacterial activity. The presence of an active superoxide dismutase gene prevented isoniazid-induced-killing of an *M. smegmatis* exposed to non-toxic levels of a superoxide-generating agent (Wang et al., 1998).

5.2. The mechanism of resistance to isoniazid

Although isoniazid is very effective against isolates of *M. tuberculosis*, it is about 100-fold less effective against *M. avium*. This difference has been attributed to a decreased permeability of isoniazid through the *M. avium* cell wall. However, the rates of INH-accumulation in *M. tuberculosis* and *M. avium* cells were approximately the same. Interestingly, the total INH-influx in *M. tuberculosis* was found to be four times higher (Mdluli et al., 1998b).

One mechanism leading to isoniazid resistance (Inh^R) are mutations in the Kat*G* catalase-peroxidase gene as was described by Zhang et al. 1992. Characterization of laboratory-generated Inh^R mutants of *M. avium* and *M. tuberculosis* revealed that

a majority had a mutation in the *kat*G gene. Unlike *M. tuberculosis*, *M. avium* formed colonies in the presence of isoniazid at concentrations that inhibited mycolic acid biosynthesis. These mycolate-deficient *M. avium* cells exhibited altered colony morphologies, modified cell wall ultrastructure, and were 10-fold more sensitive to hydrophobic antibiotics, such as rifampin (Mdluli et al., 1998b).

Among laboratory-generated Inh^R isolates of *M. tuberculosis* some lack *katG* mutations and have normal KatG catalase-peroxidase. This demonstrates that mutations other than the KatG catalase-peroxidase, such as in the mycolic acid synthesis pathway, lead to isoniazid-resistance (Quemard et al., 1991). One such gene (i.e., *inhA*) encodes one proposed target of activated isoniazid, the enoyl ACP reductase. Mutations in *inhA* were shown to confer resistance to both isoniazid and ethionamide in *M. smegmatis* and *M. bovis* (Banerjee et al., 1994). The simplest hypothesis is that isoniazid is converted to an activated form by the KatG catalase-peroxidase and the activated antibiotic inhibits the enoyl ACP reductase (Figure 1).

The gene encoding the *M. intracellulare* KatG catalase-peroxidase (heat labile, HPI T-catalase) was cloned and expressed in *E. coli* (Morris et al., 1992). They found that the presence of another catalase activity appeared to be involved in antagonizing isoniazid activity. That second catalase, perhaps the heat-stable (HPII M-type catalase), may be responsible for the relative intrinsic resistance of *M. avium* complex isolates to isoniazid. The mechanism by which this catalase confers protection against isoniazid is unknown, although it possibly scavenges H_2O_2. However, that does not necessarily mean that isoniazid-toxicity is mediated by H_2O_2, but rather that the removal of H_2O_2 by catalase inhibits H_2O_2-dependent peroxidase-mediated isoniazid-oxidation (Milano et al., 1996). That possibility is illustrated in Figure 1.

A cloned copy of the *M. intracellulare katG* gene was subjected to site-directed mutagenesis in host mycobacterial organism (Rouse et al., 1996; Marklund et al., 1998). The resulting *katG* mutants of *M. intracellulare* were resistant to isoniazid, were more sensitive to H_2O_2, and had lost the heat-sensitive catalase-peroxidase activity, but retained the Kat*E* heat-stable peroxidase activity. The same authors performed experiments in mice and demonstrated that the parent *M. intracellulare* strain and an isogenic *katG* mutant had the same growth kinetics in tissues (Marklund et al., 1998). These results proved that the KatG catalase-peroxidase mediates resistance to H_2O_2 and isoniazid-susceptibility but

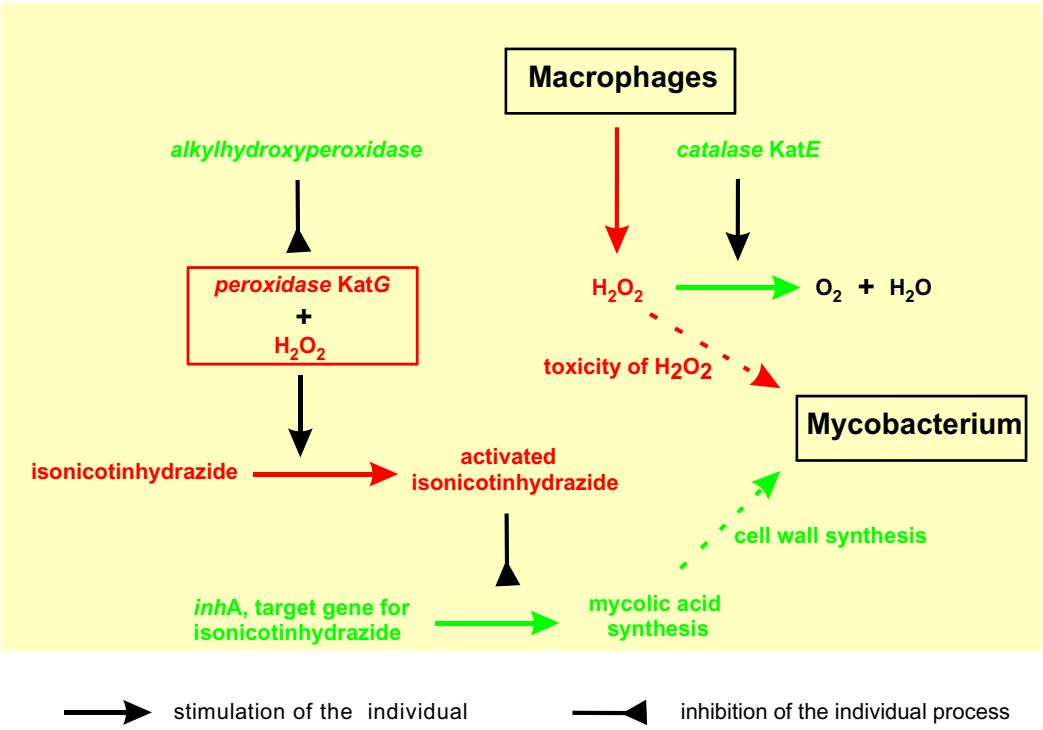

Figure 1. The mechanism of catalases and peroxidases action

is not an essential virulence factor for growth of *M. intracellulare* in mice.

6. Additional factors involving oxidative stress which participate in virulence

Other factors that participate on protection against intracellular parasites are RNI, which include nitric oxide (NO•). NO• kills mycobacteria in alveolar macrophages (O'Brien et al., 1994). Comparison of the *in vitro* susceptibility to NO• of eight *M. tuberculosis* and one *M. bovis* isolates differing in virulence showed a significant negative correlation between NO• susceptibility and virulence (O'Brien et al., 1994).

Differences in the susceptibility of *M. tuberculosis* and *M. avium* complex isolates to killing by macrophages, which may be due to differences in their susceptibility to RNI, have been reported by Doi et al. (1993) and Flesch and Kaufmann (1987). The identity of mycobacterial target of NO• is unclear. In tumour cells this radical inactivates the iron- and sulphur-dependent enzymes involved in respiration, energy production, and cell multiplication (Denis, 1991). In *Clostridium* sp. iron-nitrosyl complexes and NO• were detected due to their reaction with the iron-sulphur protein, named "ferredoxin" (Doi et al., 1993). But sensitivity of mycobacterial iron-sulphur centres to NO• is unknown. The iron-dependent superoxide dismutase (SOD) of *M. avium* was suggested as the target for NO• (Denis, 1991).

The *M. tuberculosis* alkylhydroperoxidase (AhpC) is similar to members of a family of bacterial and eukaryotic antioxidant proteins (Sherman et al., 1999). As was described by several authors (Deretic et al., 1995; Sherman et al., 1996; Wilson and Collins, 1996) the presence of AhpC is associated with resistance to isoniazid in strains of *E. coli* and *M. smegmatis*. Also several Inh^R isolates of *M. tuberculosis* have been described that carry *ahp*C promoter mutations resulting in AhpC overexpression and have reduced Kat*G* catalase-peroxidase activity (Heym et al., 1997; Sherman et al., 1999). It was proposed that because Kat*G* is only described catalase-peroxidase in *M. tuberculosis*, there is selection for compensatory *ahp*C promoter mutations in *katG* (Inh^R) *M. tuberculosis* mutants during infection, to mitigate the added burden imposed by organic peroxides (Sherman et al., 1999).

7. Conclusions

In spite of the efforts focused on elucidating the role of catalase-peroxidases, superoxide dismutases, and other enzymes in virulence and antibiotic-susceptibility in the mycobacteria, more information is required. First, it is clear that not all enzymes and genes involved in oxidant defence have been identified in the major mycobacterial pathogens (i. e. *M. tuberculosis*, *M. bovis*, *M. kansasii*, and *M. avium* complex). Further, many other proteins and genes participate in the complex process of mycobacterial survival and growth within macrophages, tissues, and infected hosts. For example, a total of 191 putative transcription regulators were identified by screening the *M. tuberculosis* complete genome sequence (Cole et al., 1998) and 380 transcripts were identified whose expression profiles appear to be altered during the general process of phagocytosis of mycobacterial cells by macrophages (Tooker et al., 2002). It is hoped that knowledge of the roles of catalase-peroxidases and superoxide dismutases in mycobacterial virulence and drug-susceptibility will lead to the development new antimycobacterial drugs.

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