Mutation rate and the emergence of drug resistance in *Mycobacterium tuberculosis*

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The emergence and spread of multidrug-resistant strains of *Mycobacterium tuberculosis* remains a major concern of tuberculosis control programmes worldwide, as treatment depends on low-efficacy, toxic compounds that often lead to poor outcomes. *M. tuberculosis* develops drug resistance exclusively through chromosomal mutations, in particular single-nucleotide polymorphisms. Moreover, in laboratory assays the organism exhibits a spontaneous mutation rate that is at the lower end of the bacterial spectrum. Despite this, whole-genome sequencing technology has identified unexpected genetic diversity among clinical *M. tuberculosis* populations. This suggests that the mycobacterial mutation rate may be modulated within the host and, in turn, implies a potential role for constitutive and/or transient mutator strains in adaptive evolution. It also raises the possibility that environmental factors might act as key mutagens during *M. tuberculosis* infection. Here we consider the elements that might influence the mycobacterial mutation rate in vivo and evaluate the potential roles of constitutive and transient mutator states in the generation of drug resistance mutations. In addition, we identify key research questions that will influence future efforts to develop novel therapeutic strategies for a disease that continues to impose a significant global health burden.

**Keywords:** genetic diversity, adaptive mutagenesis, evolution, constitutive mutator, transient mutator

### Introduction

*Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB) in humans. TB remains a massive global health problem and the emergence of multidrug-resistant (MDR) (resistant to rifampicin and isoniazid) and extensively drug-resistant (XDR) (MDR with added resistance to a fluoroquinolone and an injectable second-line agent) strains, coupled with the lack of new effective drugs to combat the disease, is of major concern.\(^1\) Recently, totally drug-resistant (TDR) cases have been described, but WHO has yet to define this form of drug-resistant TB.\(^2\) Treatment of MDR-TB and XDR-TB requires prolonged chemotherapy with expensive and less efficient drugs that often lead to undesirable side effects.\(^3\) TDR-TB might be untreatable with currently available drugs. For this reason, a greater understanding of how drug resistance arises and might be prevented is of critical importance.

In *M. tuberculosis*, genetically encoded drug resistance arises exclusively through chromosomal mutations,\(^4,5\) the majority of which are single-nucleotide polymorphisms (SNPs).\(^6\) In general, mutations occur spontaneously\(^7\) as a consequence of errors that arise during DNA replication at a rate of one mutation per 10,000–100,000 bp per round of replication.\(^8\) Correction by proof-reading exonucleases reduces the rate \(\sim 2\) log-fold,\(^9\) with post-replicative DNA mismatch repair ensuring a further \(3\) log-fold reduction.\(^9\) The bacterial mutation rate is, in turn, defined as the probability of a mutation occurring per cell division, and so is determined per bacterium per generation.\(^10\) The calculated rates for the *in vitro* evolution of resistance to various antibiotics are shown for *M. tuberculosis* and related mycobacteria in Table 1.

In this review, we discuss the different factors that might contribute to the emergence of drug resistance mutations in *M. tuberculosis* during host infection. In particular, we evaluate whether spontaneous errors during DNA replication and repair are sufficient to drive the evolution of drug-resistant *M. tuberculosis* strains within the host or if additional factors contribute to the emergence of these mutant strains. Understanding the mechanisms and pathways that influence the mutation rate might identify targets for novel agents designed to prevent the development of drug resistance\(^21\) or to potentiate the activity of existing antituberculars.\(^22\) In addition, it could aid the identification of environmental or host immune factors that could be manipulated,\(^23\) as well as inform the design of new drug regimens for the treatment of *M. tuberculosis*.
M. tuberculosis mutates at a low rate in vitro

M. tuberculosis does not exhibit an elevated mutation rate relative to most other bacteria, under in vitro conditions, it is estimated that the organism makes a mutational error at two bases in every 10,000 genomes copied. This is despite the fact that the DNA repair complement of M. tuberculosis does not include homologues of known mismatch repair (MMR) enzymes. Escherichia coli mutants lacking MMR components are considered strong heritable mutator strains; however, this does not apply to M. tuberculosis. In this review, the term ‘mutator’ is applied both to a strain that exhibits a higher mutation rate compared with its progenitor and to a gene that, when mutated, confers a higher mutation rate on the organism. Instead, it has been proposed that MMR deficiency may, in fact, exert selective pressure on M. tuberculosis, resulting in a more stable genome over time, as is suggested by the lower than expected degree of polymorphism amongst simple sequence repeats (SSRs) in the M. tuberculosis genome. SSRs (also called microsatellites) consist of repeats of 1–6 bp units and are particularly prone to polymerase slippage, which can lead to frameshift mutations. Long tracts comprising more than seven repeats of the microsatellite unit are more prone to slippage than short tracts, and may contribute to genome instability. A lack of MMR would, therefore, be expected to result in an elevated rate of mutation frequency, the proportion of mutants within a bacterial population at a given timepoint. No data were available on mutation rate.

### Table 1. Published rates for evolution of drug resistance to various antibiotics in M. tuberculosis and related mycobacteria

| Antibiotic               | Strain | Mutation rate | Reference(s) |
|--------------------------|--------|---------------|---------------|
| Isoniazid                | H37Rv  | 2.56×10⁻⁸     | 11            |
| Isoniazid                | MTB72  | 3.2×10⁻⁷      | 12            |
| Rifampicin (1 mg/L)      | H37Rv  | 2.25×10⁻¹⁰ to 3.32×10⁻⁹ | 11            |
| Rifampicin (2 mg/L)      | H37Rv  | 2.9×10⁻⁹ to 2.4×10⁻⁷ | 13–15         |
| Rifampicin (2 mg/L)      | Harlingen | 1.4×10⁻⁷ | 13            |
| Rifampicin (2 mg/L)      | Beijing clinical isolates | 7.9×10⁻⁹ to 1.3×10⁻⁸ | 13            |
| Rifampicin (2 mg/L)      | Erdman | 2.1×10⁻⁹      | 16            |
| Rifampicin (2 mg/L)      | H37Rv with reduced catalase and peroxidase activity, and no mutation in katG | 8.76×10⁻⁷ to 2.24×10⁻⁶ | 14            |
| Rifampicin (2 mg/L)      | H37Rv with katG mutation or deletion | 2.7×10⁻⁷ to 3.3×10⁻⁶ | 14            |
| Rifampicin (2 mg/L)      | H37Rv with katG mutation or deletion, exposed to H₂O₂ | 4×10⁻⁷ to 6×10⁻⁷ | 14            |
| Rifampicin (2 mg/L)      | H37Rv mutants with DinB1, DinB2 and double knockouts | 2.3×10⁻⁹ | 15            |
| Rifampicin (5 or 10 mg/L)| H37Rv  | 6×10⁻⁷        | 17            |
| Rifampicin (8 mg/L)      | MTB72  | 9.81×10⁻⁹ to 6.45×10⁻⁸ | 12,18         |
| Rifampicin (8 mg/L)      | typical Beijing strains (erg, mut2 and mut74 mutations) | 7.73×10⁻⁸ to 2.49×10⁻⁷ | 18            |
| Rifampicin (8 mg/L)      | atypical Beijing strain | 5.76×10⁻⁷ to 1.21×10⁻⁷ | 18            |
| Rifampicin (8 mg/L)      | LAM strain (Ag85C mutation) | 8.18×10⁻⁸ | 18            |
| Rifampicin (8 mg/L)      | LAM strain (Ag85C mutation and katG mutation) | 6.28×10⁻⁸ to 7.2×10⁻⁸ | 18            |
| Rifampicin (8 mg/L)      | T1 spoligotype family | 2.85×10⁻⁸ | 18            |
| Rifampicin (8 mg/L)      | T1 spoligotype family (with katG mutation) | 1.33×10⁻⁸ | 18            |
| Rifampicin (8 mg/L)      | MTB72 with katG deletions at amino acids 315 and 463 | 9.69×10⁻⁸ | 18            |
| Rifampicin (8 mg/L)      | MTB72 with partial katG deletion | 5.71×10⁻⁷ | 18            |
| Ethambutol               | H37Rv  | 1×10⁻⁷ to 6.4×10⁻⁷ | 11            |
| Streptomycin             | H37Rv  | 2.95×10⁻⁸     | 11            |
| &-cyclosinerine          | H37Rv  | 9×10⁻¹¹       | 11            |
| R207910/TMC207 (diarylquinolone ATP synthase inhibitor) at 0.3 mg/L | H37Rv and clinical isolates | 4×10⁻⁷ to 8.9×10⁻⁸ | 4            |
| R207910/TMC207 (diarylquinolone ATP synthase inhibitor) at 0.9 mg/L | H37Rv and clinical isolates | 2.4×10⁻⁹ to 3.9×10⁻⁸ | 4            |
| Ciprofloxacin            | Mycobacterium fortuitum | 5.1×10⁻⁹ | 19            |
| Levofoxacin              | M. fortuitum | 3.8×10⁻⁹ | 19            |
| Moxifloxacin             | M. fortuitum | 4.2×10⁻⁹ | 19            |
| Pyrazinamide             | M. tuberculosis clinical isolates | 1×10⁻⁵c | 20            |

*a*All strains are of M. tuberculosis unless stated otherwise.

*b*Units are per bacterium per generation.

*c*Mutation frequency, the proportion of mutants within a bacterial population at a given timepoint. No data were available on mutation rate.

Published rates for evolution of drug resistance to various antibiotics in M. tuberculosis and related mycobacteria.
N-terminal region—are not associated with a higher rate of mutation relative to base substitutions.

The absence of MMR in \textit{M. tuberculosis} might nevertheless contribute to genetic variation. Amongst other functions, MMR is known to prevent recombination between non-identical sequences during repair of double-stranded DNA breaks in other bacterial systems. In \textit{Mycobacterium smegmatis}, abrogation of nucleotide excision repair (NER) function results in an increased number of gene conversion events arising from mismatch errors during homologous recombination, suggesting that NER may (partially) compensate for the loss of MMR in mycobacteria. However, the fact that NER does not distinguish between the parent and daughter strands, whereas MMR does, raises the possibility that mutation fixation—rather than avoidance—might be a common outcome. The lack of an MMR system might also promote exchange of divergent DNA sequences, thus driving genome evolution, since evidence suggests that recombination can occur in \textit{M. smegmatis} when divergent loci are flanked by sequences of perfect homology. The mechanisms ensuring genomic stability in \textit{M. tuberculosis} therefore represent an important area for future research.

**Generation of drug resistance-conferring mutations in \textit{M. tuberculosis} during infection**

As noted above, \textit{M. tuberculosis} does not display an elevated in vitro mutation rate compared with other bacteria. For this reason, drug-resistant mutants of \textit{M. tuberculosis} are expected to be rare, and are predicted to arise as a consequence of spontaneous errors in DNA replication that are subsequently selected under applied drug pressure. Multiple factors could influence the rate of selection of drug-resistant mutants in the host, including the relative fitness of individual mutants, patient compliance with prescribed drug regimens, pharmacokinetic variability amongst patients, spatial heterogeneity in drug distribution, and the size of the infecting bacterial population. The rate of selection of a particular mutation should, however, not be confused with the basal mutation rate. The fundamental principle remains; errors in DNA replication must occur in order to generate the base substitutions and other types of mutations that lead to drug resistance. It is not entirely clear, though, whether a relatively low mutation rate is sufficient to account for the elevated rates of acquired drug resistance observed clinically. Is the mutation rate within the host modulated in some way to bring about sufficient diversity from which drug resistance can be selected?

In a pioneering study, Ford et al. applied whole-genome sequencing (WGS) technology to detect the mutational events that arose in \textit{M. tuberculosis} during experimental infection of non-human primates. Using a flexible range of predicted mycobacterial replication rates, the authors estimated the rate at which the observed mutations must have arisen in vivo. Unexpectedly, this estimated in vivo mutation rate was very similar to that which was inferred from in vitro fluctuation analyses, and, moreover, did not differ for active versus latently infected animals. In other words, \textit{M. tuberculosis} does not appear to enter into a hypermutable state within the host. Work by Saunders et al. reinforced the idea that drug resistance mutations are rare. In this case, the authors sequenced the genomes of serial isolates obtained from a TB patient over 12 years, a period that correlated with the emergence of strains resistant to isoniazid and, subsequently, rifampicin. WGS analysis suggested that only two SNPs—in \textit{katG} and \textit{rpoB}—differentiated the drug-resistant isolates from susceptible strains, indicating that the mutation rate in the host is very low. The reference genome employed in this study was assembled from pooled data from all the serial isolates. It is possible, therefore, that other SNPs present at low frequencies were not detected. In addition, the authors estimated the bacillary population to be in the order of $10^{13}$ cells/lung, which far exceeds predicted values for \textit{M. tuberculosis} infections.

In contrast, Sun et al. utilized more sensitive WGS technology to track genomic changes in serial sputum samples obtained from three patients over the course of anti-TB treatment. In this case, the authors reported a high degree of diversity in the serial clinical specimens, an observation that is consistent with the idea that mutation rates in vivo might be higher than previously proposed; for example, between 8 and 41 SNPs arose during treatment in each sample, and as many as 34 SNPs were unique to a single sample. Interestingly, the majority of the SNPs were detected at frequencies below 20% in each sample, indicating that the sputum bacillary population was characterized by significant microheterogeneity at each timepoint. These observations are supported by other studies that employed Sanger sequencing to investigate the resistance-determining regions of specific target genes. For example, serial \textit{M. tuberculosis} isolates obtained from patients over a period of 12 years revealed a diversity of mutations within \textit{rpsL}. Similarly, Mariam et al. detected different \textit{rpsL} and \textit{rrs} mutations in serial isolates obtained from a single patient. These authors also reported transient co-existence of some mutations as well as successive clonal sweeps of other mutations, suggesting a dynamic interplay between the genetic variability of the infecting bacillus and the selective pressures associated with host infection.

The extent to which the sputum bacillary population can be considered representative of the total infecting mycobacterial population is not known. For this reason, the above studies might be limited by their dependence on organisms obtained from sputum. In a key study, bacilli isolated from discrete pulmonary lesions in patients with chronic TB were shown to possess different drug resistance allelic. It seems likely, therefore, that the micro-environments represented by each lesion may contribute to the heterogeneity observed within the TB population in the host. However, WGS analyses in the non-human primate model revealed significant heterogeneity among \textit{M. tuberculosis} bacilli isolated within single lesions.

**The mycobacterial replication rate**

The levels of genetic diversity identified in the studies above imply that \textit{M. tuberculosis} might have an elevated mutation rate within the host compared with that calculated in vitro. However, it is important to remember that the fixation of spontaneous mutations occurs during replication; the mutation rate is, therefore, dependent on the rate of replication. This raises the possibility that the replication rate may be higher in the host than previously thought. Until recently, the prevailing dogma held that the rate of acquisition of dual resistance (required for MDR-TB) was the product of the individual mutation rates for rifampicin and isoniazid; that is, in the order of $10^{-16}$. This requires that, for the evolution of MDR strains, a total population of at least $10^{16}$ bacilli must
be present within any infected individual prior to the initiation of treatment. A recently proposed mathematical model suggests otherwise. In developing the model, Colijn et al.38 allowed for the possibility that a single drug-resistant mutant may arise early following infection, and could replicate to a large enough population from which the probability of the emergence of a second drug-resistance mutation would not be so low. Critically, they also considered the possibility that a greater number of replication events might occur during the initial infection period than has previously been assumed from in vitro estimates, an idea that is supported by recent evidence from experimental infections of macrophages47 and mice.48 Applying this set of revised parameters, Colijn et al.38 showed that the likelihood of emergence of drug resistance prior to initiation of anti-TB therapy is much higher than previously expected, even when basing the calculation on published in vitro mutation rates for specific TB drugs.

Human TB can be usefully divided into three broad phases: active, chronic and reactivated TB.49 Based on data from animal models of infection, it is assumed that the total number of M. tuberculosis bacilli remains stable during chronic TB and that these are in a state of slow replication or non-replication.50,51 Recent evidence suggests, however, that the apparently stable bacillary numbers conceal a population that is cycling continuously between active replication and death.48 Utilizing a ‘clock’ plasmid that is lost from daughter cells during division, Gill et al.48 demonstrated that, during chronic infection in the mouse model, a balance is established between bacillary replication and death. This implies that the replication rate may be higher than previously thought during chronic TB in humans and is consistent with an emerging appreciation of latent TB as a continuum of mycobacterial growth states.52,53 To date, the clock plasmid has only been assessed in the mouse model,48 which fails to recapitulate key features of human disease.54,55 It will be interesting, therefore, to see whether an alternative model, such as the non-human primate,16,56 yields similar results.

Is the mycobacterial mutation rate altered during host infection, and might this explain the observed rates of acquired drug resistance? From the above discussion, it is clear that additional research is required to address this question. There are many factors that might influence the mutation rate; these are considered below and summarized in Table 2, together with the corresponding knowledge gaps.

### Table 2. Factors potentially affecting the mutation rate in M. tuberculosis (Mtb) and corresponding knowledge gaps

| Cellular mechanisms                            | Knowledge gaps                                                                 |
|-----------------------------------------------|--------------------------------------------------------------------------------|
| Lack of MMR                                    | Does the lack of this system play a role in generating genetic variation in Mtb? |
| SSRs                                          | Are SSRs hypermutable?                                                         |
| Mutations in 3R\(^{\text{a}}\) genes           | Does the location of SSRs in Mtb have any effect on mutation rate?             |
| Do genetically stable mutator strains occur   | Do sublineages of certain spoligotype families have varying mutation rates?   |
| within populations of TB in the host?          | How do the polymorphisms detected in 3R genes of various Mtb strains\(^{\text{a}}\) affect their mutation rate? |
| Do different drug-resistant Mtb mutants       | If certain spoligotype families are associated with drug resistance, why are they better able to adapt to drug pressure? |
| have different mutation rates?                |                                                                              |
| Existing drug resistance-conferring mutations | Do Mtb use mistranslation as a means to adapt to environmental stress and, if so, how does it do so? |
| Mistranslation                                 | Does transcriptional mutagenesis play a role in the emergence of drug resistance in Mtb? |
| Transcriptional mutagenesis                   | Does Mfd, or the process of transcription-coupled repair, play a role in the emergence of drug resistance? |
| Error-prone DNA polymerases                    | How important a role does DnaE2 play in the emergence of drug resistance in the host? |

| External factors                               |                                                                 |
|-----------------------------------------------|-----------------------------------------------------------------|
| Antibiotics                                   | Do certain antibiotic combinations/regimens increase the probability of acquired drug resistance? |
| Antiretroviral drugs                           | Do antibiotics, other than fluoroquinolones, used to treat Mtb, increase its mutation rate in vitro? |
| Host environment                              | Do antiretrovirals, especially NRTIs, increase the mutation rate of Mtb in vitro or in vivo? |
| Is the exposure of Mtb to UV radiation long   | Is desiccation a stress relevant to acquired drug resistance in Mtb? |
| enough to cause up-regulation of the SOS     | Does oxidative stress drive the evolution of drug resistance? |
| response?                                     | How could the down-regulation of DNA repair enzymes contribute to the up-regulation of the mutation rate? |
| Is desiccation a stress relevant to acquired | Do different DNA-damaging stresses in the host contribute to the up-regulation of the mutation rate, e.g. |
| drug resistance in Mtb?                       | alkylative stress, low pH, hypoxia?                             |
| Does tobacco smoke affect the mutation rate   | Is there a strong association between burning of fossil fuels/smoking and drug resistance? |
| of Mtb?                                       | Does tobacco smoke affect the mutation rate of Mtb?              |

\(^{\text{a}}\)3R genes are involved in DNA replication, recombination and repair.
Spontaneous mutations in genes coding for DNA metabolic proteins can lead to the emergence of mutator strains that carry a short-term selective advantage owing to their capacity to produce a higher number of adaptive mutations. In some cases, these mutators are maintained owing to the linkage of the mutator allele with other beneficial mutations, but only as long as the fitness gain counterbalances (or exceeds) the cost inherent in the increased risk of generating deleterious mutations. For this reason, mutations associated with elevated mutation rates are expected to be lost from bacterial populations over time. The adaptive mutations may, however, become fixed if the mutator alleles revert to wild-type, or if suppressor mutations occur.

There has been considerable interest in the potential association of mutator alleles with the emergence of drug resistance in M. tuberculosis. For example, mutations in mutT4 and mutT2 appeared to define certain sublineages of Beijing isolates. Beijing strains have been linked with the emergence of drug resistance in some settings, however, the apparent association of specific strains with drug resistance could reflect transmission, a possibility that is consistent with the fact that no direct link has been demonstrated between the reported DNA repair mutations and the emergence of drug resistance. Instead, biochemical evidence suggests that the observed mutations might not impact DNA repair function at all. Moreover, although mutT2 and mutT4 are annotated as DNA repair-type Nudix hydrolases, the substrate specificity of these enzymes in vitro suggests a role separate from DNA repair, perhaps in regulating nucleotide availability. Consistent with this idea, MutT2 and MutT4 do not function as antimutator proteins. It is possible, however, that the observed mutations confer other phenotypes that indirectly affect the acquisition of drug resistance, but not via a DNA repair pathway—e.g. the G58R mutation in mutT2 has been proposed to increase the replication rate in macrophages.

The evidence for the association of Beijing strains with an elevated mutation rate is mixed. In one study, various Beijing strains as well as non-Beijing strains were subjected to fluctuation analyses to determine their respective mutation rates. The assumption that mutator strains would exhibit at least a 10-fold higher mutation rate than corresponding ‘non-mutator’ strains; however, the authors observed no difference between the principal regulator and key mutagenic polymerase of the mycobacterial SOS response, respectively—expression of dnaE2 was modestly, but significantly, up-regulated in S522L, H526D and S531W rpoB mutants, with S522L showing the highest constitutive expression. This is an intriguing observation; however, given that DnaE2 depends on the activity of the imuA′- and imuB-encoded accessory factors—which, like dnaE2, are included in the mycobacterial SOS regulon—the functional consequences of increased levels of DnaE2 alone remain unclear. In other work, the same authors demonstrated that an isoniazid-resistant Haarlem strain harbouring an S315T mutation in katG was also associated with an increased mutation rate. Again, this is an intriguing observation, and suggests that other unidentified strain-dependent mutations could interact with drug resistance-conferring mutations to confer a mutator phenotype.

Transient mutagenesis

In contrast to constitutive mutator strains, a transient mutator phenotype results in a temporary increase in the mutation rate. There are a number of mechanisms by which this may occur: mis-translation of proteins, especially those involved in accurate
transcriptional mutagenesis and the up-regulation of error-prone DNA polymerases. Consistent with the multiplicity of mechanisms that can generate this phenotype, it is likely that transient mutator strains are responsible for the majority of adaptive mutations in bacterial populations.

Transcriptional mutagenesis occurs when RNA polymerase bypasses DNA lesions, inserting incorrect nucleotides into the mRNA. If one of the resulting mutant proteins confers an altered growth phenotype, it may in turn lead to DNA replication past the mutagenic lesion, which could lead to the fixation of the mutation. Alternatively, the mRNA containing an error may encode a protein involved in DNA repair or replication, which could lead to a transient mutator phenotype. The transcription-repair coupling factor, Mfd, recruits the NER repair machinery to sites where RNA polymerase is stalled. Mfd was shown to play a role in mutagenesis in Bacillus subtilis and to mediate point mutations conferring fluoroquinolone resistance in Campylobacter jejuni. Currently, nothing is known about transcriptional mutagenesis in M. tuberculosis, suggesting this as a potentially useful area of future research.

Mistranslation describes any error that affects the accurate translation of mRNA into protein. A major mechanism by which this occurs is through mischarging of tRNAs, which can happen when the tRNA gene is mutated or when aminoacyl-tRNA synthetases are defective in their editing capabilities. Mistranslation was associated with an elevated mutation rate under conditions of stress in Candida albicans, Acinetobacter baylyi and E. coli. To date, there are no published data on the impact of mistranslation on the mycobacterial mutation rate.

Perhaps the most intensively studied transient mutator system is the bacterial SOS response, which is induced by DNA damage. In most bacteria, the SOS response is regulated by the RecA/LexA system; during normal growth, SOS genes are negatively regulated by the LexA repressor protein. Following genotoxic damage, RecA protein binds to single-stranded DNA at sites of DNA lesions and replicon arrest to form RecA–ssDNA filaments that can interact with LexA, stimulating its cleavage and de-repressing SOS regulon genes. Unlike bacteria such as E. coli, whose SOS regulons include multiple Y-family DNA polymerases capable of translesion synthesis (‘TLS’), the DNA damage response in M. tuberculosis is limited to the dnaE1- and dnaE2-encoded catalytic (α) subunits of the C-family DNA polymerase III. M. tuberculosis encodes two DinB-like Y-family polymerases, however, neither is induced as part of the mycobacterial SOS response. Critically, deletion of dnaE2 has been shown to reduce the frequency of rifampicin resistance emergence in the mouse model, thereby implicating the mutagenic cassette as a major mechanism driving adaptive evolution during chronic infection. It is tempting, therefore, to speculate that DnaE2-dependent translesion synthesis functions in mycobacterial evolution; however, this requires further investigation.

Another low-fidelity repair system recently characterized in M. smegmatis, a model organism for M. tuberculosis, is that of non-homologous end joining (NHEJ). Three proteins seem to be key to this process: Ku, LigD and LigC. Even though NHEJ cannot strictly be considered a transient mutator system, it has the potential to elevate mutagenesis if up-regulated under certain conditions, a possibility that requires further investigation.

Environmental mutagens

Antibiotics

Antibiotics target essential functions and therefore impose a strong selective force for genetic resistance. At subinhibitory concentrations, antibiotics can also act as mutagens, driving the emergence of drug-resistant mutants, a phenomenon that has been demonstrated in vitro for different bacterial species and a variety of antibacterial classes. M. tuberculosis may encounter subinhibitory drug concentrations as a result of patient non-compliance, poor absorption by the gastrointestinal tract, poor penetration of—or activity in—certain parts of the lung and host genetic factors that impact drug metabolism and clearance. M. tuberculosis might also inadvertently be exposed to broad-spectrum antibiotics (e.g. fluoroquinolones) in patients undergoing treatment for other respiratory infections (e.g. community-acquired pneumonia). Subinhibitory concentrations of ciprofloxacin, a fluoroquinolone whose mechanism of action results in the induction of the mycobacterial SOS response, have been shown to elevate in vitro mutagenesis in related mycobacterial species. Moreover, there is some evidence to suggest that fluoroquinolone use prior to diagnosis of TB may be associated with first-line drug resistance, especially if multiple prescriptions have been given. However, this topic is controversial owing to conflicting evidence.

It is likely, too, that antibiotics that do not act directly on DNA metabolic processes can lead to DNA damage. For example, multiple studies have reported that bactericidal antibiotics of very different classes—including β-lactams, fluoroquinolones and aminoglycosides—are united by a common mechanism of killing that results in the generation of hydroxyl radicals. Although very recent studies have questioned the general applicability of the model, there is some evidence implicating the antibiotic-dependent formation of reactive oxygen species (ROS) in an elevated mutation rate. In M. tuberculosis, the production of ROS has been directly correlated with the ability to eliminate an antibiotic-tolerant bacillary population in vitro; however, to our knowledge, the link between ROS and mutagenesis has not been explored.

Genome-wide expression studies demonstrated that fluoroquinolones induce up-regulation of SOS clusters in M. tuberculosis, whereas inhibitors of translation do not. Consistent with the prediction that isoniazid might potentially generate ROS, genome-wide studies have detected up-regulation of the SOS response and recA following isoniazid exposure. Similarly, the ligD gene, essential for mycobacterial NHEJ, was also induced. To date, however, the effect of subinhibitory concentrations of anti-TB antibiotics on the mutation rate of the organism has not been investigated in vitro, or in patients developing drug-resistant TB. Although challenging, an analysis of the drug regimens or drug combinations that could lead to an increased rate of emergence of drug resistance in M. tuberculosis-infected patients could, therefore, be of...
great value in estimating the risk of acquisition of drug resistance during chemotherapy.

**Antiretroviral drugs**

It was previously suggested that antiretroviral drugs may affect the mutation rate of *M. tuberculosis*. However, the evidence for an association between HIV status and *M. tuberculosis* drug resistance on an individual level is mixed. One class of antiretroviral comprises nucleoside reverse transcriptase inhibitors (NRTIs), which have been associated with mutagenic effects in animal models and humans. NRTIs are nucleoside analogues and may be genotoxic in bacteria owing to their ability to be incorporated into bacterial DNA, causing chain termination, which itself may lead to the induction of the SOS response. Alteration of nucleotide pools and conformational changes in DNA polymerase might similarly also lead to reduced replication fidelity. However, while NRTIs are known to elevate the mutation rate of the HIV genome, there are no published studies that have assessed their potential effects on *M. tuberculosis*.

**TB pathogenesis and the host environment**

The discrepancies observed between apparent mutation rates (and frequencies) in vivo and those measured in vitro suggest that the factors modulating the mutation rate during host infection might be very different from those operating under laboratory conditions. For example, a Beijing strain was associated with an elevated *in vitro* frequency of rifampicin resistance—but not isoniazid resistance—compared with EAI strains; yet the converse was observed in a mouse model of infection. Similarly, Bergal et al. detected different types of isoniazid resistance-conferring mutations in vitro versus those generally observed clinically. Therefore, determining the mutagenic stimuli and DNA-damaging events encountered *in vivo* may be critical to the identification of those factors that drive adaptive evolution and the emergence of drug resistance during host infection.

*M. tuberculosis* encounters many DNA-damaging influences during the infectious lifecycle (reviewed by Gorna et al.). For example, during aerosol transportation, bacilli are likely to encounter UV radiation and desiccation. UV radiation induces expression of lexA, dnaE2 and recA, while *M. smegmatis* mutants defective in *ku* and ligD are susceptible to desiccation. Expression of *ku* was also higher in granulomas than in broth. In addition, the *mutT2* gene, which may be involved in hydrolysis of 8-oxo-dGTP, was down-regulated in response to lung surfactant. This could lead to an increase in 8-oxo-dGTP in nucleotide pools and elevated mutagenesis.

Oxidative stress is a major mutagenic influence encountered by *M. tuberculosis* in the host. Macrophages, as an antibacterial defence mechanism, produce ROS and reactive nitrogen intermediates (RNI). These reactive species interact with nucleotides, resulting in chemical modifications that can lead to base mispairing and DNA damage. DNA damage can, in turn, lead to an up-regulation of error-prone DNA repair. *M. tuberculosis* is well armed against oxidative stress; moreover, mechanisms to detoxify ROS and RNI are essential for the survival of this pathogen in the host. The high levels of redundancy in these and specific DNA repair pathways might, therefore, indicate a critical role in pathogenesis. The high GC content of the genome also suggests that *M. tuberculosis* may be especially vulnerable to oxidative damage. Similarly, observations from the non-human primate model suggest a role for ROS in the spectrum of mutations observed.

In contrast, analyses by O’Sullivan et al. of mutations in *rpoB* and *pncA* in clinical isolates suggest a pattern inconsistent with oxidative stress as the major driver of bacillary evolution. Their study was based on the assumption that resistance to isoniazid most often occurs before resistance to rifampicin (conferring by mutations in *rpoB*) or pyrazinamide (conferring by mutations in *pncA*). Isoniazid resistance is conferred by mutations in *katG*, encoding an enzyme that functions in the oxidative stress response. Mutations in *katG*, including the clinically dominant S315T mutation, have been shown to have a detrimental effect on the activity of this enzyme. Therefore, *katG* mutants are expected to undergo mutagenesis driven by oxidative stress. However, O’Sullivan et al. did not observe the expected overall increase in G to A or C to T mutations in the genes analysed.

It is possible, though, that fitness plays a greater role in defining the mutation spectra of resistance genes in clinical isolates. This interpretation is consistent with evidence from another study that investigated the effect of lowering the pH on mutational events during *in vitro* culture of *M. tuberculosis*. Although the decrease in pH impacted the mutation frequency only slightly, there was a major effect on the mutation spectrum, in which rare, ‘less-fit’ mutations were observed. As discussed above, there is the possibility that less-fit mutants are associated with up-regulated expression of *dnaE2*, which might provide the opportunity for the development of compensatory mutations, a possibility that requires further investigation.

**Smoking and air pollution**

An association between drug resistance and smoking or tobacco use has been observed in some cases. Cigarette smoke contains mutagenic chemicals and smoking and environmental pollutants could also alter the redox balance, in turn affecting the mutation rate. A compound typically generated during combustion, 1,6-dinitropyrene (1,6-DNP), increased the incidence of drug resistance in *Pseudomonas aeruginosa*. Whether this compound, and other similar compounds, are important mutagens in the development of drug resistance in *M. tuberculosis* remains to be determined.

**Conclusions**

*M. tuberculosis* has a low mutation rate *in vitro*, yet seems capable of generating surprisingly high levels of genomic diversity within the host. However, determining whether the mutation rate is modulated during host infection is complicated by the fact that it is difficult to distinguish between the rate of selection for drug resistance and the rate at which the genotypic diversity for selection is itself generated. Moreover, the extent to which the mycobacterial replication rate itself is modulated during host infection remains unknown. Therefore, it is not clear whether the mutation rate of *M. tuberculosis* is higher in the host than *in vitro* or whether there are factors specific to the *in vivo* environment that might drive mutagenesis. Knowledge of the contribution made by transient and constitutive mutator strains to the mutation rate and, therefore, the evolution of drug resistance in *M. tuberculosis* represents an
important area for future research as part of continued efforts to inhibit—and prevent—the acquisition of drug resistance.

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