Hypersensitivity of hypoxia grown *Mycobacterium smegmatis* to DNA damaging agents: Implications of the DNA repair deficiencies in attenuation of mycobacteria

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**A B S T R A C T**

Mycobacteria are an important group of pathogenic bacteria. We generated a series of DNA repair deficient strains of *Mycobacterium smegmatis*, a model organism, to understand the importance of various DNA repair proteins (UvrB, Ung, UdgB, MutY and Fpg) in survival of the pathogenic strains. Here, we compared tolerance of the *M. smegmatis* strains to genotoxic stress (ROS and RNI) under aerobic, hypoxic and recovery conditions of growth by monitoring their survival. We show an increased susceptibility of mycobacteria to genotoxic stress under hypoxia. UvrB deficiency led to high susceptibility of *M. smegmatis* to the DNA damaging agents. Ung was second in importance in strains with single deficiencies. Interestingly, we observed that while deficiency of UdgB had only a minor impact on the strain’s susceptibility, its combination with Ung deficiency resulted in severe consequences on the strain’s survival under genotoxic stress suggesting a strong interdependence of different DNA repair pathways in safeguarding genomic integrity. Our observations reinforce the possibility of targeting DNA repair processes in mycobacteria for therapeutic intervention during active growth and latency phase of the pathogen. High susceptibility of the UvrB, or the Ung/UdgB deficient strains to genotoxic stress may be exploited in generation of attenuated strains of mycobacteria.

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1. Introduction

Mycobacteria constitute an important group of pathogens that include causative agents of tuberculosis (TB) and leprosy namely *Mycobacterium tuberculosis* and *Mycobacterium leprae*, respectively. Annually, ~1.4 million deaths occur due to tuberculosis (including people with AIDS) and 8.7 million new cases of TB are reported worldwide (Global Tuberculosis report, WHO 2012). The pathogen enters the respiratory tract and is taken up by the alveolar macrophages where it replicates. Subsequently, a robust immune response, characterized by generation of nitric oxide, and reactive oxygen species (RNI and ROS) is mounted by the host to destroy the pathogen. The RNI and ROS have high cytotoxicity due to their ability to inflict damages to DNA, protein and lipids. Due to the high G + C content in their genomes, mycobacteria are inherently sensitive to DNA damages involving deamination of cytosines and oxidation of guanines. It is noteworthy to mention that in this group of organisms, DNA mismatch repair pathway is missing (Springer et al., 2004; Davis and Forse, 2009). Therefore, the base excision repair (BER) and nucleotide excision repair (NER) pathways may be more critical to safeguard DNA under variety of genotoxic stress. Deficiencies of BER, and NER affect survival of mycobacteria in animal models (Sassetti and Rubin, 2003; Darwin and Nathan, 2005; Dutta et al., 2010). Further, double stranded DNA break repair pathways (non-homologous end joining, homologous recombination, and single strand annealing) have been found important in mycobacteria (Della et al., 2004; Rachman et al., 2006, Schnappinger et al., 2003; Stephanou et al., 2007; Gupta et al., 2011).

One of our long-standing interests has been to understand the importance of the base excision repair pathways in mycobacteria, more specifically, the uracil excision repair pathway (Parmapatre and Varshney, 1998; Venkatesh et al., 2003, Srinath et al., 2007; Malshetty et al., 2010) using *Mycobacterium smegmatis* as model. We reported that loss of Ung in the non-pathogenic *M. smegmatis* led to increased mutation rates and susceptibility to RNI (Venkatesh et al., 2003; Kurthkoti et al., 2010). Subsequently, a family V uracil DNA glycosylase was predicted in mycobacteria (Sartori et al., 2002). This protein encoded by rv1259 (now named UdgB) was biochemically characterized. It was shown that in addition to its ability to act on uracil, the enzyme displayed...
broader substrate specificity capable of excising hypoxanthine, and ethenocytosine (Srinath et al., 2007). Such a broad specificity may be crucial for bacterial survival considering the fact that the macrophage environment is potent enough to inflict multiple DNA lesions on the bacterial genome.

One of the hallmarks of human tuberculosis is its latency. During the course of infection, the host mounts a strong immune response characterized by generation of nitric oxide and ROS to kill the bacteria. However, under these conditions, the bacterium stops replicating and enters into a quiescent state. During the later stages of the immune response, cells from the host form a fibrotic mass called granuloma surrounding the bacteria. In addition to RNI and ROS stress, the bacteria experience nutritional and oxygen limitation in the granuloma and but remain viable in a metabolically sedentary state, a condition referred to as latency.

With one third of the human population being latently infected with M. tuberculosis, it becomes necessary to understand the physiology of bacteria in its latent or dormant state. More importantly, conditions that compromise the immune system such as aging or infection with HIV, lead to reactivation of dormant bacteria within the granuloma causing active disease. Unfortunately, it is extremely difficult to replicate the exact conditions of granuloma for studying the bacterial physiology. However, culturing bacteria in sealed containers that lead to a gradual anaerobiosis establishes dormancy in M. tuberculosis (Wayne and Hayes, 1996). These bacteria subjected to hypoxia display features such as persistence without multiplication, increased tolerance to drugs, etc. (Wayne and Hayes, 1996). The Wayne's model of hypoxia has been widely used to understand changes in bacterial physiology during dormancy (Muttucumaru et al., 2004).

Using the Wayne's model of hypoxia, we reported that loss of DNA repair especially the NER and uracil excision repair pathways significantly affected bacterial survival (Kurthkoti et al., 2008). Similarly, loss of UvrA in M. smegmatis led to its reduced survival during starvation and hypoxia (Cordone et al., 2011). Further, we observed that under hypoxic condition expression of DNA repair enzymes was down-regulated (Kurthkoti and Varshney, 2010). Interestingly, subjecting these bacteria to aerobic condition (recovery) resulted in restoration of expression of these enzymes. Hence, we questioned if mycobacteria become more susceptible to DNA damaging agents during hypoxia. In the present work, we have compared the survival of M. smegmatis mutants when subjected to genotoxic stress (hydrogen peroxide and acidified sodium nitrite) under aerobic, hypoxic and recovery conditions. Our results show that there is increased susceptibility of DNA repair deficient (particularly the UvrB, Ung and Ung/UdgB) bacteria to genotoxic stress under hypoxia conditions and reveal an interdependence of different DNA repair pathways in safeguarding genomic integrity.

2. Materials and methods
2.1. Strains, media and growth conditions

The details of various strains generated from M. smegmatis mc2155 (Snapper et al., 1990) are provided in Table 1. Glycerol stocks of M. smegmatis strains were streaked on TH10 medium (Difco) containing 0.5% (v/v) glycerol and 0.05% Tween 80, and grown in LB containing 0.2% (v/v) Tween 80 (LB) or Middlebrook 7H9 (Difco) containing 0.2% glycerol and 0.2% Tween 80. For growth on solid surfaces, 1.3% agar was included in the media. Media were supplemented with hygromycin (Hyg) and kanamycin (Kan) at 50 μg ml⁻¹, respectively, as needed. For hypoxia, cultures were grown in Dubos medium containing 5% (v/v) glycerol and 0.2% Tween 80 together with 10% ADC.

2.2. Growth of M. smegmatis strains under aerobic conditions, and effect of hydrogen peroxide and acidified sodium nitrite

 Cultures from isolated colonies of M. smegmatis strains were grown in triplicate in 7H9 medium with appropriate antibiotics to saturation and, inoculated at 1% in Dubos medium (with 10% ADC and appropriate antibiotics), grown overnight (~OD₅₆₀ of 0.5–0.7; ~2.0–3.5 × 10⁸ cfu ml⁻¹) and centrifuged at 8000 rpm in S54 rotor (Kubota 3700) for 2 min to pellet the cells. To study the effect of hydrogen peroxide, cells were resuspended in phosphate buffered saline (PBST, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, and 0.2% Tween 80; pH 7.0), at OD₅₆₀ of ~0.2, aliquots from this were either not supplemented or supplemented with desired concentration of hydrogen peroxide. At different time intervals, aliquots were withdrawn, serially diluted and plated in triplicate to determine total viable counts. To examine the effect of acidified sodium nitrite, the same procedure was followed except that the harvested cells were resuspended in PBST adjusted to pH 5.5, and the aliquots were either not supplemented or supplemented with various concentrations of sodium nitrite.

2.3. Growth of M. smegmatis strains under hypoxia, and effect of hydrogen peroxide and acidified sodium nitrite

 Cultures in Dubos medium (with 10% ADC and appropriate antibiotics) were grown to an OD₅₆₀ of 0.5–0.7 as described above, and diluted 1:100 in screw-cap flat-bottom culture tubes in 20 ml of the same medium containing methylene blue (1.5 μg ml⁻¹) as an indicator of oxygen depletion and 10 ml of air space (head space ratio 0.5; Dick et al., 1998). The tubes were sealed and subjected to slow stirring using a multipoint magnetic rod at 37 °C for 10 days. Oxygen depletion was noted by de-coloration of methylene blue (blue to colorless) in the tubes. To investigate the impact of genotoxic stress, cells from cultures (at the end of hypoxic growth) were harvested directly (hypoxia cultures) or after opening the caps under sterile condition and vigorously aerating the cultures for 1 h at 37 °C (recovery cultures). The recovery cultures were marked by re-appearance of blue color. Harvested cells were resuspended in PBST, as above (for aerobic conditions) for examining the effect of hydrogen peroxide and acidified sodium nitrite at desired concentrations.

3. Results

3.1. Experimental design

In our earlier studies (Kurthkoti et al., 2008, Kurthkoti et al., 2010, Malshetty et al., 2010), we used growth curve analysis assays in the absence or presence of hydrogen peroxide or acidified sodium nitrite under aerobic conditions of growth to study the impact of various DNA repair function deficiencies. We observed that compared to the mc2155 (L5 att::kan) strain (referred to as wild-type), strains deficient for Ung, Fpg, UvrB, or simultaneously for Ung and UdgB (ung, fpg, uvrB, ung/udgB, respectively) were

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**Table 1**

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Relevant details</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>M. smegmatis (or mc²155)</td>
<td>mc²155, a high-efficiency transformation strain.</td>
<td>Snapper et al., 1990</td>
</tr>
<tr>
<td>mc²155 L5att::kan (or WT)</td>
<td>mc²155 harboring pKB20 (kan⁴) at the L5 att site in the chromosome</td>
<td>Venkatesh et al., 2003</td>
</tr>
<tr>
<td>uvrB :: kan (or uvrB)</td>
<td>mc²155 where uvrB (MSMEG_3816) has been disrupted with kan cassette</td>
<td>Kurthkoti et al., 2008</td>
</tr>
<tr>
<td>fpg :: hyg (or fpg)</td>
<td>mc²155 where fpg has been replaced with hyg cassette</td>
<td>Jain et al., 2007</td>
</tr>
<tr>
<td>ung :: kan (or ung)</td>
<td>mc²155 where ung has been disrupted with kan cassette</td>
<td>Venkatesh et al., 2003</td>
</tr>
<tr>
<td>mutY :: kan (or mutY)</td>
<td>mc²155 where mutY has been disrupted with kan cassette</td>
<td>Kurthkoti et al., 2010</td>
</tr>
<tr>
<td>udgB :: hyg (or udgB)</td>
<td>mc²155 where udgB (MSMEG_3816) has been disrupted with hyg cassette</td>
<td>Malshetty et al., 2010</td>
</tr>
<tr>
<td>ung :: kan/udgB :: hyg (or ung/udgB)</td>
<td>mc²155 where ung and udgB have been disrupted with kan and hyg cassette, respectively</td>
<td>Malshetty et al., 2010</td>
</tr>
</tbody>
</table>
sensitive to DNA damaging agents to various extents. However, strains lacking MutY or UdgB activities (mutY and udgB, respectively) generally showed a minimal effect in these experiments. To obtain a better assessment of the relative sensitivities of the strains to hydrogen peroxide (to generate ROS) and acidified sodium nitrite (to generate nitrous acid which decomposes to nitric oxide and other forms of RNI, O’Brien et al., 1994) we performed comparative analysis of all the strains available with us, using a more quantitative assay of survival curves. In addition, we examined the effect of hydrogen peroxide, and acidified sodium nitrite on the survival of the strains grown in hypoxia, as well as those subjected to recovery under aerobic conditions (Fig. 1).  

3.2. Susceptibility of M. smegmatis strains deficient in different DNA repair proteins to hydrogen peroxide

As shown in the time course of the impact of 2.5 mM hydrogen peroxide on aerobically grown cells (Fig. 2A), the uvrB and the double knockout (ung/udgB) strains were most susceptible to the peroxide treatment (Fig. 2A, curves 6 and 7); ung, udgB, mutY and fpg strains showed intermediate sensitivity (curves 2–5) while the wild-type strain (curve 1) showed the least sensitivity. As a control, in strains untreated with oxidative stress (UT, curves 1–7), uniform viability was observed in PBST over the duration of the experiment. Subjecting the bacterial strains to hypoxia led to their increased susceptibility (including the wild-type strain) to peroxide (compare the viable counts at the end of 2 h and 3 h in Fig. 2B, panels i–iii representing treatments with 0.5, 1.5 and 2.5 mM hydrogen peroxide, respectively with Fig. 2A). The observation that even the wild-type strain is more sensitive to killing by peroxide treatment is consistent with our report (Kurthkoti and Varshney, 2010) that during hypoxia, many of the DNA repair functions are down-regulated. Further, consistent with the observation of restoration of DNA repair functions upon subjecting the hypoxia grown cultures to aerobic conditions (Kurthkoti and Varshney, 2010), treatment of the recovery cultures showed decreased killing of the strains by hydrogen peroxide (compare Fig. 2C with Fig. 2B, panel iii).  

3.3. Effects of acidified sodium nitrite on survival of M. smegmatis strains deficient in different DNA repair proteins

Under acidic conditions, sodium nitrite forms nitrous acid, which decomposes to give rise to various forms of RNI. RNI in turn
causes deamination of bases in DNA, and also reacts with oxygen to form peroxynitrite which causes oxidative damage to DNA. The results of the impact of acidified sodium nitrite treatment for 3 h are shown in Fig. 3. Under the aerobic conditions, similar to the peroxide treatment, the ung/udgB and uvrB strains showed maximum susceptibility (Fig. 3A, curves 6 and 7). The ung strain showed intermediate effect (curve 4). However, the udgB, fpg and mutY strains showed least effect (compare curves 2, 3, and 5 with 1). As shown in Fig. 3B, when the strains were subjected to hypoxia, they became hypersensitive to acidified sodium nitrite. For example, while under aerobic conditions (Fig. 3A), viable counts could be recovered in most strains (except uvrB) even at 10 mM
concentration of the reagent, in hypoxia cultures, even a concentration of 2.5 mM reagent was highly toxic (Fig. 3B). And, as was the case for peroxide treatment, compared to the hypoxia cultures, the recovery cultures showed better tolerance to acidified sodium (compare Fig. 3C with Fig. 3B). As a control, nitrite untreated cells (UT, curves 1–7) remained fully viable under the experimental conditions, suggesting that the killing of the various strains was due to the genotoxic stress of the acidified nitrite.

For a better understanding of the impact of the acidified nitrite, we repeated the survival curves for hypoxia cultures at lower concentrations of the nitrite (1, 2 and 3 mM) and followed viability at different time points (1, 2 and 3 h). As shown in Fig. 4, even at a concentration of 1 mM nitrite (panel i), the udgB/ung and the uvrB strains showed maximal sensitivities. Expectedly, the killing of the strains increased with increasing concentration of the reagent (panels ii and iii).

3.4. Important roles of UvrB, Ung and UdgB in DNA repair in mycobacteria

Based on the observation that UvrB deficiency (uvrB strain) led to high susceptibility of M. smegmatis to the DNA damaging agents (Figs. 2–4), and thus among the pathways studied, the nucleotide excision repair pathway is the most crucial repair pathway in maintenance of the genomic integrity in mycobacteria. Single deficiencies of MutY (mutY strain), Fpg (fpg strain) had the minimal effects on the susceptibility of the strains under the conditions tested. For Fpg, there are multiple orthologs in mycobacteria (Davis and Forse, 2009, Sidorenko et al., 2008; Guo et al., 2010), which may serve as back-up for Fpg. It is unknown if there are any back-up activities for MutY. However, Ung deficiency, even as a single deficiency (ung strain) does confer a significant susceptibility to M. smegmatis, at least under the conditions of acidified nitrite (Figs. 3 and 4). In these assays, the impact of UdgB deficiency, as a single deficiency, does not strike as a significant one. Intriguingly, however, when combined with Ung deficiency it results in a severe consequence on the survival of the resulting strain (ung/udgB strain) when exposed to DNA damaging agents (Figs. 2–4, curve 6). UdgB is known to excise uracil, hypoxanthine and ethenocytosine from DNA (Srinath et al., 2007). Thus, a possible interpretation of this observation is that when the backup uracil excision activity is knocked out, the presence of uracil in the genome becomes highly toxic. In addition, accumulation of hypoxanthine (deamination product of adenosine)/other damaged bases in DNA, UdgB excises may contribute to the observed toxicity.

4. Discussion

The ability to repair damaged DNA forms a crucial component of cellular machinery. Especially, in the pathogens that are constantly exposed to reactive environments, successful repair of damaged DNA may determine their virulence. M. tuberculosis is a pathogen that is constantly exposed to myriad of DNA damaging stress within the host macrophage. Using a non-pathogenic model M. smegmatis we have earlier reported the importance of DNA repair proteins in survival of the bacteria under conditions that mimic ROS and RNI stresses experienced by the pathogen in the host macrophages (Venkatesh et al., 2003, Kurthkoti et al., 2008). In the present study, we have employed survival curves (which provide a better assessment of the strains susceptibility to DNA damaging stress) to investigate the relative importance of the various DNA repair pathways. These studies have, from among the strains tested, identified nucleotide excision repair pathway as the most important repair pathway in mycobacteria (Kurthkoti et al., 2008). Earlier studies showed compromised survival of UvrB mutant of M. tuberculosis in mouse competent to produce nitric oxide (Darwin and Nathan, 2005). This observation suggests that DNA damages induced by RNI are detrimental to the pathogen and
NER contributes to the repair of such damages. In our studies, the next important repair pathway is represented by the uracil excision repair pathway. In fact, a combination of Ung and UdgB deficiencies severely compromise strain viability under the DNA damaging conditions of hydrogen peroxide and or acidic nitrite.

Importance of NER may not only be for its activity in direct recognition of the DNA lesions and their repair but also in repairing the lesions recognized by other DNA repair proteins. Under conditions of excessive DNA damage by various forms of genotoxic stress, NER may be of general importance. For example, it was reported that an alkyltransferase like (ATP) protein (in yeast and bacteria) binds to O6-methylguanine or O4–(3-pyridyl)–4-oxo-butyguanine but cannot excise it. Interestingly, complex formation of ATP with the damaged base distorts the DNA structure and facilitates recruitment of NER proteins for damage repair (Tubbs et al., 2009; Latypov et al., 2012).

Based on our earlier observation, DNA repair functions are down regulated during growth in hypoxia (Kurthkoti and Varshney, 2010). Hence, we reasoned that bacterial strains grown under hypoxic conditions (such as those experienced by the pathogenic M. tuberculosis during its latent state in the host) may be more susceptible to DNA damaging agents. Indeed, we observed that susceptibility of even the wild-type strain to DNA damaging agents was significantly enhanced upon growth in hypoxia (compared to cells grown in aerobic conditions) under conditions of ROS and RNI stress. Of particular interest are the udgB and mutY mutants that had shown only mild effects in earlier growth curve experiments, showed substantial increase in their sensitivities to RNI/ROS stress under hypoxia. We had earlier observed that expression of UdgB and MutY are down regulated in hypoxia but are restored during recovery in aerobic growth conditions (Kurthkoti and Varshney, 2010). These observations raise a possibility of these proteins being more important during certain stressful conditions. Another point of consideration is the fact that both UdgB and MutY are iron sulfur cluster containing proteins. Sequestering iron by these proteins and/or transitions of the oxidation states of iron in the iron sulfur cluster may provide these proteins with yet another mechanism to regulate aspects of DNA repair.

Taken together our present data and those reported earlier (Kurthkoti et al., 2008) suggest that targeting DNA repair pathway may not only control actively dividing bacteria but also potentially control the non-replicating bacteria. Indeed a lead compound PA-824 belonging to the class of bicyclic nitroimidazole shows potency against non-replicating bacteria through its ability to generate nitric oxide (Singh et al., 2008). Further, a chemical molecule 2-(5-amino-1,3,4-thiadiazol-2-yl-benzo[f]chromen-3-one) (ATBC) which inhibits the incision activity of NER complex has been identified (Mazloum et al., 2011). Also, it appears that oxidative damage on DNA could facilitate bacterial killing brought about by antibiotics (Foti et al., 2012). These findings reinforce the idea of using DNA repair pathways as new drug targets. Finally, our studies reinforce the idea of use of DNA repair mechanisms as a potential target for therapeutic intervention for inhibition of mycobacterial growth on one hand and exploitation of the DNA repair deficient strains in generation of attenuated strains to modulate immune responses of the host on the other.

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References


