ABSTRACT

Title of Document: A NEW INSIGHT INTO MYCOBACTERIUM RESISTANCE TO REACTIVE OXYGEN INTERMEDIATES.

Rabab Kish, Master of Science, 2006

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The genes encoded by the RD1 locus are known to be important for intracellular survival of pathogenic mycobacterium, however their role in counteracting host defense is not known. I hypothesize that RD1 is involved in counteracting host oxidative response by secreting ROI-neutralizing enzymes such as catalase and superoxide dismutase. In support of this hypothesis I have shown that *M. marinum* RD1 mutants are more sensitive than wild type to ROIs both *in vitro* and in BMDM. Western blot analysis on the KatG protein levels within the bacterial cells grown in 7H9 rich medium demonstrated that wild type bacteria produced significantly higher amount of KatG than the RD1 mutants. When the bacteria were exposed for 2 hours to H_2O_2, wild type showed a significant reduction of the KatG level, while the RD1 mutants maintained constant levels of KatG, suggesting that RD1 genes might be involved in the secretion of KatG upon exposure to H_2O_2. These results together demonstrate an important unknown function of RD1 in resistance to ROIs.

A NEW INSIGHT INTO MYCOBACTERIUM RESISTANCE TO REACTIVE OXYGEN INTERMEDIATES

By

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Dedication

I would like to dedicate this work to my husband, Gregory Kish, who always offers support and encouragement when I need it most.

Acknowledgements

I would like to thank my mentor, Dr. Gao, for his guidance and support. I would also like to thank Ekua Abban for her work on the *in vitro* assays, Amro Bohsali for creating the *Δesat-6* and Dr. Seema Madhaven for being a great teacher. Finally, I would like to thank Joanna Manoranjan for her tireless dedication to Western blotting and for being an excellent lab companion and friend.

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List of Abbreviations

BMDM – Bone marrow derived macrophages

IFN-γ – Gamma interferon

MapK – Map kinases

NAC – N-acetyl cysteine

NO – Nitric oxide

ROI – Reactive oxygen intermediate

RNI – Reactive nitrogen intermediate

SOD – Superoxide dismutase

UCP2 – Uncoupling protein 2

Chapter 1: Introduction

It is estimated that one third of the world's population is infected by *Mycobacterium tuberculosis*. *M. tuberculosis* is a facultative intracellular pathogen that survives and grows within host macrophages. An important characteristic of *M. tuberculosis* infection is the formation of granulomas at the site of bacterial implantation. It is the necrotization of these granulomas that aids in the aerosol spread of the bacterium. Of those infected with *M. tuberculosis*, only 10% develop active tuberculosis, the remaining 90% may have a low level of infection capable of reactivating at later times (North, 2003).

One of the characteristics that make *M. tuberculosis* such a successful pathogen is its ability to survive within the toxic environment of the host cell. After phagocytosis, a macrophage will activate numerous defense mechanisms in order to eliminate bacteria as well as present antigens to elicit an acquired immune response (Kaufmann, 1988). *M. tuberculosis* has developed mechanisms to derail the antimicrobicidal processes of the host cell and thus persist within the macrophage. During the process of phagocytosis, mycobacterium initiate the transcription of several genes. This sudden change in gene expression likely represents the upregulation of virulence genes necessary for overcoming host defenses (Ehrt, 2001).

One mechanism utilized by mycobacterium to overcome host defense is the inhibition of phagolysosome fusion. Typically a phagosome will undergo a maturation process that ends with acidification and fusion to the lysosome. Mycobacterium block this process by inhibiting the preceding increase in cytosolic calcium levels (Malik, 2000). The inhibition of a cytosolic rise in calcium also inhibits another host defense mechanism, apoptosis. Apoptosis is induced by the macrophage as another means of killing the

bacterium and presenting its antigens. By inhibiting calcium fluxes, mycobacterium indirectly inhibits the activation of the caspase cascade that leads to the formation of the apoptosome (Briken, 2004).

The inhibition of phagolysosome fusion and apoptosis ensure mycobacterium survive inside the phagosome. However, to survive within the phagosome, they must overcome other aspects of host defense such as reactive oxygen and nitrogen intermediates.

Reactive Oxygen Intermediates

Upon phagocytosis, a complex known as NADPH oxidase assembles on the phagosome. NADPH oxidase is composed of two membrane-bound components, $gp91^{phox}$ and $p22^{phox}$, and four cytosolic components, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and RacGTPase. The position of $gp91^{phox}$ and $p22^{phox}$ on the plasma membrane ensures that upon phagosome formation, superoxide is generated directly into the phagosome. This process occurs when $p47^{phox}$ is phosphorylated allowing it to transport the complex to the membrane (Heyworth, 1991). The NADPH oxidase complex accepts electrons from NADPH and donates them to molecular oxygen to generate superoxide (Babior, 1999).

In 1983, Nathan *et al.* showed that macrophages activated with gamma interferon increased their capacity to release peroxides. Further research showed that gamma interferon activated transcription factors which increased transcription of NADPH cytosolic components resulting in a greater number of complexes assembled. In resting cells this transcription level is noticeably lower, however, the process of phagocytosis alone can activate the assembly of the NADPH complex (Cassatalla, 1990).

The superoxide generated by NADPH oxidase is the starting material for the formation of several different reactive oxygen intermediates (ROIs). Superoxide can undergo

spontaneous dismutation to form hydrogen peroxide (H_2O_2). Hydrogen peroxide can then combine with superoxide to form the hydroxyl radical (OH^-). Transition metal catalysts enhance this reaction rate via the Haber-Weiss reaction. Phagocyte-derived peroxidases can interact with H_2O_2 to form HOCl. Superoxide reduction of HOCl can also yield OH^- (Miller, 1997).

The numerous ROIs produced vary in their toxicity. Superoxide itself is relatively unreactive although it may be able to target specific enzymes. Hydrogen peroxide, OH^-, and HOCl are much more toxic and can penetrate membranes. These ROIs are capable of extensive damage such as lipid peroxidation; enzyme inactivation and DNA strand breaks (Hampton, 1998).

NADPH oxidase-generated ROIs play an extremely important role in host defense. Individuals with chronic granulomatous disease are particularly susceptible to a number of infections because they have an inactive NADPH oxidase complex, making them unable to generate ROIs which typically kill many intracellular pathogens. Also, mice with $p47^{phox-/-}$ show a similar phenotype, characterized by the reduction of the respiratory burst and increased susceptibility to infection (Jackson, 1995).

In addition to the direct effect of ROIs on bacteria within the phagosome, ROIs play a significant role in cell signaling within the cytosol. The ROIs localized to the cytosol has two origins, one being from the NADPH oxidase complex and the other from the mitochondria (Park, 2004) (Woo, 2004). Recent evidence has shown that mitochondria are induced to produce ROIs in response to bacterial LPS suggesting an important role for mitochondrial ROIs in the immune response, most likely through the initiation of signaling cascades. Furthermore, research has shown that the ROI-mediated signaling

may be a unique attribute of mitochondrial ROIs (Asehnoune, 2004). In support of this theory, the specific inhibition of mitochondrial ROIs following viral infection leads to reduced NFκB activation while the inhibition of NADPH oxidase has no effect (Mogensen, 2003). Thus inhibition of cytosolic ROIs by the ROI inhibitor NAC will block NFκB activation, presumably by inhibition of mitochondrial ROIs (Sim, 2005).

The role of the mitochondria in generating ROIs has been linked to the uncoupling protein 2 (UCP2). This protein is believed to be involved in negatively regulating the release of ROI from the mitochondria. LPS decreases *ucp2* expression leading to an increase in mitochondrial ROI. Hence $ucp2^{-/-}$ mice are more resistant to pathogens such as *Toxoplasma gondii* because of an inability to increase mitochondrial ROI levels upon infection (Arsenijevic, 2000). The down regulation of *ucp2* occurs through Map kinases (MapK) and p38 pathways and the subsequent increase in cytosolic ROIs leads to increased downstream signaling events such as apoptosis (Emre, 2006). Consequently, bacteria would benefit two fold by eliminating ROIs, decreasing intracellular signaling and eliminating the direct toxic effects of ROI on the bacteria. Given the deleterious effects of ROIs, it is clear why many pathogens have developed mechanisms to counteract them.

Defense Mechanisms against ROIs

Some examples of intracellular pathogens that inhibit ROIs include *Leishmania donovani, Salmonella enterica, Mycobacterium leprae* and *Brucella abortus*. Leishmania promastigotes can express a surface glycoconjugate lipophosphoglycan (LPG), which can impair monocyte oxidative responses (Brandonision, 1994). *M. leprae* produces a similar lipid, phenolic glycolipid 1, which is also capable of scavenging ROIs (Chan, 1989). *S.*

enterica have multiple mechanisms for inhibiting ROIs including the production of antioxidant scavengers, heat shock proteins and superoxide dismutase (Diepen, 2002). Another mechanism employed by *S. enterica* is the inhibition of NADPH oxidase recruitment to the phagosome, a mechanism controlled by *Salmonella* Pathogenicity Island 2 (SPI-2). Research to demonstrate this phenotype showed that in SPI-2 mutants NADPH oxidase localized to the phagosome but not in wild type (Vazquez-torres, 2000).

One ROI-neutralizing mechanism common to many intracellular pathogens is the expression of superoxide dismutase (Sod), an enzyme that breaks down superoxide. *E. coli* that had been genetically altered to allow invasion of epithelial cells were unable to survive intracellularly, however their survival increased when they were made to express increased levels of periplasmic superoxide dismutase (Battistoni, 2000). Similarly, Salmonella mutants lacking the periplasmic SOD were shown to be much less virulent than wild type (de Groote, 1997). A comparable phenotype is seen in infections with *Brucella abortus* mutants that lack periplasmic SOD (Gee, 2005).

The mechanisms mentioned above demonstrate the importance of neutralization of ROIs for intracellular pathogen survival. In the case of *M. tuberculosis*, experimental evidence suggests that this intracellular pathogen has developed potent mechanisms for inhibiting ROIs (Chan, 1992). Typically, activated macrophages can inhibit the growth of mycobacterium through several mechanisms including the production of ROIs. Yet ROI-deficient macrophages did not allow any significant growth recovery for *M. tuberculosis*, suggesting that the bacteria are insensitive to ROIs (Chan, 1992). Research done by Chan *et al.* demonstrated that when macrophages are treated with superoxide dismutase or catalase, there is no reduction in the antimycobacterial activity of the host cell and that

M. tuberculosis is insensitive to H_2O_2 *in vitro*. This suggests that *M. tuberculosis* must have mechanisms to inhibit or neutralize ROIs.

Mycobacterial ROI defense mechanisms

One mechanism by which *M. tuberculosis* neutralize ROIs is via the enzymes superoxide dismutase and catalase. Superoxide dismutase (Sod) breaks down superoxide to H_2O_2 and water; catalase breaks down H_2O_2 to water and oxygen. *M. tuberculosis* has two Sod genes, superoxide dismutase A (SodA) which is manganese associated and superoxide dismutase C (SodC) which is copper-zinc associated. Previous studies detail the importance of Sod in neutralizing ROIs. SodC mutants are more sensitive to exogenous ROIs and are susceptible to killing by activated wild type bone marrow derived macrophages (BMDM) but not by $gp91^{phox-/-}$ BMDM (Piddington, 2001). SodA is much more difficult to study because it appears to be essential for bacteria viability, making a SodA null mutant difficult to obtain. However, studies using mutants with attenuated SodA production showed increased sensitivity to H_2O_2 and attenuated growth in the spleen and lungs of mice (Edwards, 2001).

The *M. tuberculosis* catalase, KatG, has been well studied since clinical isolates with mutations in KatG are resistant to the common antibiotic INH. These mutants are more sensitive to 10 mM H_2O_2, with killing rates from 43-67% (Manca, 1998). Also, *M. tuberculosis* Δ*katG* are more attenuated then wild type in growth in BMDM, but are indistinguishable from wild type when infecting $gp91^{phox-/-}$ BMDM (Ng, 2004). This suggests that the KatG mutant's growth is inhibited by ROIs produced by the macrophage. Further studies on KatG have shown that the integration of *katG* gene into an attenuated strain of *M. bovis* that lacked catalase expression restored bacterial

virulence (Wilson, 1995). This research suggests that KatG plays an important role in mycobacterium virulence by contributing to the inhibition of ROIs.

Taken together, the experimental evidence suggests that both catalase and superoxide dismutase are important in counteracting ROIs within macrophages. While SodC is found exclusively intracellularly and on the cell surface, both KatG and SodA have been shown to be secreted molecules (Braunstein, 2003). In addition to other secreted molecules, these two enzymes were shown to have activity in culture filtrates of pathogenic mycobacterium but not in the culture filtrates of nonpathogenic strains such as *M. smegmatis* (Raynaud, 1998). This suggests that SodA and KatG may play a role in the pathogenesis of *M. tuberculosis*. These enzymes are secreted in a SecA2 dependant manner. The deletion of SecA2 led to reduced virulence suggesting that the secretion of these two enzymes is an important part of their virulence (Braunstein, 2003).

Oxidative Stress Response of Mycobacterium

Many pathogenic bacteria exhibit an inducible oxidative stress response characterized by an upregulation of genes involved in protection against oxidative stress. Since the focus of my research involved mycobacterial response to reactive oxygen intermediates, it is important to address the issue of gene regulation in response to ROIs. Mycobacterial species possess the gene *oxyR*, a gene equivalent to the oxidative stress response gene found in *E. coli* (Farr, 1991). However, *Mycobacterium tuberculosis* have an inactive *oxyR* gene, caused by several mutations (Deretic, 1995). In other organism *oxyR* is capable of responding to oxidative stress by inducing transcription of important oxidative stress response genes. In *M. tuberculosis*, the gene operon believed to be most likely involved in an oxidative stress response is the FurA/KatG locus. *FurA* is the negative

regulator of *katG* and the promoter upstream of *furA* is required for *katG* expression (Pym, 2001).

Previous studies have shown that H_2O_2 can induce the expression of *katG* directly in an *oxyR* independent manner. However, there is some debate as to whether this increased expression is capable of protecting the bacteria upon further exposure to H_2O_2 (Sherman. 1995).

One interesting aspect of oxidative stress response is the expression of the *ahpC* gene. Alkyl hydroperoxide reductase (AhpC) is a peroxidase encoded by *ahpC*, a gene located down stream of *oxyR*, which is not interrupted by mutation in *M. tuberculosis*. Although *ahpC* expression is generally low in wild type *M. tuberculosis*, expression increases significantly in *katG* mutant strains suggesting a compensatory role of AhpC (Sherman, 1996). Presumably *ahpC* expression allows the bacterium to maintain a necessary level of peroxidase activity in the absence of KatG, which has both catalase and peroxidase activity.

Another interesting characteristic of AhpC is it's differential induction in virulent versus avirulent species of mycobacterium. In *M. bovis* BCG, *ahpC* expression increases upon exposure to H_2O_2. However, in the pathogenic strains *M. bovis* and *M. tuberculosis*, expression of *ahpC* is constitutively lower and no induction occurs (Springer, 2001). The exact role of AhpC in virulence remains unclear. Studies on *ahpC* expression during infection show that it is silenced in wild type *M. tuberculosis* and derepressed in BCG. This suggests that *ahpC* expression may not play a role in resistance to host generated ROIs or RNIs. Other experiments have supported this argument showing that Δ*ahpC* did not appear to have lost any degree of virulence (Master, 2002). However, it is still not

clear whether increased levels of AhpC in avirulent strains corresponds to decreased levels of KatG and whether this correlation could explain decreased virulence associated with increased *ahpC* expression. The increase in *ahpC* expression in *katG* negative *M. tuberculosis* isolates would support this claim.

Reactive Nitrogen Intermediates

Although the focus of this research is on ROIs, reactive nitrogen intermediates (RNIs) and ROIs often work synergistically and both are important mechanisms for counteracting bacterial growth. Activated macrophages upregulate the production of nitric oxide (NO), a product of enzymes known as NO synthases. Similar to the effects of ROIs, NO can damage DNA and enzymes. NO can also combine with superoxide to form peroxynitrite, another damaging chemical (Chan, 2001). Nitric oxide plays an important role in inhibiting mycobacterial growth. *M. tuberculosis* infection of $iNOS^{-/-}$ mice shows an increased dissemination of infection (MacMicking,1997). Despite this apparent sensitivity to RNIs, bacteria have developed mechanisms to overcome RNIs in the host cell. For example, the *M. tuberculosis* noxR1 gene appears to contribute to resistance against the toxic effects of RNI although the mechanism is unknown (Ehrt, 1997).

RD1 gene loci

Region of Difference 1 (RD1) is a region of genes that is present in *M. tuberculosis* but absent in the vaccine strain BCG. RD1 is believed to contribute to the virulence of *M. tuberculosis* and its absence is the reason for attenuation of BCG. When the vaccine strain BCG is complemented with RD1 it acquires increased virulence (Pym, 2002).

RD1 encodes a novel secretion system known as secretion in mycobacterium (snm). Two proteins known to be secreted by RD1 are ESAT-6 and CFP-10, both of which lack classical secretion signals that would target them to Sec secretion. Some RD1 genes proposed to make up a core part of the secretion system are Rv3870, 3871, 3877. These genes encode a triple ATPase and 12 transmembrane domain. Deletion of any of these genes eliminates ESAT-6 and CFP-10 secretion and results in reduced intracellular growth of the mutant (Stanley, 2003).

Many other RD1 genes have also been shown to be important for secretion of ESAT-6 and CFP-10 and knocking out any of the essential genes leads to reduced virulence (Brodin, 2006). Similarly, deletion of either *esat-6* or *cfp-10* results in reduced virulence. The precise role these two secreted molecules play is unknown. *M. marinum* contains all the nine RD1 genes present in *M. tuberculosis* and experiments on *M. marinum* have shown that several genes outside the RD1 locus, deemed Extended RD1, are also required for ESAT-6 secretion (Figure 1) (Gao, 2004).

In order to further study the role of RD1 in pathogenesis, I will be working with the organism *Mycobacterium marinum*. There are several advantages in approaching the research through *M. marinum* experiments. Whereas *M. tuberculosis* requires stringent bsl-3 conditions and has a replication time of 24 hours, *M. marinum* can be handled in bsl-2 conditions and replicates at the much faster rate of six to eight hours. This difference between the two strains will allow the research to proceed at a faster rate. The two strains are similar genetically so research on *M. marinum* is useful in understanding the pathogenesis of *M. tuberculosis*. These two pathogens also have a similar pathology in their primary hosts. Whereas *M. tuberculosis* causes granuloma formation in humans,

M. marinum causes similar pathology in zebrafish suggesting similar pathogenic mechanisms.

The goal of this thesis project was to identify the role of RD1 genes in intracellular growth. To achieve this goal RD1 *M. marinum* mutants were used. The *in vitro* sensitivity of RD1 mutants to ROIs was examined followed by cell infection assays to determine the role of ROIs in limiting RD1 mutant growth within macrophages. Western blots were then used to identify the role of RD1 in the secretion of catalase and superoxide dismutase. Combined, the results of these experiments can be used to confirm my hypothesis that RD1 contributes to intracellular growth via the secretion of synthesis of the enzymes KatG and SodA.

Figure 1

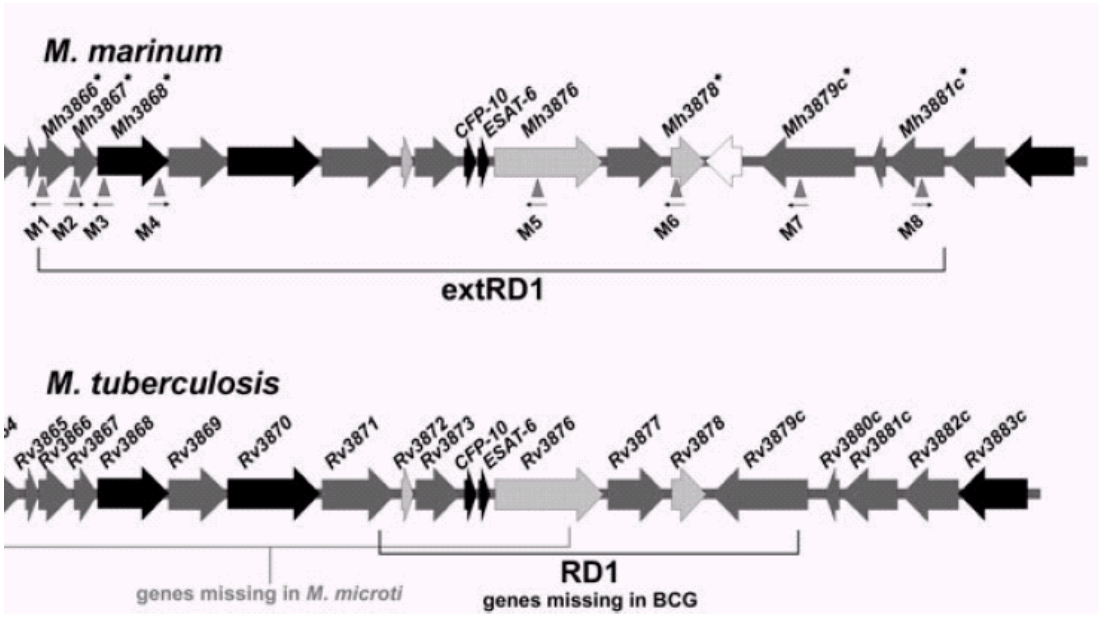


Figure 1. The RD1 and extended RD1 region of *M. tuberculosis* and *M. marinum*. Filled areas represent extRD1 genes with directions of transcription, and filling colors indicate sequence similarities: black, >90%; dark gray, 70–89%; light gray, 55–69%; and open, <54%. RD1indicates *M. tuberculosis* genes deleted in BCG. *Mh* denotes *M. marinum* genes corresponding to *M. tuberculosis* homologues[1].

[1]Gao, L.Y *et al*. A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and Esat6 secretion. 2004 Mol. Microbiology. 53(6)1677-1693.

Chapter 2: Results

RD1 mutants are more sensitive to exogenous ROIs

In a preliminary study I showed that certain RD1 mutants exhibited growth inhibition when grown in 7H9 broth medium lacking catalase. However, this growth inhibition was not obvious for the wild type strain. This requirement for catalase to grow led us to speculate that the RD1 mutants would have increased susceptibility to exogenously added H_2O_2. To test this possibility, we examined the growth of wild type and RD1 mutants that were exposed to 1 mM H_2O_2, by measuring OD_{600} at 3, 4, and 5 days after the exposure. This growth was then compared to that in 7H9 medium lacking H_2O_2 to get the value of percent inhibition. As seen in Figure 2, wild type showed an average growth inhibition of 35%, while the RD1 mutant that had a deletion of both the *esat-6* and *cfp-10* genes (Δ*esat-6*+*cfp-10*) showed much greater growth inhibition averaging 65%.

Figure 2

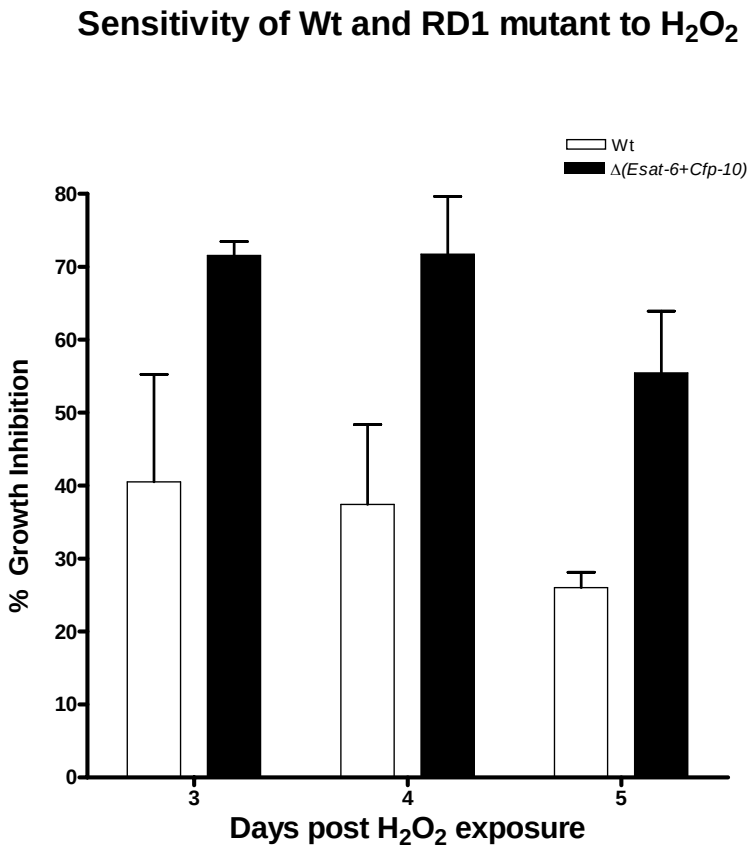


Figure 2. Sensitivity of wild type and RD1 mutant to 1mM H_2O_2. All strains were grown in 7H9 medium containing catalase. Then the cultures were passaged into 7H9 medium lacking catalase, with addition of 1 mM H_2O_2. At 3, 4, and 5 days post H_2O_2 exposure, growth was analyzed by measuring the OD at wavelength 600nm. Results are representative of one experiment performed in duplicate. Statistical significance was examined with the Student *t* test, and *P* values were recorded. P values for 3, 4, and 5 days post H_2O_2 exposure are .0491, .0347 and .0207 respectively.

RD1 mutants are resistant to ROIs generated from within the bacteria

To determine whether the hypersensitivity of the RD1 mutant to H_2O_2 was due to its deficiency in neutralizing ROIs from within the bacterial cell or in the extracellular milieu, we exposed wild type and the RD1 mutant to paraquat. Paraquat is a reagent that enters the bacterium and combines with dioxygen to produce superoxide; an ROI that does not escape from the bacterium yet can be converted to H_2O_2 and other forms of ROIs. The paraquat assays were performed following the same procedures as those in the H_2O_2 sensitivity assays. Bacterial growth was determined by measuring OD_{600} at 3, 4, and 5 days after exposure to 1 mM paraquat and the growth inhibition was analyzed similarly as in the H_2O_2 assay. As seen in Figure 3, wild type and the RD1 mutant were equally resistant to paraquat, indicating that there were no significant differences between the two strains in their capacity to neutralize intracellular ROIs. The results suggest that wild type and the RD1 mutant may have similar levels of intracellular ROI neutralizing enzymes, which implies that the difference between the two in sensitivity to exogenous ROI might be due to their difference in the production of ROI neutralizing enzymes into the extracellular milieu.

Figure 3

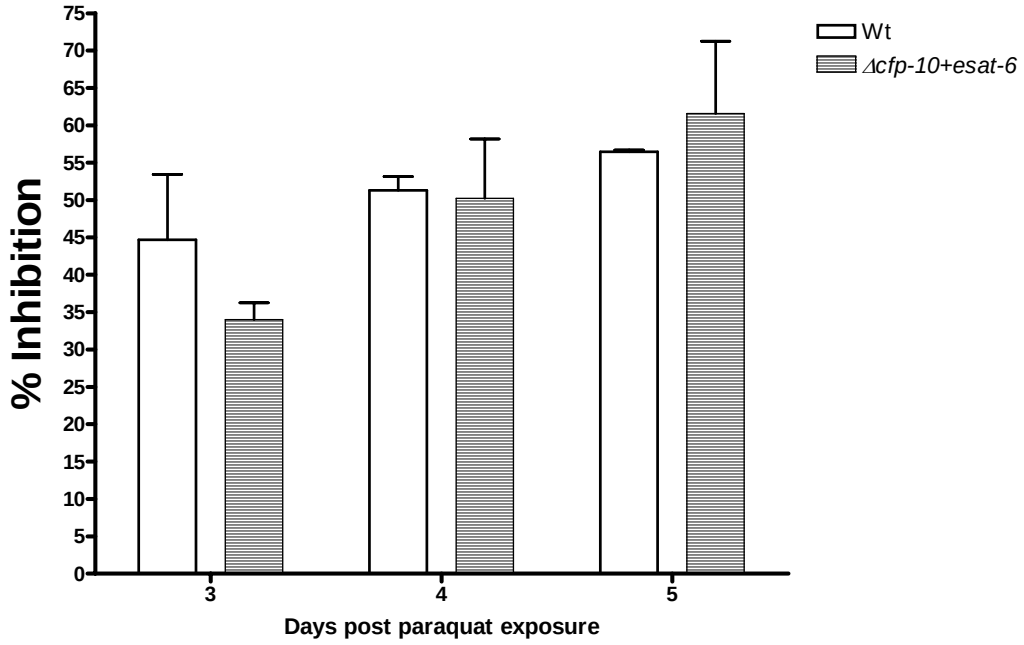


Figure 3. Sensitivity of wild type and RD1 mutant to 1mM paraquat. Wild type and RD1 mutants were initially grown in 7H9 medium containing catalase. Cultures were then passaged into 7H9 medium without catalase containing 1 mM paraquat. At 3, 4, and 5 days post inoculum, growth was analyzed by measuring the OD at wavelength 600nm. Results are representative of one experiment performed in duplicate. Statistical significance was examined with the Student *t* test, and *P* values were recorded. P values for 3, 4, and 5 days post H_2O_2 exposure are .0.1793, .4534 and .3239 respectively.

RD1 mutants are deficient for intracellular growth in activated macrophages

Activated macrophages are known to produce large amount of ROIs to limit microbial growth. To examine whether the role of RD1 genes in resistance to the exogenous ROI is biologically significant during *Mycobacterium* infection of host cells, I performed a series of macrophage infection studies. In these studies, wild type or RD1 mutants were used to infect activated BMDM at an MOI of 1, and bacterial intracellular growth was determined at 0, 24 and 48 hours after infection. The RD1 mutants used in these cell infection studies were Δ*esat-6* (deletion of *esat-6*) and Δ*cfp-10*+*esat-6* (deletion of both *cfp-10* and *esat-6*). Both RD1 mutants displayed approximately 3-4 fold growth inhibition at 48 hours post infection when compared to wild type (Figure 4). The intracellular growth defect has been previously shown for multiple RD1 mutants, including Δ*esat-6*, of both *M. tuberculosis* and *M. marinum*. However, the cause of the defect remains unknown.

Figure 4

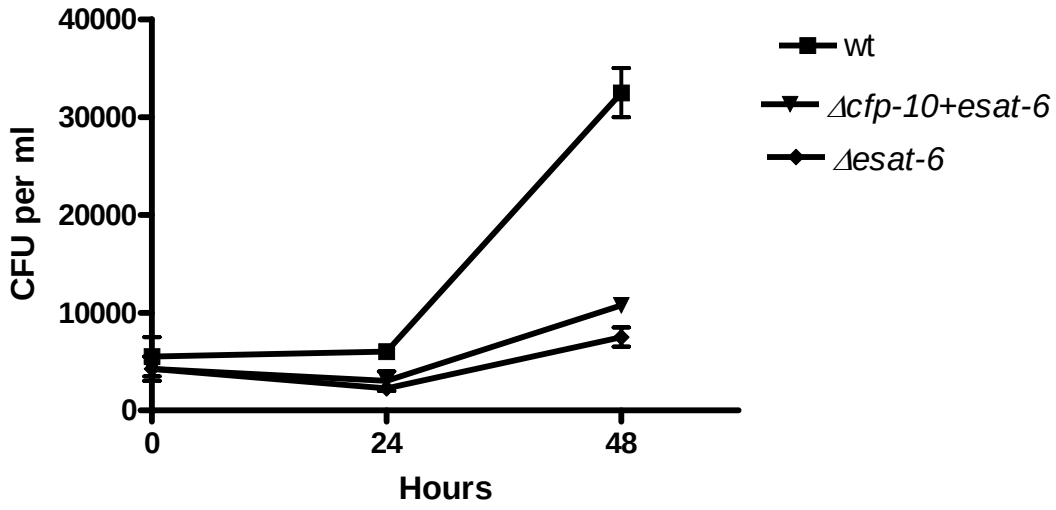


Figure 4. Quantification of the intracellular growth of wild type and RD1 mutants in activated BMDM. Macrophages were activated with both LPS and gamma interferon and infected at an MOI of 1. At 0, 24, and 48 hours post infection, the cells were lysed, and the bacteria were serially diluted and spotted on to 7H10 plates for enumeration of bacterial CFU. Bars indicate standard deviation. Results are representative of one experiment performed in duplicate.

The intracellular growth defect of the RD1 mutants can be recovered by the removal of ROIs

In order to determine whether ROIs were involved in inhibiting the intracellular growth of the RD1 mutants, BMDM were treated with ROI-neutralizing enzymes to remove ROIs and the bacterial intracellular growth was examined. The first approach I took was to treat the activated BMDM with superoxide dismutase and catalase (50 U/ml for each) to remove the ROIs generated by the macrophages. Superoxide dismutase breaks down superoxide to hydrogen peroxide and catalase works by breaking down hydrogen peroxide to water and oxygen. The intracellular growth of *M. marinum* in those cells was compared to that without the treatment of the enzymes. As shown in Figure 5, both RD1 mutants had significantly increased intracellular growth in BMDM treated with the ROI-neutralizing enzymes compared to that without the enzyme treatment. On the other hand, the growth recovery for the wild type bacteria in the enzyme-treated cells was not evident. These results suggest that the production of ROIs by the activated macrophages contributed to the inhibition of the intracellular growth of the RD1 mutants. It was noted that although the RD1 mutants showed significantly recovered growth in the enzyme-treated cells, their growth still did not reach to the level of the wild type strain, suggesting that other factor(s) besides ROIs might have been involved in inhibiting the growth of the RD1 mutants within the activated macrophages.

The second approach I took was to treat the activated BMDM with 5 mM N-acetyl cysteine (NAC) to remove the ROIs produced by the cells. Unlike superoxide dismutase or catalase which remove a specific species of ROI, NAC is able to remove multiple species of ROIs by increasing the levels of the antioxidant scavengers within the cell,

making it a more potent neutralizer of ROIs than the addition of the above two enzymes. As shown in Figure 6, the RD1 mutants showed an even higher growth recovery in the cells treated with NAC than in the cells treated with the two enzymes. Interestingly, even wild type bacteria exhibited slightly increased growth in NAC-treated cells. Although the increased growth of wild type is not statistically significant, there is still the implication that high levels of ROIs produced by activated macrophages can limit the intracellular growth of both wild type and the RD1 mutants but with a much higher inhibitory effect on the RD1 mutants than wild type.

Figure 5

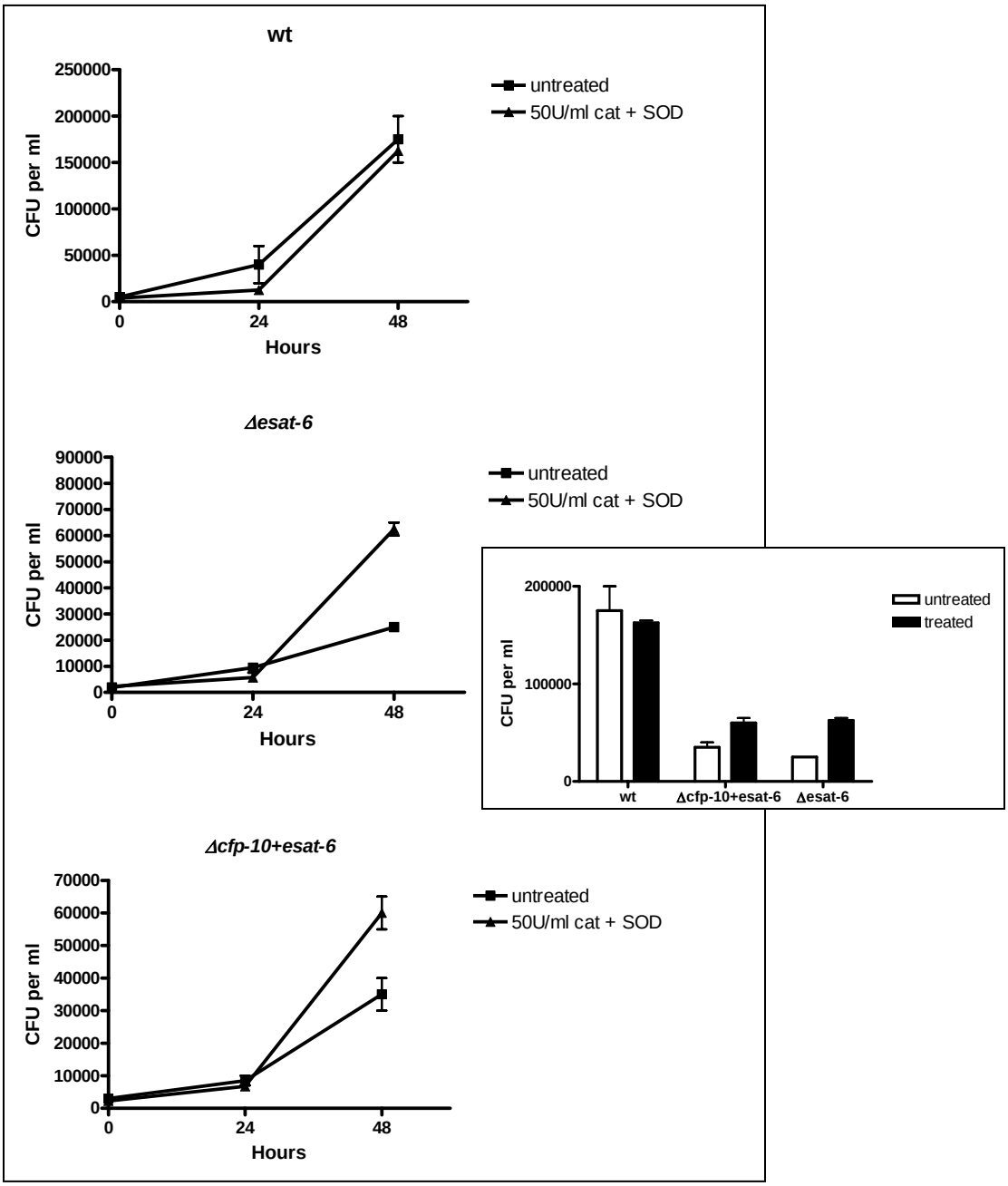


Figure 5. Intracellular growth of wild type and RD1 mutants in activated BMDM treated or not treated with 50U/ml SOD and 50U/ml catalase. BMDM were activated with gamma interferon and LPS and infected at an MOI of 1. At 0, 24 and 48 hours post infection, the cells were lysed and the bacteria were serially diluted and spotted on to 7H10 plates for enumeration of bacterial CFU. Inset figure shows the recovery of the RD1 mutants when treated with SOD and catalase at the 48-hour time point. Results are representative of one experiment performed in duplicate. Error bars indicate standard deviation. Statistical significance for results of inset figure was examined with the Student *t* test, and *P* values were recorded. *P* values for wild type and *Δcfp-10+esat-6* are .3341and .0358 respectively. *P* values not applicable for *Δesat-6*

Figure 6

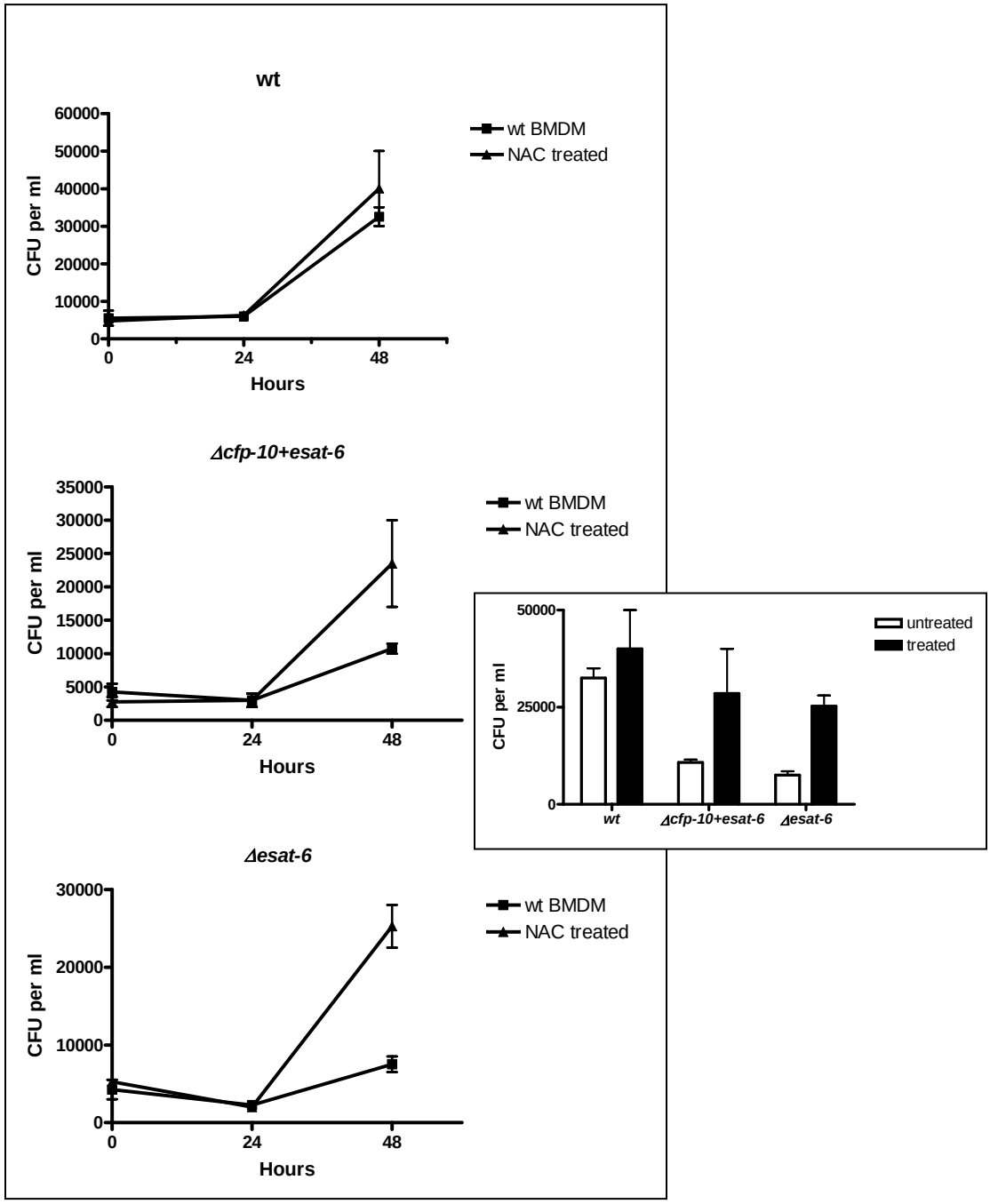


Figure 6. Intracellular growth of wild type and RD1 mutants in activated BMDM treated or not treated with 5mM NAC. BMDM were activated with gamma interferon and LPS and infected at an MOI of 1. At 0, 24 and 48 hours post infection, the cells were lysed, and the bacteria were serially diluted and spotted on to 7H10 plates for enumeration of bacterial CFU. Inset figure shows the recovery of the RD1 mutants when treated with NAC at the 48-hour time point. Results are representative of one experiment performed in duplicate. Error bars indicate standard deviation. Statistical significance for results of inset figure was examined with the Student *t* test, and *P* values were recorded. *P* values for wild type, *Δesat-6* and *Δcfp-10+esat-6* are .2713, .0131 and .1317 respectively.

RD1 mutants show no recovery in BMDM deficient in NADPH oxidase

The NADPH oxidase enzyme complex is involved in producing abundant ROIs in activated neutrophils or macrophages. To investigate whether the ROIs produced by the NADPH oxidase complex played a role in inhibiting the intracellular growth of the RD1 mutants, I compared the growth of the mutants in BMDM from the $gp91^{phox}$ or $p47^{phox}$-knockout mice to that from the wild type mice. Both gp91 and p47 are essential subunits of the NADPH enzyme complex. The intracellular growth assays were performed similarly as described above. The results showed unexpectedly that the RD1 mutants and wild type did not gain growth recovery (Figure 7). These results suggest that the ROIs produced by NADPH oxidase do not play a major role in limiting the intracellular growth of RD1 mutants.

Figure 7

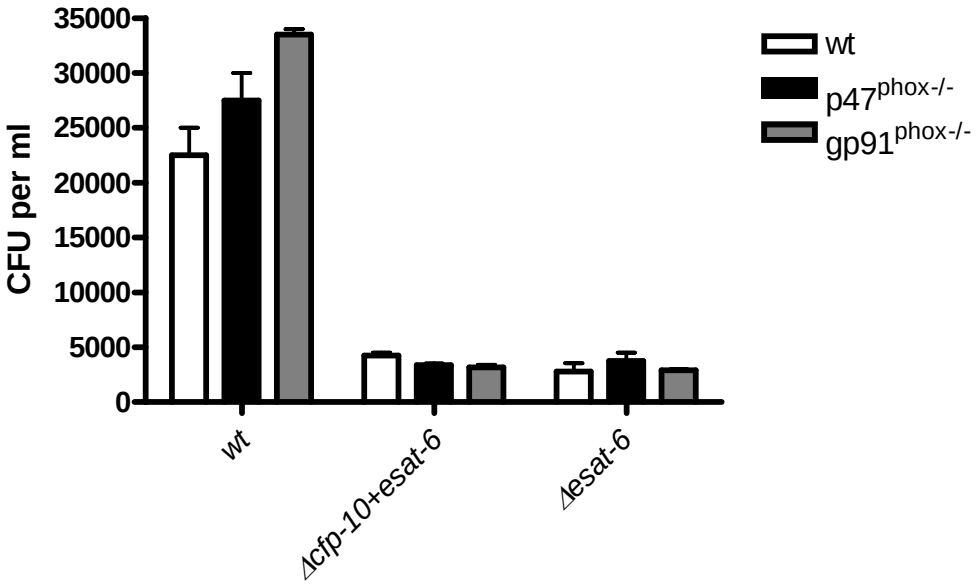


Figure 7. Intracellular growth of wild type and RD1 mutants in activated BMDM from gp91$^{phox-/-}$ and p47$^{phox-/-}$ mice. BMDM were activated with gamma interferon and LPS and infected at an MOI of 1. At 0, 24 and 48 hours post infection, the cells were lysed, and the bacteria were serially diluted and spotted on to 7H10 plates for enumeration of bacterial CFU. Results are representative of one experiment performed in duplicate. Error bars indicate standard deviation.

RD1 mutants show no growth recovery in BMDM deficient for iNOS

Nitric oxide produced by the iNOS enzyme can react with superoxide to form an even more toxic radical, peroxynitrite, which is a potent antimicrobial agent. I showed above that the removal of the ROIs produced by the NADPH oxidase complex did not benefit the growth of the RD1 mutants. One possible explanation is that the superoxide produced by an NADPH oxidase-independent source reacted with nitric oxide generated by iNOS to form peroxynitrite, which might have produced a potent growth inhibition for the mutants. To test this possibility, I examined the growth of the RD1 mutants in BMDM from iNOS-knockout mice and compared it to that from the wild type mice. Activation and infection of BMDM were performed similarly as described above. The results showed that wild type or the RD1 mutants did not gain grow recovery in the iNOS-knockout macrophages (Figure 8). In fact, both bacteria somehow showed slightly reduced growth in the knockout cells. Therefore, the reactive nitrogen intermediates did not seem to play a role in inhibiting the intracellular growth of the RD1 mutants in activated macrophages.

Figure 8

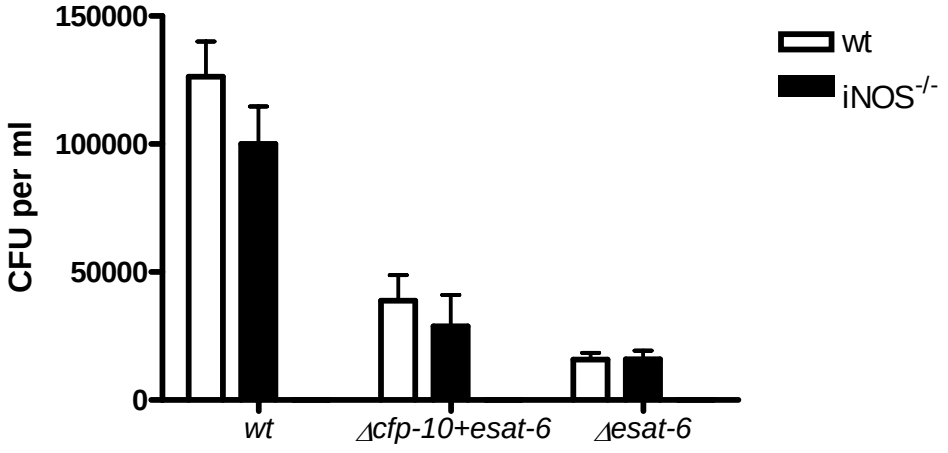


Figure 8. Intracellular growth of RD1 mutant in BMDM from iNOS$^{-/-}$ mice. BMDM were activated with gamma interferon and LPS and infected at an MOI of 1. At 0, 24 and 48 hours post infection, the cells were lysed, and the bacteria were serially diluted and spotted on to 7H10 plates for enumeration of bacterial CFU. Results are representative of two experiments performed in duplicate. Error bars indicate standard deviation. Statistical significance was examined with the Student *t* test, and *P* values were recorded. *P* values for wild type, *Δesat-6* and *Δcfp-10+esat-6* are.1201, .4890 and .2763 respectively.

RD1 genes may play a role in the synthesis and/or secretion of KatG

To determine whether the increased sensitivity of the RD1 mutants to ROI was due to their defect in the synthesis and/or secretion of KatG, I examined the production of KatG in both the bacterial cell lysates and culture filtrates by Western blot analysis. In my initial analysis, I analyzed KatG production by the bacteria grown in the Sauton's minimum defined medium. No KatG was detected in the culture filtrates of both wild type and RD1 mutants, suggesting that the amount of KatG secreted into the Sauton's medium was under the detectable level (data not shown). Since a difference in the intracellular level of KatG may also explain differential sensitivity of wild type and RD1 mutants to ROIs, the cell lysates of wild type and RD1 mutants were probed for KatG. The results presented in Figure 9A showed that the RD1 mutants had varying levels of KatG within the crude cell lysates. Quantification of the KatG levels within the crude cell lysates showed that the differences between wild type (set at 100%) and the majority of the RD1 mutants might not be significant, except that the *Mh3878::kan* mutant produced strikingly more KatG than wild type (Figure 10).

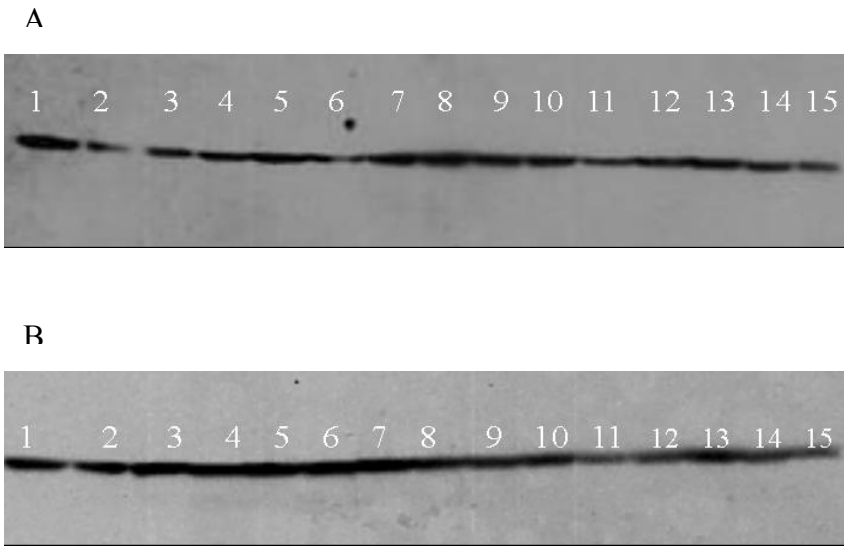
Previous research has suggested that KatG may be a cell wall associated protein, hence possible differences between wild type and the RD1 mutants in the amount of KatG associated with the cell wall might also explain the differences between them in the sensitivity to ROIs. To analyze the differences in the cell wall-associated KatG, the cytosol plus cytoplasmic membrane fraction was separated from the cell wall fraction by centrifugation, and the cytosol plus cytoplasmic membrane fraction was probed for KatG using anti-KatG antibodies. The results in Figures 11 and 12 showed that the RD1

mutants had varying levels of KatG in the cytosol plus cytoplasmic membrane fraction, for example the *Mh3876::kan* and *Mh3879::kan* mutants had much reduced levels of KatG compared to wild type. Many other RD1 mutants showed more or less similar levels of KatG compared to wild type. The *Mh3878::kan* mutant again showed much higher level of KatG than wild type. However, wild type bacteria clearly did not show significantly reduced level of KatG when compared to most of the RD1 mutants, implicating that there was no differential localization of KatG on the wild type bacterial cell wall.

The speculation that wild type bacteria grown in the 7H9 rich medium, rather than Sauton's medium, might contain higher level of KatG than the RD1 mutants is intriguing, because all of the above described *in vitro* and cell infection experiments examining the sensitivity of RD1 mutants to ROIs were performed using the bacteria grown in 7H9 medium. Therefore, I wondered whether the different growth conditions might have caused the differences in the KatG levels in wild type or the RD1 mutant bacteria. To further evaluate this possibility, I examined the KatG level in the cell lysates of wild type and RD1 mutants grown in 7H9 medium. The results presented in Figures 13 and 14 showed that wild type bacteria produced significantly more KatG than the RD1 mutants when grown in the 7H9 medium. Quantification of the KatG levels indicated that the KatG level of wild type bacteria was approximately 3- and 2-times higher than that of the Δ*esat-6* and the Δ*cfp-10+esat-6* mutants, respectively. Therefore, the results indicated that wild type bacteria grown in the 7H9 rich medium produced a significantly higher level of KatG than the RD1 mutants, suggesting that the RD1 genes are possibly involved in the optimal production/synthesis of KatG.

Another important aspect to look into was the dynamics of the KatG level in response to the exposure to ROIs. Interestingly, after exposure to 1 mM H_2O_2, the KatG level of wild type bacteria dropped significantly within 2 hours by 150% (Figure 14). This rapid drop of the KatG protein level in wild type bacteria can be accounted for by two possibilities, one is the degradation and the other the secretion of the KatG protein. Intriguingly, the drop of KatG for both RD1 mutants after exposure to 1 mM H_2O_2 was not as evident as for wild type, implying that the drop of KatG level in wild type bacteria probably was not due to degradation but rather the secretion of KatG. The above experiment was performed only once. If the results can be reproduced, the data would suggest that the RD1 genes might be involved in both the production/synthesis and the secretion of KatG when grown in the rich medium, consistent with the observation that wild type bacteria are more resistant than the RD1 mutants to H_2O_2.

Figure 9



1. ***Mh3878::kan***
2. ***Mh3868a::kan***
3. ***Mh3879::kan***
4. ***Mh3876::kan***
5. ***Mh3868b::kan***
6. ***Mh3867::kan***
7. ***Mh3881::kan***
8. ***Mh3866::kan***
9. ***Δesat-6***
10. ***ΔsecA_2***
11. ***wt***
12. ***Mh3868c::kan***
13. ***Δesat-6***
14. ***Δesat-6***
15. ***Δcfp-10+esat-6***

Figure 9. Analysis of KatG within the crude cell lysate of wild type and RD1 mutants grown in Sauton's minimum defined media by Western blot. Cell lysates were processed and proteins separated on a 4-20% gradient SDS gel. Proteins were probed with anti-KatG (A) and anti-GroEL (B) antibodies. Lanes are indicated on the side of the blot. These results are representative of a single Western blot experiment

Figure 10

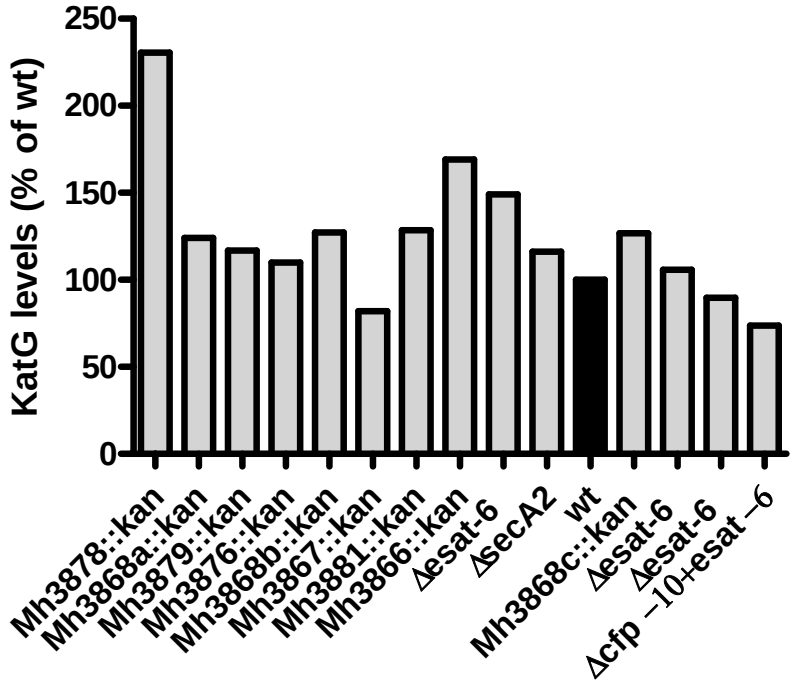
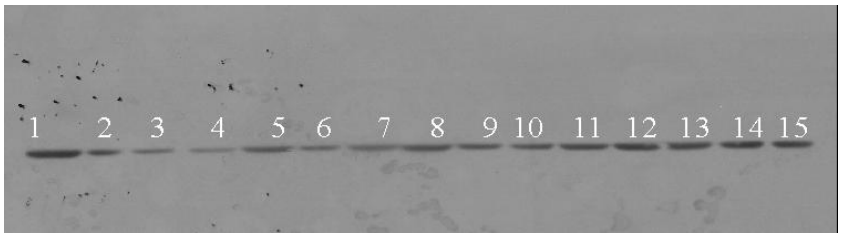


Figure 10 Quantification of KatG levels present in the samples presented in Figure 9. Quantification was performed using ImageJ software. The KatG levels were normalized according to the levels of GroEL. Wild type levels of KatG are set to 100% so RD1 mutant levels of KatG are expressed as a percent of the wild type level.

Figure 11



1. ***Mh3878::kan***
2. ***Mh3868a::kan***
3. ***Mh3879::kan***
4. ***Mh3876::kan***
5. ***Mh3868b::kan***
6. ***Mh3867::kan***
7. ***Mh3881::kan***
8. ***Mh3866::kan***
9. ***Δesat-6***
10. ***ΔsecA_2***
11. ***wt***
12. ***Mh3868c::kan***
13. ***Δesat-6***
14. ***Δesat-6***
15. ***Δcfp-10+esat-6***

Figure 11. Analysis of KatG localized to cytosol and plasma membrane for bacteria grown in Sauton's minimum defined media. Crude cell lysates were centrifuged to remove cell wall extract. The remaining cytosolic and plasma membrane proteins were separated on a 4-20% gradient gel and KatG was probed with an anti-KatG antibody. Lanes are indicated on the side of the blot. These results are representative of a single Western blot experiment

Figure 12

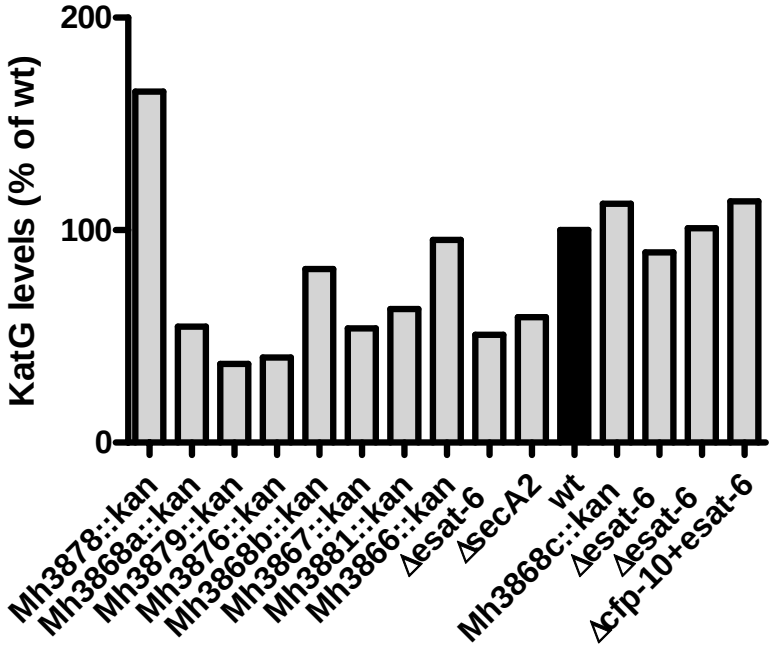
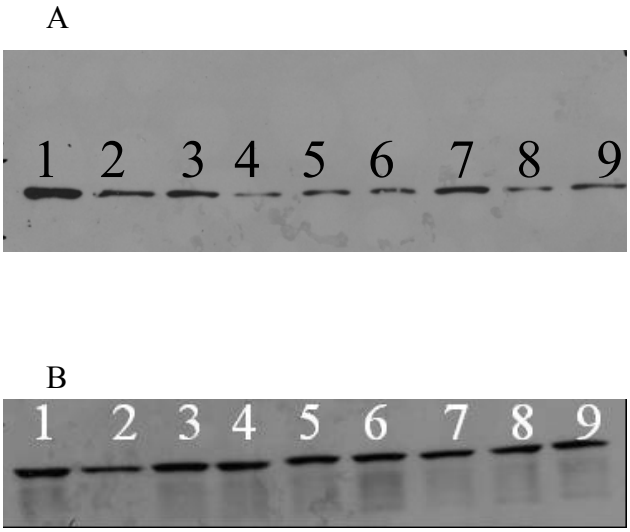


Figure 12. Quantification of KatG levels present in the samples presented in Figure 11. Quantification was performed using ImageJ software. The KatG levels were normalized according to the levels of GroEL. Wild type levels of KatG are set to 100% so RD1 mutant levels of KatG are expressed as a percent of the wild type level.

Figure 13



1. **Wt – 0 H_2O_2**
2. **Wt – 1 mM H_2O_2**
3. **Wt – 20 mM H_2O_2**
4. **Δ*esat-6* – 0 H_2O_2**
5. **Δ*esat-6* – 1 mM H_2O_2**
6. **Δ*esat-6* – 20 mM H_2O_2**
7. **Δ*cfp-10*+*esat-6* – 0 H_2O_2**
8. **Δ*cfp-10*+*esat-6* – 1 mM H_2O_2**
9. **Δ*cfp-10*+*esat-6* – 0 H_2O_2**

Figure 13. Analysis of KatG expression in the cell lysates of RD1 mutants grown in 7H9 medium following exposure to H_2O_2. Wild type and RD1 mutants were initially grown in 7H9 medium then exposed to H_2O_2 for 2 hours prior to processing of cell lysate. Proteins the cell lysates were separated on a 4-20% gradient gel and probed with anti-KatG antibodies (A) and anti-GroEl (B). Lanes are indicated on the side of the blot. These results are representative of a single Western blot experiment

Figure 14

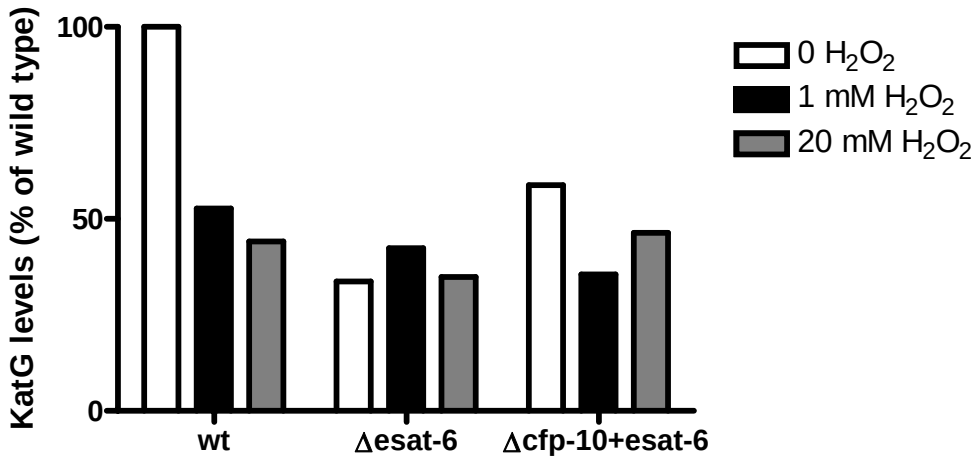


Figure 14. Quantification of the KatG protein levels for the samples presented in Figure 13. Quantification was performed using ImageJ software. The KatG levels were normalized according to the levels of GroEL. Wild type levels of KatG are set to 100% so RD1 mutant levels of KatG are expressed as a percentage of wild type levels.

RD1 may play a role in the regulation of AhpC

Published data has demonstrated a clear link between the production of KatG and AhpC, in which there is an induction of AhpC as compensation for a decrease of KatG. Therefore I examined whether the RD1 mutants, which as described above may be deficient in the production/synthesize and secretion of KatG, would express an increase in AhpC after exposure to ROIs such as H_2O_2. AhpC protein was detected by Western blot, using the same cell lysates described above from bacteria grown in 7H9 medium for KatG analysis. The results shown in Figures 15 and 16 showed that wild type bacteria expressed relatively constant levels of AhpC following exposure to H_2O_2. However, the RD1 mutants showed markedly increased levels of AhpC following the H_2O_2 exposure. After exposure to 1 mM H_2O_2 Δ*esat-6* and Δ*cfp-10*+*esat-6* appeared to induce AhpC by approximately 125% and 100% more, respectively. The results are consistent with the previously described link between KatG and AhpC and further support my above observation that the RD1 mutants have deficiency in KatG synthesis and secretion.

Figure 15

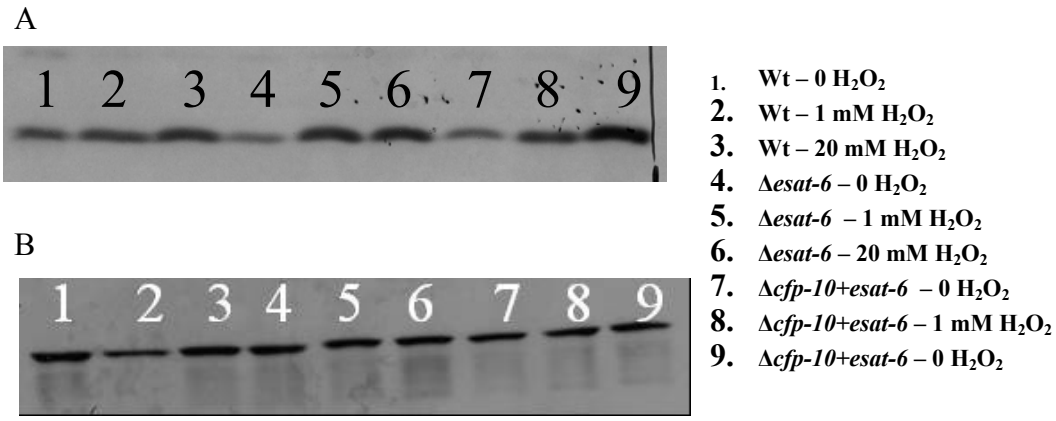


Figure 15. Analysis of AhpC expression in the cell lysates of RD1 mutants grown in 7H9 medium following exposure to H_2O_2. Wild type and RD1 mutants were initially grown in 7H9 medium then exposed to H_2O_2 for 2 hours prior to processing of cell lysate. Proteins the cell lysates were separated on a 4-20% gradient gel and probed with anti-KatG antibodies (A) and anti-GroEl (B). Lanes are indicated on the side. These results are representative of a single Western blot experiment

Figure 16

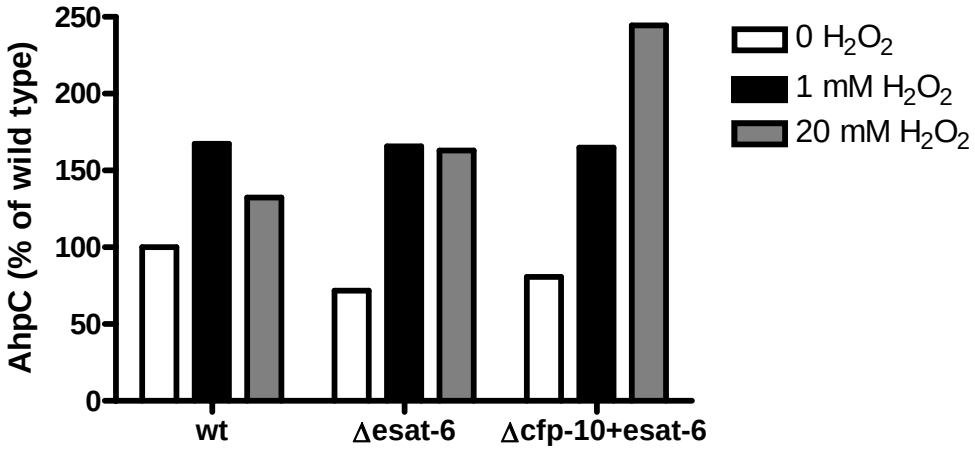


Figure 16. Quantification of the AhpC protein levels for the samples presented in Figure 15. Quantification was performed using ImageJ software. The AhpC levels were normalized according to the levels of GroEL. Wild type levels of AhpC are set to 100% so RD1 mutant levels of AhpC are expressed as a percentage of wild type levels.

The role of RD1 genes in the synthesis and/or secretion of superoxide dismutase is inconclusive

Here I examined another ROI-scavenging enzyme, superoxide dismutase. My preliminary study focused on the secretion of this enzyme by wild type and the RD1 mutants. I first examined the enzyme activity in the culture filtrates of wild type and the RD1 mutants grown in Sauton's minimum defined medium. The culture filtrate proteins were separated by a 15% non-denaturing agarose gel and the superoxide dismutase enzyme activity was detected by incubation with nitroblue tetrozolium. As seen in Figures 17A and 18A, the RD1 mutants and wild type bacteria all showed the clear band for superoxide dismutase at varying degrees of intensity. In general, the wild type bacteria produced little superoxide dismutase activity compared to the RD1 mutants. Some RD1 mutants, such as *Mh3868a::kan, Mh3868b::kan, Mh3868c::kan*, *Mh3867::kan*, and the Δ*esat-6* mutants secreted much more SodA compared to wild type. The above results of the enzymatic activity were further confirmed by Western blot assays shown in Figures 17B and 18B. It is also interesting to note that the SecA2 mutant appears to secrete superoxide dismutase despite previous studies that imply that SodA may be secreted via the SecA2 secretion system (Braunstein, 2003). However, the secretion of SodA is not likely to be exclusive to SecA2 given the fact that when secretion is analyzed under different growth phases, the SecA2 mutant has been shown secrete SodA, which is confirmed in by our Western blot.

Generally, the results demonstrate that RD1 mutants secrete equivalent or even higher levels of superoxide dismutase than wild type when the bacteria are grown in the Sauton's medium. As I described above, RD1 genes are likely involved in the synthesis and secretion of KatG when grown in 7H9, but not Sauton's medium. Therefore it is not

conclusive whether these genes play a similar role in the synthesis and/or secretion of superoxide dismutase when the bacteria are grown in 7H9 medium.

Figure 17

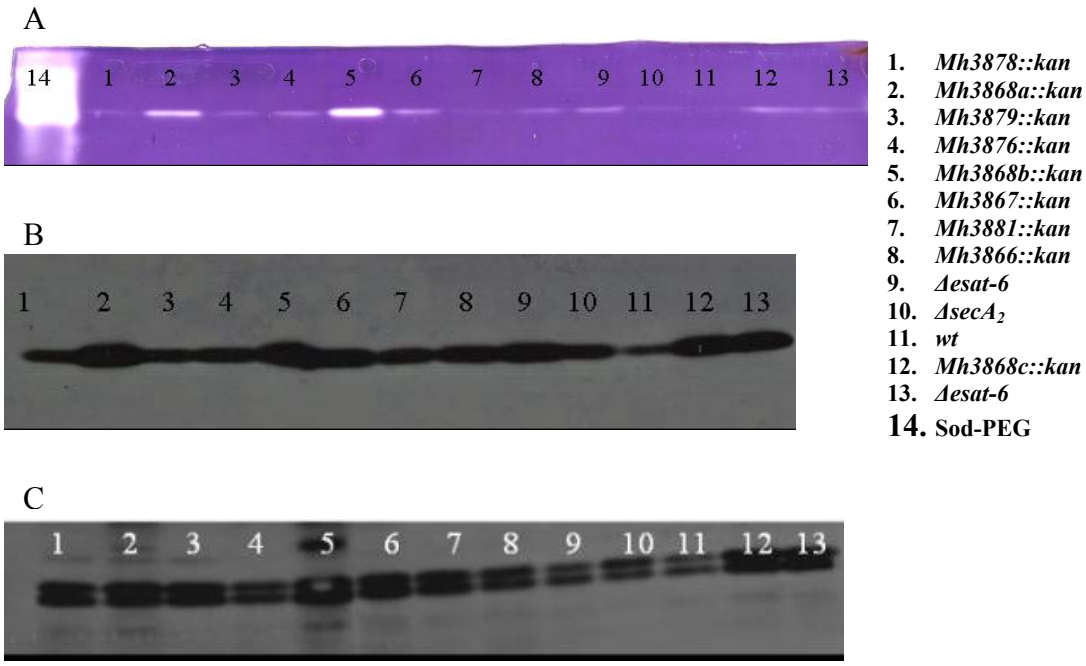
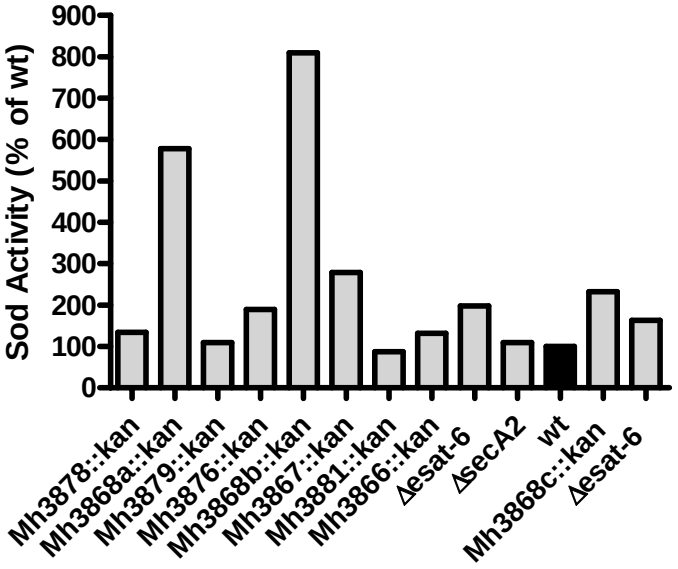


Figure 17. Analysis of the protein levels of superoxide dismutase in the culture filtrate of wild type and RD1 mutants grown in Sauton's defined minimal media. A) SodA enzymatic activity. The nondenatured culture filtrate was separated by a 15% gel. Activity was measured by incubation with nitroblue tetrozolium followed by a solution of TEMED, riboflavin and potassium phosphate. B) Western blot probing for SodA. Proteins of the culture filtrates were separated by a 4-20% gradient gel and SodA was probed with anti-SodA antibodies. C) Western blot probing for Antigen 85, which is protein secreted via the SecA1-dependant pathway. Lanes are indicated on the side. These results are representative of a single Western blot experiment.

Figure 18

A



B

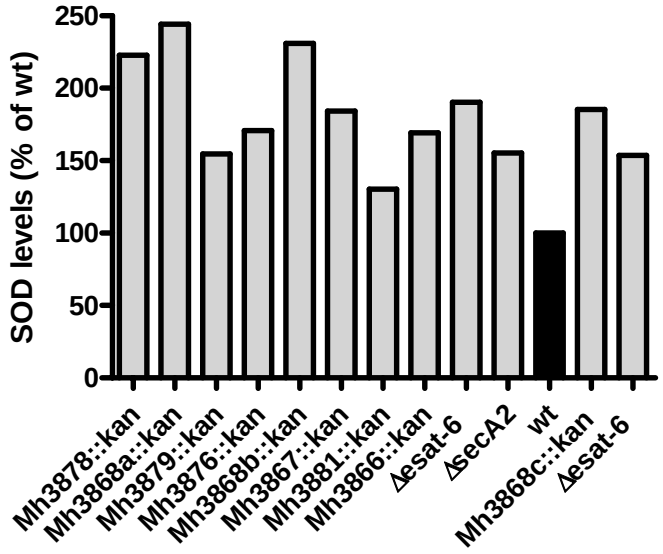


Figure 18. Quantification the enzymatic activity (A) and protein levels (B) of SodA for the samples presented in Figures 17 A and B. Quantification was performed using ImageJ software. Wild type level of SodA was set to 100% so SodA levels of were expressed as a percent of wild type levels

Chapter 3: Experimental procedures

Bacterial Strains and Media

RD1 mutant strains were created by Gao et al, 2003. Bacteria were cultures in Middlebrook 7H9 (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% albumin-dextrose-catalase enrichment (7H9 broth) or on Middlebrook 7H10 agar (Difco), supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase enrichment. Antibiotics used for RD1 mutant media was kanamycin (30 μg/ml) Initial inoculums were cultured at 32˚C with shaking (100RPM) to confluence. Cultures were then concentrated in 7H9 containing 30% glycerol and stored at –80C for subsequent use.

Cell Culture Lines

Bone marrow derived macrophages were derived from bone marrow exudates of cells extracted from the femurs of female 6-week-old C57BL/6 mice obtained from Jackson laboratories (Bar Harbour, Maine). Cells were flushed using 2% FBS in PBS. Following isolation and washes, cells were cultured for four days at 37˚C in 5% CO_2. The media used for culturing BMDM was Dulbecco's Modified Eagle Media (DMEM) modified with 10% fetal bovine serum, 15% L929 cell supernatant, 1% glutamine and 2% HEPES buffer. After four days, fresh media was added to the cells and culture was maintained four more days before further processing. Bone marrow derived macrophages were also isolated from $p47^{phox-/-}$, $gp91^{phox-/-}$ and $iNOS^{-/-}$ mice.

Hydrogen Peroxide and Paraquat (methyl violegen) *in vitro* Assays

Wild-type *M. marinum* and RD1 mutants Δ*esat-6* and Δ*cfp-10+esat-6* were grown in 7H9 liquid medium with catalase to late log phase (OD_{600}≈1.2-1.5). All bacterial cultures were standardized to OD_{600}= 1.2 by diluting with 7H9 without catalase medium. 130ul of the standardized bacterial culture were further diluted with 1ml 7H9 without catalase medium. 125 µl of this solution was then inoculated into Erlenmeyer flasks containing 30ml 7H9 without catalase. Hydrogen peroxide and paraquat solutions with specific concentrations were also prepared with 7H9 without catalase culture medium and then inoculated into flasks with cultures. Cultures were grown in 32˚C with constant shaking (100RPM). Three days after the initial inoculum, OD_{600} readings were taken to measure bacterial survival after initial exposure to extracellular hydrogen peroxide and paraquat. The statistical analysis of the data were performed with Student's *t* test using Graph Prism software.

Bone Marrow Derived Macrophage Infection

BMDM cells were seeded at a density 2.5 x 10^5 per ml or 5x10^4 per well in 96 well plates. Cells were activated one day prior to infection by treatment with BMDM infection medium containing interferon gamma at a concentration of 100 U/ml. BMDM infection media consists of DMEM, 10% FBS, 1% glutamine and 2% Hepes buffer. Cells were conditioned at 32˚C for 24 hours prior to infection. Two hours prior to infection, cells were treated with infection medium containing 10 ng/ml LPS. For recovery assays, ROI-inhibitors were also added at this time. Cells were treated with either 50 U/ml superoxide dismutase-PEG and 50 U/ml catalase-PEG, or 5 mM N-acetyl cysteine (NAC).

Prior to infection bacteria were grown to OD_{600} of 1.2-1.5. Bacteria were processed by washing with PBS followed by isolation of single bacteria by passage through a 26G needle two times. Bacteria concentrations were adjusted in BMDM infection media with the required concentration of superoxide dismutase /catalase and NAC. Bacteria were added to BMDMs to establish a multiplicity of infection of 1. After a two-hour incubation at 32ºC at 5% CO_2 pressure, cells were washed three times with PBS. Cells were then incubated with infection media containing 4 μg/ml streptomycin as well as the appropriate concentration of the ROI inhibitor used for that infection.

At each time point, 0 (immediately after two hour infection), 24 and 48, cells were lysed using cell lysis media triton. For the time 0 and 24-hour time points, cells were lysed by the addition of 200 μl of 0.1% triton followed by a ten-minute incubation at 32˚C. After resuspending, 20 μl was transferred to 180 μl of PBS. Six 1:10 serial dilutions were made and 10 μl of each dilution was spotted onto a 7H10 plate. For the 48-hour time point, the 200 μl supernatant was combined with 20 μl of 4% triton. The adherent cells were treated with 200 μl of 0.1% triton. After a 10-minute incubation both supernatant and adherent cells were resuspended and 20 μl from each was transferred to 160 μl PBS for serial dilutions.

For each infection, untreated wild type BMDM were infected simultaneously with the BMDM treated with ROI inhibitors. Cell infections with untreated BMDM followed the same procedures with the exception being the addition of the ROI neutralizing reagents. Infections performed on mutant cell lines ($iNOS^{-/-}$, $gp91^{phox-/-}$, and $p47^{phox-/-}$) followed the same procedure as those used to infect wild type untreated BMDM. The statistical analysis of the data were performed with Student's *t* test using Graph Prism software.

Culture filtrate and cell lysate preparation of RD1 mutants

RD1 mutants were inoculated from frozen stock into 7H9 medium. This culture was passed two times into Sauton's minimal media. Each passage was done at the point that the cultures had reached confluence as determined by OD_{600} of 1.2 to 1.4.The final cultures were grown for 6 days. The culture filtrate was separated via centrifugation at 3800 RPM for ten minutes followed by filtration through a filter cup. The remaining pellet containing whole cells was frozen at –80˚C. EDTA (pH 7-8) was added to the culture filtrate at a concentration of 1 mM. The culture filtrate was then concentrated to 1ml by ultracentrifugation at 4˚C using centriplus ultracentrifugation tubes. Cocktail proteinase inhibitor was added to concentrated culture filtrate. Culture filtrates were normalized to the weight of the pellets and adjusted to 10^{11} bacteria/ml.

Prior to preparation of whole cell lysates, pellets were measured and concentrations were normalized by resuspending in 20 mM Tris buffer (pH=7.5), containing a cocktail of proteinase inhibitors. Cells were then subjected to four rounds of bead beating performed at maximum speed for thirty seconds per round. Between each round cells were kept on ice. Tubes were then centrifuged two times at 3000xg at 4˚C for 10 minutes. The remaining supernatant was crude cell lysate containing cell wall, cytosol, and plasma membrane.

To remove the cell wall portion, the crude cell lysate was centrifuged at 16,000xg for 60 minutes. The pellet was washed two times and saved as the cell wall fraction. Prior to use, culture filtrates and cell lysates were normalized using the BCA™ Protein Assay Kit (Pierce).

For induction experiments bacteria were grown from frozen stock in 7H9 liquid medium with catalase to late log phase (OD_{600}≈1.2-1.5). Cultures were adjusted to OD_{600} 1.0 prior to treatment with 1 mM or 20 mM H_2O_2 . Cultures were incubated with H_2O_2 for two hours prior to processing of cell lysates. Processing of cell lysates occurred as previously described.

Immunoblot analysis of culture filtrate proteins and whole cell lysates

Culture filtrate proteins and whole cell lysates were subject to SDS-PAGE. 20 µl of normalized protein was loaded onto 4-20% gradient gel for each strain. This was followed by transfer to nitrocellulose membrane followed by visualization by chemiluminescence (BioRad) Antibodies used to probe membrane include rabbit polyclonal anti-M. marinum KatG (titer 1:2500), Rabbit polyclonal anti-Mtb SodA antibody (titer 1:2000), rabbit polyclonal antigen 85 antibody (titer 1:2000), rabbit polyclonal AhpC antibody (titer 1:500) and mouse monoclonal GroEL antibody (titer 1:50).

The SodA and Antigen 85 antibodies were obtained from Marcus Horwitz's lab at UCLA. The KatG antibody was obtained from NIH. The GroEL antibody was obtained from Colorado State University. The AhpC antibody was obtained from Dr. Steward Cole at the Institut of Pasteur.

Superoxide dismutase Activity Assay

For superoxide dismutase enzymatic assay culture filtrate was run through a non-denaturing agarose gel made up of a 15% stacking gel and a 15% separating gel at 4˚C. The culture filtrate used was not subjected to boiling and was combined with loading dye

deficient in SDS prior to loading. Once the gel was run, it was incubated for 30 minutes in a solution of 2.5 mM nitro blue tetrazolium. This was followed by a 20-minute incubation with a solution made up of 30 mM potassium phosphate, 30 mM TEMED and 30 μM riboflavin at pH 7.8. Bands were then illuminated on a light box.

Chapter 4: Discussion

Although RD1 is well documented to play a critical role in mycobacterium growth within host macrophages (Stanley, 2003), it is not understood what host defense mechanism RD1 counteracts. It is also not clearly known how the proteins are secreted by RD1 and what functions the secreted proteins have. I hypothesize that the virulence function of RD1 is achieved by secreting neutralizing enzymes, such as catalase and/or superoxide dismutase, that counteract the reactive oxygen and nitrogen intermediates produced by the host cell. This hypothesis was supported in part by my initial observation that the RD1 mutants grew slower than wild type in 7H9 medium with no supplemental catalase. The hypothesis was further supported by the observation that the RD1 mutants had significantly reduced growth compared to wild type in 7H9 medium containing 1 mM H_2O_2. These results suggested that somehow the RD1 mutants were unable to effectively neutralize H_2O_2. However, this does not exclude other possibilities. For example, one possibility is that the mutants might be less able to repair the oxidative damage by H_2O_2, another possibility is that the mutants' cell wall might be more permeable to H_2O_2. These possibilities are discussed in more detail below and elsewhere in this chapter.

In order to determine whether the mutants' defect in neutralizing ROI is due to lack of secretion of neutralizing enzymes or their synthesis within the bacterial cell, we examined the sensitivity of wild type and RD1 mutants to paraquat, a chemical that generates superoxide within the bacterial cell, which then is converted to other forms of ROIs in the bacteria. The results showed that wild type and RD1 mutants had comparable growth in the presence of paraquat, suggesting that RD1 may play a role either in the secretion of the ROI neutralizing enzymes or in maintaining the cell wall

permeability to H_2O_2. These results argue against the possibility that RD1 is involved in repairing damage caused by ROIs. Together, the above *in vitro* assays support my initial hypothesis that RD1 may be involved in the secretion of catalase and/or superoxide dismutase that participate in the neutralization of ROIs.

To further support my hypothesis, I performed macrophage cell infection studies, in which I demonstrated that RD1 played an important role in counteracting ROIs produced by the host cell. Consistent with published results, I showed that RD1 mutants had a marked defect in intracellular growth within activated BMDM as compared to wild type. Importantly, I showed that upon removal of ROIs produced by activated macrophages, using the exogenously added superoxide dismutase and catalase or NAC, RD1 mutants exhibited a significant recovery of growth, to a greater extent than wild type. The results indicate that the negative pressure that limits the intracellular growth of the RD1 mutants in part comes from ROIs suggesting that the mutants are ineffective in neutralizing ROIs produced by the host cell. The fact that the wild type did not recover to the same extent as the mutants suggests that the ROIs within the macrophages were not affecting the wild type as much as the mutants. Therefore, I conclude that RD1 genes play an important role in the neutralization of ROIs within activated macrophages.

In order to determine the source of ROIs that was involved in limiting the growth of RD1 mutants, I examined the intracellular growth of wild type and RD1 mutants in $p47^{phox-/-}$ or $gp91^{phox-/-}$ BMDM. Interestingly, the mutants did not show any growth recovery in both knockout macrophages, implicating that an NADPH oxidase-independent source of ROIs was enough to limit the mutant's intracellular growth. There are three possibilities that may account for the observations. One possibility is that an NADPH oxidase-

independent pathway produces the predominant source of ROIs that directly affect the intracellular growth of RD1 mutants within activated BMDMs. The second possibility is that the NADPH oxidase-independent source of ROIs limits the mutants' growth by an indirect mechanism. The third possibility is that the NADPH oxidase-independent source of ROIs is sufficient to react with nitric oxide to produce the highly toxic compound, peroxynitrite, which limits the intracellular growth of the RD1 mutants. Below are the in-depth discussions on those possibilities.

With respect to the possibility that an NADPH oxidase-independent pathway produces the predominant source of ROIs that directly limits intracellular growth of RD1 mutants, it has been reported that oxidases other than NADPH oxidase play a significant role in inhibiting the intracellular growth of certain pathogenic bacteria. For example, xanthine oxidase has been reported to play a role in inhibiting the dissemination of Salmonella, possibly by inhibiting the intracellular growth of the bacterium (Umezawa, 1997). Although NADPH oxidase has been considered the primary source of ROIs for phagocytes, it is not known whether it definitely plays a primary role in limiting the intracellular growth of RD1 mutants in our experiments due to the following two possible reasons. One reason is that it is not clearly understood whether NADPH oxidase generates the primary source of ROIs in BMDM during the cell activation by IFN-γ. Measuring and comparing the amount of ROIs produced by BMDMs derived from wild type and the phox-knockout mice should provide some insight into this possibility. It would also be beneficial to include the *katGΔ* as a positive control in phox$^{-/-}$ infection experiments since published data demonstrates that the *katGΔ* shows increased growth in cells lacking NADPH oxidase (Ng, 2004). In addition, it is necessary to determine the

ROI levels in the activated BMDM before and after the treatments with superoxide dismutase and catalase or NAC to in order to determine whether the ROI levels in treated BMDM are similar to those within the phox$^{-/-}$ BMDMs.

The other reason is that it is not known whether NADPH oxidase localizes to the mycobacterium-containing phagosome. Salmonella has been shown to inhibit the recruitment of the NADPH oxidase subunits to the Salmonella-containing vacuole (Vazquez-torres, 2000). Although inhibition of the recruitment of NADPH oxidase to the mycobacterium-containing vacuole has not been experimentally tested, it has been shown that mycobacterium inhibit the recruitment of iNOS to the bacteria containing phagosome (Miller, 2004). Examining the recruitment of NADPH oxidase to mycobacterium-containing phagosome should provide insight into this possibility.

With respect to the possibility that the NADPH oxidase-independent source of ROIs may limit the mutant's growth by an indirect mechanism, it is noteworthy that the cytosolic ROIs derived from mitochondria may play a role. In order to determine whether mitochondria-derived ROIs play a role in inhibiting the RD1 mutants' growth, specific inhibitors to these ROIs could be utilized. If the mitochondria-derived ROIs are the main source of ROIs to inhibit bacterial growth then the inhibition of these ROIs should allow the RD1 mutants to recover to a level similar to that seen by the inhibition of ROIs by the addition of NAC.

Mitochondria-derived ROIs are increased upon exposure to LPS and this increase is believed to trigger many signaling pathways including NFκB. Published data suggest that the addition of NAC to activated macrophages can block NFκB activation (Sim, 2005). Inhibition of NFκB may then inhibit apoptosis, which is often triggered by this

transcription factor (Liang, 2004). Thus it is possible that the addition of NAC or superoxide dismutase/catalase to BMDM would inhibit ROIs that are involved in the signaling pathways, and this inhibition would allow the RD1 mutants to recover their deficient intracellular growth in activated macrophages. This would suggest that the role of RD1 in neutralizing ROIs might be more important for the neutralization of mitochondria-derived ROIs for the purpose of inhibiting cell signaling, which possibly leads to reducing apoptosis. However our *in vitro* studies suggest that the role of RD1 in neutralizing ROIs is more important in directly inhibiting the effects of the exogenous ROIs. Therefore, it is equally possible that the mitochondria-derived ROIs may be involved in direct inhibition of the intracellular growth of RD1 mutants. Thus, RD1 may play either or both roles, one is to directly protect the bacteria by reducing the levels of ROIs in the bacteria-containing vacuole, and the other is to indirectly protect the bacteria by reducing the overall ROI level within the cell leading to dampening of the ROI-mediated signaling. If the inhibition of cell signaling were more crucial, it would be anticipated that the removal of only the proximal ROIs from the bacteria-containing vacuole via inactivation of the NADPH oxidase might not provide a significant benefit to the RD1 mutant bacteria. An important experiment to test this possibility would be to selectively inhibit the mitochondria-derived ROIs and then observe the ability of RD1 mutants to grow within these cells. Since mitochondria-derived ROIs have been linked to cell signaling, then the recovery of RD1 mutants after the removal of these ROIs would indicate that RD1 mutants are more susceptible to the effect of the ROI-mediated signaling pathways rather than the direct effect of the ROIs on the bacteria. The next step to confirm this would be to measure the signaling molecules in cells treated with the

mitochondria-derived ROI inhibitors. In order to demonstrate that the inhibition of the ROI-mediated signaling might have accounted for the growth recovery of the RD1 mutants, it would then be necessary to further examine the growth of RD1 mutants in the cells upon treatment with an inhibitor for a particular cell signaling pathway, such as an inhibitor for the NFκB signaling pathway.

To examine the possibility that the NADPH oxidase-independent source of ROIs may react with nitric oxide to produce peroxynitrite, wild type and RD1 mutants were used to infect $iNOS^{-/-}$ cells and the results showed no apparent recovery for the RD1 mutants. Therefore, RNI apparently is not involved in limiting the intracellular growth of RD1 mutants in activated macrophages. Interestingly, wild type bacteria also showed no recovery. This result is unusual since published data suggest that the removal of RNI provides a significant benefit to wild type *M. tuberculosis* (MacMicking, 1997). It is possible that *M. marinum* are less sensitive to RNI as compared to *M. tuberculosis* although further experimentation is necessary to figure this out.

Based on the results of the above experiments and the discussions, it is reasonable to conclude that RD1 genes contribute to the bacteria's ability to survive the toxic effects of the reactive oxygen intermediates both *in vitro* and in activated macrophages. However, the precise mechanism by which RD1 works in this process is not clear. I hypothesize that the RD1 secretion system is required to secrete the enzymes superoxide dismutase and/or catalase, which neutralize ROIs. My initial attempts to testing this hypothesis were unsuccessful. In those initial studies, I examined by Western blots the SodA and KatG protein levels in culture filtrates or whole cell extracts from wild type and RD1 mutants grown in the Sauton's minimal defined medium. This analysis showed that RD1

mutants were not significantly different from wild type in the synthesis of KatG and the secretion levels of the enzyme were too low to be detected, although the secretion of SodA was more prominent for the mutants than wild type. The results of this analysis were rather contradicting to the observed phenotypes of the mutants both *in vitro* and in cell infections studies. However, as will be discussed below, when the bacteria were grown in the 7H9 rich medium, the opposite phenotypes were observed compared to the Sauton's minimum defined medium. At this moment, we do not know how the different growth conditions caused the significantly different phenotypes. This will be a direction of future studies.

There is a potential problem with using the bacteria grown in the Sauton's minimum defined medium to measure enzyme secretion and synthesis. It is important to note that in all our *in vitro* and cell infection experiments; the bacteria were grown in 7H9 but not Sauton's medium, indicating that experimentation on the 7H9-grown bacteria would be more relevant to the observed phenotypes. In support of this notion, I demonstrated that wild type bacteria grown in 7H9 did produce much higher levels of KatG in the bacterial cell than the RD1 mutants grown under the same conditions. This result is consistent with the observation that the RD1 mutants are more sensitive than wild type to ROIs *in vitro* and within activated macrophages. These results suggest that RD1 genes may be involved in the production/synthesis of KatG. However, our paraquat experiment suggested the opposite, that RD1 genes likely are not involved in the intracellular levels of the ROI-neutralizing enzymes. A possible mechanism that can explain the opposite observations would be that the RD1 genes are involved in the secretion of KatG, which contributes to the resistance of wild type to the exogenous ROIs. To provide an indirect

support to this possibility, I examined the KatG levels in the bacterial cell after exposure to H_2O_2. I observed that after 2 hours of exposure to H_2O_2, wild type bacteria showed a significant reduction of its KatG level in the cell lysates by approximately 150%. On the other hand, this reduction of KatG level was not significant for the RD1 mutants. These results suggest that RD1 genes may be involved in the secretion of KatG after the exposure of wild type bacteria to exogenous ROI such as H_2O_2, consistent with all our observations in the *in vitro* and cell infection studies. A further study is imperative to examine the KatG levels in the culture medium of wild type and RD1 mutants after the induction with H_2O_2. Exposing the bacteria to H_2O_2 for longer time points and measurement of KatG expression levels at various growth stages may provide further insight into the differences between wild type and RD1 mutants in terms of KatG synthesis and secretion. Another useful experiment to establish the function of KatG secretion in contributing to intracellular growth would be to create an RD1 mutant capable of secreting KatG through an RD1 independent mechanism. This mutant should show increased survival in activated BMDM, confirming the role of KatG secretion in counteracting host defense mechanisms. The results of these studies should further establish an important unknown link between RD1 and KatG.

One possible explanation for the link between RD1 and KatG could be explained by the involvement of RD1 in a unique oxidative stress response. In *M. bovis* BCG, *ahpC* expression increases upon exposure to hydrogen peroxide. However in the pathogenic strains *M. bovis* and *M. tuberculosis*, expression of *ahpC* is constitutively lower and no induction occurs (Springer, 2001). Since BCG lacks RD1 it is possible that RD1 is involved in the negative regulation of *ahpC*. In order to pursue this hypothesis, the cell

lysates of the RD1 mutants grown in 7H9 and exposed to H_2O_2 were probed for AhpC. The results showed that RD1 mutants, but not wild type, exhibited significant upregulation of AhpC 2 hours after exposure to H_2O_2. AhpC induction is somewhat linked to avirulence, the mechanism of this AhpC-mediated attenuation is not understood. It has been shown that AhpC induction compensates for the lack of KatG in the bacterial cell (Sherman, 1996). Therefore, it is possible that the induction of AhpC in the RD1 mutants represented a deficiency of KatG, explaining indirectly why the RD1 mutants were more sensitive to the exogenous ROIs. This difference between wild type and RD1 mutants in the induction of AhpC remains an area that demands increased attention and future experimentation.

The results of the Western blot analysis of wild type and RD1 mutant KatG expression under 7H9 conditions indirectly support the hypothesis that RD1 may be involved in the secretion of KatG. While the results of SodA secretion are inconclusive, it remains a possibility that analysis of secretion under more relevant growth conditions; RD1 may be implicated in the secretion of this enzyme as well. However, previous studies suggest that both KatG and Sod A secretion is SecA2 dependant (Braunstein, 2003). The basis of this claim comes from analysis of SodA and KatG secretion by a SecA2 mutant. However, this mutant only shows a defect in SodA secretion when the culture filtrate is analyzed after 3 days and again at 7 and 14 days. However, when secretion is measured at 5 days, the mutant appears to secrete SodA. Similarly, the SecA2 mutant appears to be defective in secretion of KatG only at 3 days, when the culture filtrate is collected and probed for KatG at later time points, there is not a significant difference between mutant and wild type. The combined results clearly indicate that even mutants lacking SecA2

are able to secrete the enzymes SodA and KatG depending on their growth phase. Thus it is likely that SodA and KatG secretion are not mediated exclusively by SecA2. Therefore, it remains possible that RD1 may also play a role in the secretion of these enzymes, possibly by working in concert with the SecA2 secretion system. Further research will be necessary to establish this possible link.

Overall the results of this project are extremely promising in predicting the role of RD1 genes in virulence. The *in vitro* assays suggest a direct role for RD1 in neutralizing ROIs. The cell infection assays confirm this role, indicating that the neutralization of mitochondria-derived ROIs may be more important for bacteria than the neutralization of ROIs generated by NADPH oxidase. From what is known regarding the importance of mitochondrial ROIs in macrophage activation, it is not surprising that mycobacterium would seek to eliminate these chemicals. The mechanism by which RD1 eliminates ROIs may be through the production of KatG to the cell wall and extracellular milieu, particularly following the exposure to H_2O_2. This would suggest a novel role for the secretion system, possibly contributing to regulation as well as export of important ROI neutralizing molecules.

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