Experimental Evolution Reveals Redox State Modulates Mycobacterial Pathogenicity

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Understanding how *Mycobacterium tuberculosis* has evolved into a professional pathogen is helpful in studying its pathogenesis and for designing vaccines. We investigated how the evolutionary adaptation of *M. smegmatis* mc²51 to an important clinical stressor H₂O₂ allows bacteria to undergo coordinated genetic mutations, resulting in increased pathogenicity. Whole-genome sequencing identified a mutation site in the *fur* gene, which caused increased expression of katG. Using a Wayne dormancy model, mc²51 showed a growth advantage over its parental strain mc²155 in recovering from dormancy under anaerobic conditions. Meanwhile, the high level of KatG in mc²51 was accompanied by a low level of ATP, which meant that mc²51 is at a low respiratory level. Additionally, the redox-related protein Rv1996 showed different phenotypes in different specific redox states in *M. smegmatis* mc²155 and mc²51, *M. bovis* BCG, and *M. tuberculosis* mc²7000.

In conclusion, our study shows that the same gene presents different phenotypes under different physiological conditions. This may partly explain why *M. smegmatis* and *M. tuberculosis* have similar virulence factors and signaling transduction systems such as two-component systems and sigma factors, but due to the different redox states in the corresponding bacteria, *M. smegmatis* is a nonpathogen, while *M. tuberculosis* is a pathogen. As mc²51 overcomes its shortcomings of rapid removal, it can potentially be developed as a vaccine vector.

Keywords: mycobacterial pathogenicity, KatG, Fur, H₂O₂ resistant, TB

INTRODUCTION

Tuberculosis (TB) is caused by the pathogen *Mycobacterium tuberculosis* and remains a public health threat, resulting in 1.4 million deaths in 2020 (WHO, 2021). The prevalence of multidrug-resistant *M. tuberculosis* and the rising cases of co-infection with HIV increase this health concern. The World Health Organization (WHO) has estimated that a quarter of the world’s population is infected with *M. tuberculosis* (WHO, 2021). This latent state may be extended as long as the life of the infected host, but unfortunately, the reactive rate is approximately 5–10% of infected individuals (Flynn and Chan, 2001). TB is treated with chemotherapy, and the latent state of mycobacteria prolongs the time of treatment, which is one of the causes for the development of mycobacterial resistance.

As one of the world’s most successful human pathogens, *M. tuberculosis* has evolved elegant strategies to escape the immune defensive system of the host. For example, D’Arcy et al. observed that *M. tuberculosis*-containing phagosomes do not fuse with the lysosome inside infected macrophages (Brown et al., 1969; Armstrong and Hart, 1971). Several studies have indicated that *M. tuberculosis*...
being an intracellular pathogen is partially due to its ability to survive and persist in macrophages, in hostile environments with oxidative stress, in low pH, and under starvation and other stresses (Cohen et al., 2018; Nauseef, 2019). When *M. tuberculosis* infects macrophages, mycobacteria must overcome exogenous reactive oxygen species (ROS), a classical innate defense mechanism against infection (Pieters, 2008). In addition, during latency, *M. tuberculosis* continues to be exposed to oxidative stress, and thus, the accumulation of mutations caused by oxidative DNA damage is predicted as a potential risk for resistance to antibiotics (Ford et al., 2011). Clinical investigation has shown that cells from TB patients produce less ROS than those of healthy individuals (Jaswal et al., 1992; Kumar et al., 1995). In addition, ROS can also be associated with the treatment of TB (Piccaro et al., 2014; Hu et al., 2021). To avoid ROS attack, an evolved detoxified system is essential for *M. tuberculosis* survival, persistence, and subsequent reactivation (Piccaro et al., 2014).

As a mycobacterial model of *M. tuberculosis*, *M. smegmatis* has contributed to understanding the functions of mycobacteria (Aldridge et al., 2012; Kieser et al., 2015; Gray et al., 2016). The essential genes in *M. tuberculosis* have a corresponding ortholog gene in *M. smegmatis* (Dragset et al., 2019; Judd et al., 2021). *M. smegmatis* mc²155 and *M. tuberculosis* H37Rv share 2547 mutually orthologous genes (Dragset et al., 2019). At least under certain conditions, *M. tuberculosis* and *M. smegmatis* have similar growth mechanisms. The assumed virulence factors identified in *M. tuberculosis* such as PhoPR and DosR/S/T (Gonzalo-Asensio et al., 2014; Mehra et al., 2015) are also present in *M. smegmatis*. However, compared to virulent *M. tuberculosis*, *M. smegmatis* is a nonpathogenic mycobacterium. *M. tuberculosis* can persist in the infected host, while the host can quickly remove *M. smegmatis* (Anes et al., 2003; Anes et al., 2006). Considering that *M. tuberculosis* with high resistance to hydrogen peroxide (H₂O₂) persists in the lungs, while *M. smegmatis* with lower resistance to H₂O₂ exists in the soil, we hypothesized that the redox state of *M. tuberculosis* and *M. smegmatis* may adapt to the corresponding redox environment. The differences between the two bacteria are due to their corresponding degree of resistance to H₂O₂. To test this hypothesis, we selected a series of H₂O₂-resistant mutant strains using a clinically important stressor H₂O₂ and identified the highly H₂O₂-resistant mycobacterial strain mc²51 (Li et al., 2014b). Compared to the wild-type *M. smegmatis* mc²155 strain, the minimum inhibitory concentration (MIC) of H₂O₂ was more than 80-fold higher, while the MIC of mc²51 to H₂O₂ was 3.125 mM, similar to that of *M. bovis* BCG (0.625 mM) (Li et al., 2014a) and *M. tuberculosis* (0.625 mM), while that of mc²155 was 0.039 mM (Li et al., 2014b). mc²51 exhibited a slow growth rate similar to *M. tuberculosis*.

In this study, we first showed that H₂O₂-resistant mc²51 had a growth advantage both in mice and macrophages compared with wild-type mc²155, which indicated that the higher resistance to H₂O₂ of mycobacteria is related to higher virulence. Similar to *M. tuberculosis* that can survive under hypoxia in the lungs and resuscitate under appropriate conditions, mc²51 presented a growth advantage to recover from dormancy using the Wayne dormancy model. Furthermore, we showed that Fur mutant carrying the A28V point mutation dysregulated katG levels, which was the main cause for resistance to H₂O₂ and low levels of ATP. Additionally, the redox-related protein Rv1996, responsible for regulating gene expression, exhibited different phenotypes associated with isoniazid susceptibility in *M. smegmatis* mc²155 and mc²51, *M. bovis* BCG, and *M. tuberculosis* mc²7000. Thus, the same protein presents different phenotypes under different physiological conditions. Our results suggest that the difference in the corresponding redox status causes the difference in pathogenicity between *M. tuberculosis* and *M. smegmatis*.

## RESULTS

### The Mycobacterium tuberculosis-Like *M. smegmatis* Mutant Strain mc²51 Displayed Improved Virulence

Previous studies showed that *M. smegmatis* with the gain of H₂O₂ resistance, named mc²51, improves growth fitness in mycobacteria under stress (Table 1) (Li et al., 2014a; Li et al., 2014b). Prior to selecting H₂O₂-adapted mycobacterial mutants, we first measured the MIC of wild-type mc²155 to H₂O₂, which was 0.039 mM. We used 0.0293 mM H₂O₂ for the initial screening, which was lower than the MIC of mc²155 to H₂O₂ (Table 1). The whole process was performed as follows: cultures were started from glycerol-frozen stocks and grown to log phase (OD₆₀₀ of 0.6–0.8). Then, the culture was diluted 1,000 times and grown under 0.0293 mM H₂O₂ until the OD₆₀₀ reached logarithmic (log) phase. Cultures were then further diluted 1:1,000, and an additional 0.0293 mM of H₂O₂ was added to the culture. This process was repeated until the H₂O₂ concentrations reached 0.4395 mM. In further rounds of culture, H₂O₂ was added in steps of 0.0879 mM instead of 0.0293 mM until a concentration of 1.5 mM was reached (Supplementary Figure S1; Supplementary Table S1). The actual MIC of H₂O₂ of the mutant strain, named mc²51, selected at 1.5 mM, was 3.125 mM. In a previous study, the mutated mycobacterial strain, mc²51, evolved into an *M. tuberculosis*-like strain that presented slow growth and improved growth fitness under stress (Li et al., 2014b), which H₂O₂ resistance in mycobacteria linked to virulence. Previous studies including ours have associated isoniazid (INH) with H₂O₂ resistance (Timmins and Deretic, 2006; Hu et al., 2021). The MIC of isoniazid (INH) for mc²51 was dramatically reduced to ~1% of the MIC for mc²155 (Table 1). To test our hypothesis that the H₂O₂-resistant strain mc²51 would be more persistent in the host than its parental strain mc²155, we performed non-invasive intranasal infections, which can induce respiratory mucosal immune responses and is a promising way of vaccination for respiratory infection diseases. As shown in Figure 1A, intranasal infection of C57BL/6 mice with ~1 x 10⁶/50 μl and bacterial colony-forming units (CFUs) were counted 1 day after infection.
infection. Compared to mc²155 with the percentage of survival in the infected lung of 0.03653 ± 0.01462% (n = 3), the mc²51 strain with that of 0.7153 ± 0.2597% (n = 3) has significantly higher survival percentage (p = 0.0451) (Figure 1B). After infection with live mycobacteria, the bacilli enter alveolar macrophages (AMΦs) and persist within them. To exclude the possibility that the higher CFUs were caused by the larger number of infected bacilli being captured by AMΦs, macrophage-killing assays were explored using the THP-1 cell line. The survival percentage of mutant mc²51 is 0.3158 ± 0.1502 (n = 9), while the survival of wild-type mc¹⁵⁵ was 0.01921 ± 0.01223 (n = 6) (p = 0.0003) (Figure 1C). These results indicated that mc²51 exhibited enhanced virulence.

Mutant fur Altered the Intracellular Redox State and Was Conductive to Latency and Resuscitation via Modulation of KatG Levels

*M. tuberculosis* has evolved to survive in hypoxic conditions. Over more than 100 years of research, *M. tuberculosis* has been confirmed to be an obligate aerobic bacterium that cannot replicate under hypoxic conditions. However, *M. tuberculosis* has incredible survivability in long-term anaerobic environments.

### TABLE 1 | List of bacteria in this study.

| Strains        | Genotype or relevant characteristics                      | Source or references | H₂O₂ (mM) | INH (µg/ml) |
|----------------|----------------------------------------------------------|---------------------|-----------|-------------|
| mc²155         | *Mycobacterium smegmatis*                                | Snapper et al. (1990) | 0.099     | 10          |
| mc²51          | *Highly H₂O₂-resistant Mycobacterium tuberculosis-like Mycobacterium smegmatis* | Li et al. (2014a); Li et al. (2014b) | 3.125     | 0.1         |
| ΔkatG          | *Mycobacterium smegmatis ΔkatG::hygR*                   | This study          | N/A       | N/A         |
| pMV261-katG/mc²155 | *Mycobacterium smegmatis harboring pMV261-katG*               | This study          | N/A       | N/A         |
| mFur/mc²155    | *Mycobacterium smegmatis mFur at the T28A site*          | This study          | 0.64      | N/A         |
| pMV261/mc²1996/mc²155 | *Mycobacterium smegmatis harboring pMV261-rv1996, KanR* | This study          | N/A       | 2.5         |
| pMV261/mc²51   | *Highly H₂O₂-resistant Mycobacterium smegmatis harboring pMV261 KanR* | This study          | N/A       | 10          |
| pMV261/mc²51   | *Highly H₂O₂-resistant Mycobacterium smegmatis harboring pMV261-rv1996, KanR* | This study          | N/A       | 0.1         |
| ΔkatG          | *Mycobacterium smegmatis ΔkatG::hygR*                   | This study          | N/A       | 0.1         |
| pMV261/BCG     | *Mycobacterium bovis BCG Pasteur harboring pMV261, KanR* | This study          | N/A       | 0.05        |
| pMV261-rv1996/BCG | *Mycobacterium bovis BCG Pasteur harboring pMV261-rv1996, KanR* | This study          | N/A       | 0.025       |
| pMV261/mc²7000 | *Mycobacterium tuberculosis ΔpanCD harboring pMV261, KanR* | This study          | N/A       | 0.05        |
| pMV261/mc²7000 | *Mycobacterium tuberculosis ΔpanCD harboring pMV261-rv1996, KanR* | This study          | N/A       | 0.05        |
| pMV261/Δmc²7000 | *Mycobacterium tuberculosis ΔpanCD harboring pMV261, KanR* | This study          | N/A       | 0.05        |
| pMV261/BCG     | *Mycobacterium bovis BCG Pasteur harboring pMV261, KanR* | This study          | N/A       | 0.025       |
| pMV261-rv1996/BCG | *Mycobacterium bovis BCG Pasteur harboring pMV261-rv1996, KanR* | This study          | N/A       | 0.025       |
| pMV261/Δmc²7000 | *Mycobacterium tuberculosis ΔpanCD harboring pMV261-rv1996, KanR* | This study          | N/A       | 0.05        |

N/A: not available.
Evidence suggests that *M. tuberculosis* has the ability to reduce the respiratory system to low levels and maintain vitality (Loebel et al., 1933). Due to respiratory depression, ATP is maintained at low levels, which guarantees minimal metabolic activity to ensure membrane integrity under hypoxic conditions. We first compared the survival of mc2^151 and mc2^51 strains under hypoxic conditions. We established a Wayne dormancy model (Wayne and Hayes, 1996), consisting of mycobacterial strain cultures grown in the 7H9 medium to an OD_{600} of 1.0 with a headspace ratio of 0.5. The cultures were stirred at 120 rpm. Methylene blue (1.5 mg/L) was used as an oxygen tension indicator. Methylene blue changes from blue to colorless under reducing conditions. Data present results of three biological replicates. Growth rates of strains mc2^151 (navy blue) and mc2^51 (brown) were measured by measuring the OD_{600} (B) and by determination of CFUs (C) after plating on 7H10. Data are presented as the mean ± standard deviation of three independent replicates. **p < 0.01. (D) The H$_2$O$_2$-resistant mutant strain mc2^51 shows a growth advantage of recovering from dormancy in an anaerobic state over mc2^151. After the indicator methylene blue in the culture of the Wayne dormancy model became colorless, cultures of mc2^151 (navy blue) or mc2^51 (brown) were collected and reinoculated into 7H9 combined with the heart infusion medium at an OD_{600} of 0.01 and aerobic shaken at 200 rpm. The CFUs were determined 20 h after inoculation. Data are presented as the mean ± standard deviation of three independent replicates. ****p < 0.0001.
FIGURE 3 | ATP content in mc²155 is similar to that in pMV261-katG/mc²155. (A) The detection of ATP content in mc²155, ΔkatG, and pMV261-katG/mc²155. The cultures of mc²155 (navy blue), pMV261-katG/mc²155 (brown), and ΔkatG (olive drab) at an OD₆₀₀ of 0.8 were collected. ATP levels were measured in relative light unit (RLU) using a Cytation 3 Cell Imaging Multi-Mode Reader. The corresponding ATP concentrations were calculated according to the ATP standard curve and further converted to μM/mg protein. Data are presented as the mean ± standard deviation of three independent replicates. ***p < 0.001. (B) The intracellular ATP content of mc²51 and mc²155. The cultures of mc²155 (navy blue) or mc²51 (brown) at an OD₆₀₀ of 0.8 were collected. ATP levels were measured in relative light unit (RLU) using a Cytation 3 Cell Imaging Multi-Mode Reader. The corresponding ATP concentrations were calculated according to the ATP standard curve and further converted to μM/mg protein. Data are presented as the mean ± standard deviation of three independent replicates. **p < 0.01.

FIGURE 4 | A28V Fur mutant protein decreased DNA binding to the fur promoter. (A) Genetic organization of the fur-katG and the schematic diagram of Fur negative regulation of katG (upper panel). Genetic organization of the mfur-katG and the schematic diagram of mFur resulted in derepression of katG with increasing katG mRNA (bottom panel). The red line indicates the fur promoter DNA fragment. (B) Electrophoretic mobility shift assays (EMSA) of the binding of Fur/mFur protein to the fur promoter DNA fragment. Purified SMSEG_2415 protein (2415), unrelated to Fur, expressed in E. coli, was run in the first lane of a 4–20% polyacrylamide gel. Gel shift caused by Fur (lane 2) and mFur (lane 3) is shown. The image shown is representative of at least three experiments.
the abundance of ATP in mc²51 (0.1885 ± 0.0481 μM/mg) was lower than that of mc²155 (0.8138 ± 0.1324 μM/mg) (Figure 3B).

We previously performed whole-genome sequencing to compare differences in mc²155 and mc²51 at the genome level. Whole-genome sequencing revealed that there were 29 single-nucleotide polymorphisms (SNPs) in mc²51. All 29 SNPs were cloned, and each was transformed into mc²155 strains, and only the fur (msmeg_3460), located upstream of msmeg_3461, encoding KatG (Figure 4A), could restore the resistance phenotype to H₂O₂ (Li et al., 2014b). The fur-encoded protein Fur negatively regulated katG expression (Pym et al., 2001). The A28V Fur mutation (mFur) in mc²51 may also affect the expression of katG. To verify this hypothesis, we examined the binding of mFur to the target DNA (the promoter region of the fur) using electrophoretic mobility shift assays (EMSAs). Compared to wild-type Fur protein, EMSA showed that mFur decreased DNA binding (Figure 4B), which resulted in the katG transcription dysregulation by mFur. In addition, the RNA of mc²155 and mc²51 was extracted and quantified. The results showed that compared to the mc²155 strain, the expression of the catalase-peroxidase (KatG) encoding gene katG of the mc²51 strain was significantly upregulated to ~61.82-fold that of the wild-type strain. Taken together, mFur increases the KatG protein level in mc²51. KatG is a dual enzyme for catalase and peroxidase, which hydrolyzes ROS (Ng et al., 2004). Thus, the mc²51 may maintain ATP at lower levels through KatG, compared to mc²155 levels; that is, the abundance of KatG may affect the mycobacterial redox state and, thus, change the susceptibility to H₂O₂. We then constructed the ΔkatG (mc²155 with knockout katG) and pMV261-katG/mc²155 (mc²155 with overexpression of katG) strains, and their respective ATP content was tested. As shown in Figure 3A, the KatG level negatively correlated with the ATP level. Furthermore, we constructed the specific site mutant of the fur gene (mfur) in wild-type mc²155 using an amino acid change of A28V of Fur (Figures 5A,B) by using recombinase protein gp61 from Che9c mycobacteriophage (van Kessel et al., 2008), to construct mc²155-mfur (Table 1). As we expected, the Fur mutation at A28V induced high resistance to H₂O₂ with the MIC of H₂O₂ in mc²155-mfur being 0.64 mM. We showed that the point mutation of fur dysregulation of katG expression is a major factor leading to the phenotype H₂O₂ resistance.

The Same Protein Performs Different Functions in Different Redox States

As a successful human pathogen, M. tuberculosis has unique respiration properties. M. tuberculosis excretes alkaline supernatants, which is in contrast to other strains that excrete acidic supernatants (Merrill, 1930). The difference between secreted compounds with different acid–base properties suggested that M. tuberculosis has a distinctive redox state. As shown in Table 2, the comparative genomic analysis shows that PhoPR and DosR/S/T, identified as virulence factors of M. tuberculosis, are present in M. smegmatis. The signaling transduction systems such as the two-component systems and the sigma factors of M. tuberculosis are homologous in M. smegmatis (Table 3). Different phenotypes might be due to different redox states. Thus, we considered that the same redox-regulated related protein might perform different functions in different redox states. To test this hypothesis, we examined the biological function of a universal stress protein Rv1996 that increases the expression of KatG, in various mycobacterial strains (Hu et al., 2015). Both previous studies and our studies have linked isoniazid action with redox states, and H₂O₂ resistance is negatively correlated with INH susceptibility in mycobacteria (Bhaskar et al., 2014; Hu et al., 2015; Vilcheze et al., 2017). We used INH as a chemical probe for monitoring mycobacterial redox states and measured the MICs of INH to the corresponding mycobacterial strains (Figure 6). As predicted, the MICs of INH differed across the tested mycobacterial strains: the MIC of INH in pMV261-rv1996/mc²7000 was equal to that of pMV261/mc²7000; the MIC of INH in pMV261-rv1996/BCG was lower than that in pMV261/BCG; the MIC of INH in pMV261-rv1996/mc²51 was equal to that of pMV261/mc²51; and in mc²155, the opposite results were observed with the MIC of...
TABLE 2 | Conservation of M. tuberculosis H37Rv TCSS in M. smegmatis.

| Gene        | M. tuberculosis rv# | Mtb       | Msm       | References                        |
|-------------|---------------------|-----------|-----------|-----------------------------------|
| RegX3-SenX3 | Rv0491-Rv0490       | +         | +         | James et al. (2012); Parish et al. (2003b); Rifat and Karakousis (2014) |
| HK1-HK2-TcrA| Rv0600c-Rv0601c-Rv0602c | *         | –         | Srivastava and Das (2007)          |
| PhoP-PhoR   | Rv0757-Rv0758       | +         | –         | Walters et al. (2006)             |
| NarL-NarG   | Rv0844c-Rv0845c     | +         | +         | Schnell et al. (2008)             |
| PriA-PriB   | Rv0903c-Rv0902c     | +         | +         | Arora et al. (2021); Nowak et al. (2006) |
| MprA-MprB   | Rv0981-Rv0982       | +         | +         | He and Zahrt (2005); Sareka et al. (2007) |
| KdpD-KdpE   | Rv1028c-Rv1027c     | +         | +         | Parish et al. (2003a); Steyn et al. (2003) |
| TrcR-TrcS   | Rv1032c-Rv1034c     | +         | +         | Haydel et al. (2002)              |
| MtrA-MtrB   | Rv3245c-Rv3247c     | +         | +         | Fol et al. (2004); Li et al. (2010); Plocinska et al. (2012) |
| TrcR-TrcS   | Rv3245c-Rv3247c     | +         | +         | Bhattacharya et al. (2010)         |
| PdtaS-PdtaR | Rv3220c-Rv1626      | +         | +         | Boshoff et al. (2004); Srivastava and Das (2007) |

Msm, Mycobacterium smegmatis; Mtb, Mycobacterium tuberculosis CDC1551. +Genes encoding the sensor kinase and the response regulator are present and genetically linked. –Genes encoding both the sensor kinase and the response regulator are absent. *Genes encoding the two sensor kinases have been fused, and the gene encoding this fused sensor kinase is genetically linked to the response regulator.

TABLE 3 | Sigma factor genes in mycobacteria.

| Sigma | M. tuberculosis rv# | Mtb | Msm | References |
|-------|--------------------|-----|-----|------------|
| SigA (ρ^H) | Rv2703 | +   | +   | Gomez et al. (1998) |
| SigB (ρ^E) | Rv2710 | +   | +   | Lee et al. (2008b); Mukherjee and Chatterji (2005) |
| SigC (ρ^F) | Rv2069 | +   | –   | Sun et al. (2004) |
| SigD (ρ^D) | Rv2414c | +   | +   | Calamita et al. (2005); Raman et al. (2004) |
| SigE (ρ^F) | Rv1221 | +   | +   | Song et al. (2008) |
| SigF (ρ^I) | Rv2286c | +   | +   | Rodrigue et al. (2007) |
| SigG (ρ^A) | Rv0182c | +   | +   | Lee et al. (2008a) |
| SigH (ρ^I) | Rv2223c | +   | +   | Song et al. (2008) |
| SigI (ρ^I) | Rv1189 | +   | –   | Homerova et al. (2008) |
| SigJ (ρ^A) | Rv3328c | +   | +   | Homerova et al. (2008) |
| SigK (ρ^F) | Rv0445c | +   | –   | Veyrier and Behr, (2008) |
| SigL (ρ^I) | Rv0735 | +   | +   | Hahn et al. (2005) |
| SigM (ρ^I) | Rv3911 | +   | +   | Agarwal et al. (2007); Raman et al. (2006) |

+, presence of the gene; –, absence of the gene.

INH in pMV261-rv1996/mc^2155 being lower than that of INH in pMV261/mc^2155 (Figure 6).

DISCUSSION

Understanding how M. tuberculosis evolved into a professional pathogen is of benefit to the study of its pathogenesis and design of vaccines. The combination of experimental evolution and whole-genome sequencing provides a powerful method for identifying adaptive mutations and elucidating the specific genotype-phenotype relationship (Elena and Lenski, 2003; Lenski, 2017). Historically, the most successful example of continuous selective cultures is M. bovis BCG, the only anti-TB vaccine, which was attenuated after 13 years of continuous in vitro passages of M. bovis BCG. We previously used a similar adaptive evolution strategy to select H_2O_2-resistant M. smegmatis strains by using a clinically key stressor H_2O_2 (Li et al., 2014b). Preliminary results showed that the mc^251 strain was highly resistant to H_2O_2 and had greater susceptibility to INH, compared to mc^2155. The mc^251 phenotype showed an M. tuberculosis-like M. smegmatis phenotype. Altogether, the mutant M. smegmatis mc^251 exhibited higher virulence.

The whole-genome sequencing showed the presence of gene mutations in fur, and the mutant Fur resulted in katG levels (Figure 4B). In the Wayne dormancy model, mc^251 shows a growth advantage of recovering from dormancy under anaerobic conditions over mc^2155 (Figure 2B). In parallel, a high level of katG in mc^251 is accompanied by lower ATP levels, which implied mc^251 exhibited at a lower level of respiration (Figure 3B). Moreover, we showed that a redox-related protein Rv1996 exhibits a different phenotype under different redox states in M. smegmatis mc^2155 and mc^251, M. bovis BCG, and M. tuberculosis mc^27000 (Figure 6). This study indicated that the same genotype presents different phenotypes under different physiological conditions. We at least partially explain why M. smegmatis and M. tuberculosis have similar virulent factors, including a two-component system and sigma factors (Tables 2, 3), but M. smegmatis is a nonpathogen and M. tuberculosis is a pathogen.

M. tuberculosis is a successful human pathogen. It is considered to be derived from the environment (Gutierrez...
et al., 2005; Wolfe et al., 2007) and has adapted to the immune environment of the human body through long-term evolution. Its successfully established infection is partially attributed to its survival capacity and persistence in macrophages (Podinovskaia et al., 2013). To defend against mycobacterial infection, the host produces ROS, as an important innate defense mechanism. Consequently, M. tuberculosis has evolved a hierarchy and unique antioxidant function and maintains a low level of respiration, manifested by slow growth and persistence in the host. In contrast, M. smegmatis is present in the soil, which is a totally different environment from the host (Zhang and Furman, 2021). In Table 2, we show that M. tuberculosis and M. smegmatis have similar genotypes; however, they show different phenotypes, in terms of INH susceptibility, H2O2 resistance, and virulence. We believe that this striking difference is due to H2O2 resistance. The selected resistance to H2O2 of mc251 shows improved virulence in both the macrophage-killing assay and in an animal model (Figure 1). In fact, several studies have shown that abiotic stress can improve the virulence phenotype of bacterial pathogens (Sundberg et al., 2014; Li et al., 2021). Our study also supports the sit-and-wait hypothesis (Wang et al., 2017), that is, bacterial environmental abiotic stress and virulence evolution. In addition, this study also suggests that we can use mc251 as a model strain, replacing mc155, to study the regulation of redox homeostasis of M. tuberculosis.

We previously sequenced the whole genome of mc251 strain (Li et al., 2014a) and identified 29 SNPs, compared to mc251. Confirmed with our previous study (Li et al., 2014b), we found that only the fur gene can partially complement the resistant phenotype. This suggested that the fur mutation facilitated elevated H2O2 resistance, although it was not entirely responsible for the high resistance observed in mc251. We also mutated the fur in wild-type mc155 by genome editing and produced a phenotype similar to mc251, which is highly resistant to H2O2 (Table 1). Large-scale whole-genome sequencing studies on the evolutionary history of tuberculosis also show that key tract mutations at the transcription site will have a critical impact on the particular phenotype (Gagneux, 2018). For example, the change of PhoP in BCG allows infection with bovine pathogenic bacteria capable of infecting humans (Gonzalo-Asensio et al., 2014; Broset et al., 2015). This study reminds us that when designing vaccines, greater attention should be paid to regulators, which may be more efficient targets. M. smegmatis is an effective vaccine for TB and HIV (Sweeney et al., 2011; Kim et al., 2017). The disadvantage of M. smegmatis as a vaccine vector is its transient infection and difficulty to establish a persistent infection and produce adaptive immunity. The M. tuberculosis-like mutant M. smegmatis mc251 may be developed as a vaccine vector.

By comparing the survival of mc155 and mc251 in the Wayne dormancy animal model, we also found that low respiratory levels are beneficial for survival under anaerobic conditions and resurrection (Figure 2). In the future, we plan to use these strains to compare physiological indicators such as NADH/ NAD+, NADPH/NADP+, and ATP, to further understand the mechanisms underlying M. tuberculosis resurrection. This study provides insight into H2O2-resistant mechanisms in mycobacteria and has important implications for linking mycobacteria redox capacity and persistence infection in mice.

![Comparison of isoniazid susceptibility in different mycobacterial strains harboring pMV261-rv1996 and the corresponding control strain harboring pMV261. The minimum inhibitory concentrations (MICs) of isoniazid were determined by inoculating each bacterial strain in 7H9 containing serially isoniazid (INH). The values of MIC were recorded. 261 present pMV261. Rv1996 present pMV261-rv1996. The image represents the results of three independent repeats.](image-url)
In brief, INH or H2O2 was serially diluted using the 7H9 medium. The diluted fold was 1.25- or 2-fold, when required. Then, 40 μl of diluted INH or H2O2 was mixed with 40 μl of mycobacterial suspension with 1 × 10⁷ cells/ml in each well of 96-well microtiter plates and then incubated at 37°C for the indicated days. As an indicator, 0.02% resazurin was added to individual samples, and the color switches from blue to pink were recorded after 4 h. All the experiments were performed in triplicate. The abundance of the cultures was measured using a microplate reader (FLUOstar OPTIMA, BMG Labtech). A difference of two serial dilutions or more indicated a significant difference in the INH or H2O2 susceptibility of bacterial strains.

Mice Infection
Female pathogen-free C57BL/6 mice (aged 6–8 weeks) were purchased from Vital River (Beijing, China). For the intranasal infection of M. smegmatis, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg), and ~10⁷ CFU/50 μl PBS of mc²51 or mc²155 was introduced dropwise through the nostril of each mouse. The bacterial burden throughout the infection was monitored by collecting whole lung tissue at the indicated times after the mice were euthanized, and serial dilutions were then plated on 7H10 agar plates. The dose of infection was confirmed on day one after infection by plating whole lung homogenates from three mice on 7H10 agar. The percentage of survival was calculated as (CFUs after infection/CFUs before infection) × 100%.

Macrophage-Killing Assay
Human-derived cell line THP-1 (ATCC TIB-202) was cultured in RPMI medium with 10% fetal bovine serum (FBS, Gibco, United States). THP-1 cells were activated with 100 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma, United States) overnight. Infection was carried out at a multiplicity of infection (MOI) of 10 for 1 h at 37°C and 5% CO2 atmosphere. The infected THP-1 cells were washed with RPMI 3 times and then chased for 1 h. The cells with intracellular bacilli were then washed and lysed in sterile cold PBS (PBS with 0.05% Tween 20). Lysates were then vortexed, diluted, and plated on 7H10 agar plates as previously described (Chan et al., 1992). The percentage of survival was calculated as (CFUs after infection/CFUs before infection) × 100%.

Wayne Dormancy Model and Dormancy Exit
Mycobacterial strains were cultured under hypoxic conditions as described by Wayne and Hayes (Wayne and Hayes, 1996). In brief, cultures were initiated at an OD₆₀₀ of ~0.01 (1 × 10⁶) and incubated in anaerobic tubes with sealed caps. The headspace ratio of the cultures was 0.5. The cultures were stirred using an 8 mm Teflon stir bar (Fisher Scientific, United States) at 200 rpm. Methylene blue (1.5 mg/L) was used as an oxygen tension indicator. It changes in color from blue to colorless under low oxygen tension. The color transition time was recorded. All experiments were performed in triplicate. Growth was monitored by measuring the OD₆₀₀ and by determination of CFUs after plating on 7H10.

The indicator methylene blue in the culture became colorless, indicating that the bacteria entered anaerobic conditions. Then, after collecting the bacteria in the anaerobic tube, they were washed with the culture medium or PBS three times and resuspended in a culture medium (7H9 and brain–heart infusion medium), the concentration was adjusted to the same amount of OD₆₀₀ (OD₆₀₀ of ~1.0) and diluted for the CFU count, 1:100 or 1:50 into a fresh culture medium was shaken at 37°C, to monitor the status of the bacteria, and the OD₆₀₀ was measured. Three independent mycobacterial strain clones were set, and each clone set up three replicates.

Measurement of Intracellular ATP
The ATP Assay Kit was purchased from Beyotime Biotechnology (Beijing, China). The intracellular ATP assay was performed following the protocol provided by the manufacturer. In brief, the sample measurements were prepared as follows: cultures of indicated mycobacteria were obtained to an OD₆₀₀ of 0.8. Bacteria were collected by low-temperature centrifugation at the maximum speed, and the pellet was washed with precooled PBS buffer 3 times. A 300 μl volume of ATP detection lysate and 0.5 ml volume of glass beads were added for cell lysis. The lysate obtained was centrifuged at low temperature for 5 min, and the supernatant was placed on ice for later use. The preparation of the standard solution of gradient concentration ATP was performed as follows: the ATP standard solution was serially diluted into 7 concentrations of 10, 3.333, 1.111, 0.37, 0.1234, 0.04115, and 0.01371 μM and stored on ice for later use. The preparation of the working solution for the detection of ATP was performed as follows: an appropriate amount of ATP detection reagent was prepared according to the number of samples, and then, a 90% final volume of ATP detection reagent diluent was added. The prepared working fluid was placed on ice for further use. The determination of the ATP level was performed as follows: 1) the prepared ATP detection working solution was dispensed into 1.5 ml centrifuge tubes, 100 μl per tube, and was allowed to incubate at room temperature for 5 min to allow full reaction of the ATP in the centrifuge tube; 2) during the test, 20 μl of each sample (standard or total protein sample) was added to a 1.5 ml centrifuge tube containing 100 μl of ATP detection working solution and was mixed quickly with a pipette and incubated for 2 s to complete the reaction before using a Cytation 3 Cell Imaging Multi-Mode Reader to determine the relative light unit (RLU); 3) a standard curve was constructed to measure and determine the concentration of the sample by converting the RLU into an ATP concentration; and 4) in order to eliminate the error caused by the difference in the amount of protein during sample preparation, the BCA protein concentration determination kit produced by Beyotime Biotechnology (Beijing, China) was used to determine the protein concentration in the sample. The ATP concentration was converted to μM/mg protein.

Electrophoretic Mobility Shift Assay
The coding regions of fur and mfur were amplified from mc²155 and mc²51 genomic DNA and cloned into the Escherichia coli expression vector pET23b (+) (Novagen, Madison, WI, United States) in-frame fused with a C-terminal His₆-tag sequence to construct the plasmids pET23b-fur and pET23b-mfur. The final constructs were transformed into BL21 (DE3) for expression, and recombinant Fur/mFur proteins were purified using Ni-NTA.
agarose (Qiagen, California, United States). The proteins were induced by the addition of 1 mM IPTG at 16°C for 12 h. Protein purification was performed as described previously (Li et al., 2014c). The protocols of the recombinant protein purification are available on request. The recombinant protein MSMEG_2415 was purified as described previously (Li et al., 2014c) and used as a negative control for EMSA, while MSMEG_2415 is irrelevant to Fur. The DNA fragment containing the promoter region of fur for gel shift experiments was amplified by PCR with specific primers (forward: 5′-CGTTGGAAAAACACCATTGCCAG-3′, reverse: 5′-CATCCGCGATTGGCTTCGACC-3′). Binding reaction mixtures in 20 μl of binding buffer (20 mM Tris HCl pH 8.0, 1 mM dithiothreitol (DTT), 50 mM KCl, and 5 mM MgCl2) containing 0.15 pmol of the DNA fragment were incubated with purified Fur/mFur protein (0.5 nmol) for 30 min at 30°C. Reaction mixtures were loaded on a 4–20% polyacrylamide gel containing 0.5 × TBE. Gels were run at 70 V at 4°C for 3 h. The gel was stained with Good-view and photographed for the image.

**Generation of the katG Knockout and KatG Overexpression Strains**

The knockout katG strain was constructed using mycobacteriophage-based specialized transduction (Bardarov et al., 2002; Li et al., 2014c). The upstream and downstream sequences of katG were amplified from M. smegmatis genome DNA. The knockout vector was constructed using phAE159 (Hsu and Jacobs, unpublished data). The mycobacteriophage used for knockout was obtained using MaxPlax packaging extract (Epicentre Biotechnologies, Madison, WI, United States), and a katG knockout strain was obtained by phage transduction, named ΔkatG. The KatG overexpression strain was constructed using pMV261 to yield pMV261-katG, and the constructed plasmid was electroporated into mc2155, yielding pMV261-katG/mc2155. The detailed information for construction of all the mycobacterial strains and primers for plasmid construction is available on request.

**Generation of Fur Point Mutation on the Chromosome in M. smegmatis**

The single-strand (ss) DNA oligonucleotides used for recombineering were ordered from Genewiz (Suzhou, China). The site-directed mutagenesis of fur was performed using Phage Che9g gp61-mediated recombination (van Kessel et al., 2008). The detailed information on primers for construction of the fur mycobacterial strain (named mc155-mfur) is available on request. The coding region containing fur point mutation in the genome (encoding mFur) was amplified and sequenced by Genewiz (Suzhou, China).

**Generation of the rv1996 Overexpression Mycobacterial Strains**

The rv1996 gene was amplified and constructed and cloned into pMV261 to yield pMV261-rv1996. The constructed pMV261-rv1996 plasmid was transformed into mycobacterial strains, M. smegmatis mc2155 and mc251, M. bovis BCG Pasteur, and M. tuberculosis mc27000, and the corresponding strains, named pMV261-rv1996/mc2155, pMV261-rv1996/mc251, pMV261-rv1996/BCG, and pMV261-rv1996/mc27000. The empty vector pMV261 was transformed into the corresponding mycobacterial strains, named pMV261/mc2155, pMV261/mc251, pMV261/BCG, and pMV261/mc27000.

**Statistical Analysis**

Each experiment was carried out at least twice with three–nine mice or samples per group. The CFUs and OD_{600} were analyzed using an unpaired t-test (Version 8.0 for Windows GraphPad Software). The ATP content was analyzed using ANOVA tests (Version 8.0 for Windows GraphPad Software). ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05.

**Animal Ethics**

This study was performed in strict accordance with the recommendations of the Ethics Committee established in the Guide for the Care and Use of Laboratory Animals of the Institute of Microbiology, Chinese Academy of Sciences (IMCAS). The protocol was approved by the Committee on the Ethics of Animal Experiments of the IMCAS. The mice were bred under specific pathogen-free conditions at the IMCAS laboratory animal facility. All animal experiments were conducted under isoflurane anesthesia, and all efforts were made to minimize suffering.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the following: BioProject: PRJNA233977; BioSample: SAMN02951866, https://www.ncbi.nlm.nih.gov/nuccore/ AJJD00000000.

**AUTHOR CONTRIBUTIONS**

KM conceived and designed the experiments; ZJ and ZZ performed the experiments; KM wrote the manuscript; and KM, ZJ, and ZZ revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgen.2022.758304/full#supplementary-material

REFERENCES

Agarwal, N., Woolwine, S. C., Tyagi, S., and Bishai, W. R. (2007). Characterization of the Mycobacterium tuberculosis SigA Factor SigM by Assessment of Virulence and Identification of SigM-dependent Genes. Infect. Immun. 75, 452–461. doi:10.1128/IAI.01395-06

Aldridge, B. B., Fernandez-Suarez, M., Heller, D., Ambrahaneswaran, V., Irimia, D., Toner, M., et al. (2012). Asymmetry and Aging of Mycobacterial Cells lead to Variable Growth and Antibiotic Susceptibility. Science 335, 100–104. doi:10.1126/science.1216166

Anes, E., Kühnel, M. P., Bos, E., Moniz-Pereira, J., Habermann, A., and Griffiths, G. (2003). Selected Lipids Activate Phagosome Actin Assembly and Maturation Resulting in Killing of Pathogenic Mycobacteria. Nat. Cell Biol 5, 793–802. doi:10.1038/nca1003

Anes, E., Peyron, P., Staali, L., Jordao, L., Gutierrez, M. G., Kress, H., et al. (2006). Dynamic Life and Death Interactions between Mycobacterium Smegmatis and J774 Macrophages. Cell Microbiol 8, 939–960. doi:10.1111/j.1462-5822.2005.00675.x

Armstrong, J. A., and Hart, P. D. A. (1971). Response of Cultured Macrophages to Mycobacterium tuberculosis, with Observations on Fusion of Lysosomes with Phagosomes. J. Exp. Med. 134, 713–740. doi:10.1086/jem.134.3.713

Arora, G., Bothra, A., Prosser, G., Arora, K., and Sajid, A. (2021). Role of post-translational Modifications in the Acquisition of Drug Resistance in Mycobacterium tuberculosis. FEBS J. 288, 3375–3393. doi:10.1111/febs.15582

Bardarov, S., Bardarov, S., Pavelka, M. S., Sambandamurthy, V., Larsen, M., Tufariello, J., et al. (2002). Specialized Transduction: an Efficient Method for Generating Marked and Unmarked Targeted Gene Disruptions in Mycobacterium tuberculosis. Mol. Microbiol. 40, 643–657. doi:10.1111/j.1365-2958.2006.05137.x

Ford, C. B., Lim, P. L., Chase, M. R., Shah, R. R., Iartchouk, O., Galagan, J., et al. (2011). Use of Whole Genome Sequencing to Estimate the Mutation Rate of Mycobacterium tuberculosis during Latent Infection. Nat. Genet. 43, 482, 466. doi:10.1038/ng.1088

Franzblau, S. G., Witzig, R. S., McLaughlin, J. C., Torres, P., Madico, G., Hernandez, A., et al. (1998). Rapid, Low-Technology MIC Determination with Clinical Mycobacterium tuberculosis Isolates by Using the Microplate Alamar Blue Assay. J. Clin. Microbiol. 36, 362–366. doi:10.1128/JCM.36.2.362-366.1998

Gagneux, S. (2008). Ecology and Evolution of Mycobacterium tuberculosis. Nat. Rev. Microbiol. 16, 202–213. doi:10.1038/nrmicro20188

Gomez, D., Doukhan, L., Nair, G., and Smith, I. (1998). sigAIs an Essential Gene in Mycobacterium smegmatis. Mol. Microbiol. 29, 617–628. doi:10.1046/j.1365-2958.1998.00660.x

Gonzalo-asensio, J., Malaga, W., Pawlik, A., Astarie-Dequeker, C., Passemar, C., Moreau, F., et al. (2014). Evolutionary History of Tuberculosis Shaped by Conserved Mutations in the PhoPR Virulence Regulator. Proc. Natl. Acad. Sci. USA 111, 11491–11496. doi:10.1073/pnas.1406603111

Hahn, M.-Y., Raman, S., Anaya, M., and Husson, R. N. (2005). The Mycobacterium tuberculosis Exoprotein-Plasma-Function Sigma Factor SigL regulates Polypeptide Syntheses and Secreted or Membrane Proteins and is Required for Virulence. J. Bacteriol. 187, 7062–7071. doi:10.1128/JB.187.20.7062-7071.2005

Haydel, S. E., Benjamin, W. H., Dunlap, N. E., and Clark-Curtiss, J. E. (2002). Expression, Autoregulation, and DNA Binding Properties of the Mycobacterium tuberculosis Tcr Response Regulator. J. Bacteriol. 184, 2192–2203. doi:10.1128/JB.184.8.2192-2203.2002

Cohen, S. B., Gern, B. H., Delahaye, J. L., Adams, K. N., Plumlee, C. R., Winkler, J. K., et al. (2018). Alveolar Macrophages Provide an Early Mycobacterium tuberculosis Niche and Initiate Dissemination. Cell Host & Microbe 24, 439–446. doi:10.1016/j.chom.2018.08.001

Dragset, M. S., Loerger, T. R., Zhang, Y. J., Mærk, M., Ginbot, Z., Sacchettini, J. C., et al. (2019). Genome-wide Phenotypic Profiling Identifies and Categorizes Genes Required for Mycobacterial Low Iron Fitness. Sci. Rep. 9, 11394. doi:10.1038/s41598-019-47905-y

Elena, S. F., and Lenski, R. E. (2003). Evolution Experiments with Microorganisms: The Dynamics and Genetic Bases of Adaptation. Nat. Rev. Genet. 4, 457–469. doi:10.1038/nrg1038

Flynn, J. L., and Chan, J. (2001). Immunology of Tuberculosis. Annu. Rev. Immunol. 19, 93–129. doi:10.1146/annurev.immunol.19.1.93

Fol, M., Chauhan, A., Nair, N. K., Maloney, E., Moomey, M., Jagannath, C., et al. (2006). Modulation of Mycobacterium Tuberculosis Proliferation by MtrA, an Essential Two-Component Response Regulator. Mol. Microbiol. 60, 643–657. doi:10.1111/j.1365-2958.2006.05137.x

Gray, T. A., Clark, R. R., Boucher, N., Lapierre, P., Smith, C., and Derbyshire, K. M. (2005). Intercellular Communication and Conjugation Are Mediated by ESX Complex from the Viewpoint of PhoPR. Proc. Natl. Acad. Sci. USA 112, 11219–11224. doi:10.1073/pnas.1505768112

Hernandez, A., et al. (1998). Rapid, Low-Technology MIC Determination in Mycobacterium Smegmatis. J. Clin. Microbiol. 36, 362–366. doi:10.1128/JCM.36.2.362-366.1998
Shrivastava, R., and Das, A. K. (2007). Temperature and Urea Induced Conformational Changes of the Histidine Kinases from Mycobacterium tuberculosis. *Acta Cryst. Sect F* 64, 1096–1100. doi:10.1107/S1744309107035203 Shrivastava, R., and Das, A. K. (2007). Temperature and Urea Induced Conformational Changes of the Histidine Kinases from Mycobacterium tuberculosis. *Int. J. Biol. Macromolecules* 41, 154–161. doi:10.1016/j.ijbiomac.2007.01.010

Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T., and Jr, W. R. J., Jr. (1990). Isolation and Characterization of Efficient Plasmid Transformation Mutants of Mycobacterium Smegmatis. *Mol. Microbiol.* 4, 1911–1919. doi:10.1111/j.1365-2958.1990.tb02040.x

Song, T., Song, S.-E., Raman, S., Anaya, M., and Husson, R. N. (2008). Critical Role of a Single Position in the −35 Element for Promoter Recognition by Mycobacterium tuberculosis SigE and SigH. *J. Bacteriol.* 190, 2227–2230. doi:10.1128/Jb.01642-07

Steyn, A. J. C., Joseph, J., and Bloom, B. R. (2003). Interaction of the Sensor Module of Mycobacterium tuberculosis H37Rv KdpD with Members of the Lpr Family. *Mol. Microbiol.* 47, 1075–1089. doi:10.1046/j.1365-2958.2003.03356.x

Sun, R., Converse, P. J., Ko, C., Tyagi, S., Morrison, N. E., and Bishai, W. R. (2004). Mycobacterium tuberculosis ECF Sigma Factor sigC Is Required for Lethality in Mice and for the Conditional Expression of a Defined Gene Set. *Mol. Microbiol.* 52, 25–38. doi:10.1111/j.1365-2958.2003.03958.x

Sundberg, L.-R., Kuutu, H. M. T., and Vähänen, E. (2014). Starvation Can Diversify the Population Structure and Virulence Strategies of an Environmentally Transmitting Fish Pathogen. *BMC Microbiol.* 14, 67. doi:10.1186/1471-2180-14-67

Sureka, K., Dey, S., Datta, P., Singh, A. K., Dasgupta, A., Rodrigue, S., et al. (2007). Polyphosphate Kinase Is Involved in Stress-Induced mprAB-sigE-Rel Signalling in Mycobacteria. *Mol. Microbiol.* 65, 261–276. doi:10.1111/j.1365-2958.2007.05814.x

Sweeney, K. A., Dao, D. N., Goldberg, M. F., Hsu, T., Venkataswamy, M. M., Henao-Tamayo, M., et al. (2011). A Recombinant Mycobacterium Smegmatis Induces Potent Bactericidal Immunity against Mycobacterium tuberculosis. *Nat. Med.* 17, 1261–1268. doi:10.1038/nm.2420

Timmins, G. S., and Deretic, V. (2006). Mechanisms of Action of Isoniazid. *Mol. Microbiol.* 62, 1220–1227. doi:10.1111/j.1365-2958.2006.05467.x

van Kessel, J. C., Marinelli, L. J., and Hatfull, G. F. (2008). Recombineering Mycobacteria and Their Phages. *Nat. Rev. Microbiol.* 6, 851–857. doi:10.1038/nrmicro2014

Veyrier, F., Said-Salim, B., and Behr, M. A. (2008). Evolution of the Mycobacterial SigK Regulon. *J. Bacteriol.* 190, 1891–1899. doi:10.1128/Jb.01452-07

Walters, S. B., Dubnau, E., Kolesnikova, I., Laval, F., Daffe, M., and Smith, I. (2006). The Mycobacterium tuberculosis PhoPR Two-Component System Regulates Genes Essential for Virulence and Complex Lipid Biosynthesis. *Mol. Microbiol.* 60, 312–330. doi:10.1111/j.1365-2958.2006.05102.x

Wang, L., Liu, Z., Dai, S., Yan, J., and Wise, M. J. (2017). The Sit-And-Wait Hypothesis in Bacterial Pathogens: A Theoretical Study of Durability and Virulence. *Front. Microbiol.* 8, 2167. doi:10.3389/fmicb.2017.02167

Wayne, L. G., and Hayes, L. G. (1996). An In Vitro Model for Sequential Study of Shutoff of Mycobacterium tuberculosis through Two Stages of Nonreplicating Persistence. *Infect. Immun.* 64, 2062–2069. doi:10.1128/aiai.64.6.2062-2069.1996

WHO (2021). *Global Tuberculosis Report 2021*. Geneva, Switzerland: WHO.

Wolfe, N. D., Dunavan, C. P., and Diamond, J. (2007). Origins of Major Human Infectious Diseases. *Nature* 447, 279–283. doi:10.1038/nature05775

Zhang, Z., and Furman, A. (2021). Soil Redox Dynamics under Dynamic Hydrologic Regimes - A Review. *Sci. Total Environ.* 763, 143026. doi:10.1016/j.scitotenv.2020.143026

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