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Strain diversity, epistasis and the evolution of drug resistance in *Mycobacterium tuberculosis*

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Abstract

Mycobacterium tuberculosis harbours little DNA sequence diversity compared with other bacteria. However, there is mounting evidence that strain-to-strain variation in this organism has been underestimated. We review our current understanding of the genetic diversity among *M. tuberculosis* clinical strains and discuss the relevance of this diversity for the ongoing global epidemics of drug-resistant tuberculosis. Based on findings in other bacteria, we propose that epistatic interactions between pre-existing differences in strain genetic background, acquired drug-resistance-conferring mutations and compensatory changes could play a role in the emergence and spread of drug-resistant *M. tuberculosis*.

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Introduction

Tuberculosis (TB) remains an important global health problem, with close to 10 million new cases per year and a pool of 2 billion latently infected individuals worldwide [1]. Of particular concern are the ongoing epidemics of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), which threaten to make TB incurable [2]. Tuberculosis is a complex phenomenon driven by multiple biological, socioeconomic and environmental factors [3]. In this review, we discuss some of the bacteriological factors involved in TB with a particular focus on drug resistance. We start by reviewing our current knowledge of the genetic diversity in the *Mycobacterium tuberculosis* complex (MTBC), the causative agent of human TB, and how this diversity influences the emergence of drug-resistant strains. We then summarize some recent findings from other bacteria on the role of epistasis in the evolution of drug resistance, and end by discussing possible implications for our understanding of MDR-TB and XDR-TB.

Strain Diversity in MTBC

Despite exhibiting limited DNA sequence diversity compared with other bacteria [4], the extent of strain diversity in MTBC is more pronounced than previously believed [5]. Recent comparative genomic studies have shown that genomic deletions and duplications represent an important source of genomic plasticity in MTBC [6–13]. Furthermore, investigation of global collections of clinical strains revealed that human MTBC exhibits a phylogeographic population structure with different strain lineages associated with particular geographic regions and human populations [6,14–18]. In a study by Hershberg *et al.* [19], 89 genes were sequenced in each of 108 strains representative of the global diversity of MTBC. Based on these gene sequences, the authors con-

structed the most comprehensive DNA sequence-based phylogeny of MTBC to date [20]. The authors also showed that the genetic distances between the most distant human MTBC strains were equivalent to the genetic distance between an average human strain and animal-adapted MTBC [19]. Because animal-adapted MTBC such as *Mycobacterium bovis* and *Mycobacterium microti* are believed to form distinct ecotypes within MTBC [21], the findings by Hershberg *et al.* [19] support the notion that the different human-adapted lineages of MTBC might represent different ecotypes adapted to different human populations [6,18]. In 2010, Comas *et al.* [22] used 21 whole genome sequences to generate the first whole genome-based phylogeny of human-adapted MTBC. This phylogeny confirmed that human-adapted MTBC consists of six main phylogenetic lineages, two of which are also known as *M. africanum* [23].

DNA sequencing studies in MTBC clinical isolates found consistently that two-thirds of the single nucleotide polymorphisms in MTBC were non-synonymous [19,22,24]. Furthermore, the study by Hershberg *et al.* [19] showed that about 40% of these non-synonymous single nucleotide polymorphisms occurred at positions that were highly conserved in other mycobacteria, and therefore were likely to affect gene function. The authors concluded that purifying selection against non-synonymous single nucleotide polymorphisms was reduced in MTBC. Despite this high proportion of non-synonymous single nucleotide polymorphisms in MTBC, the study by Comas *et al.* [22] found that essential genes were more evolutionarily conserved than non-essential genes. Hence, even though purifying selection in MTBC might be reduced compared with other bacteria [19], natural selection is still acting on MTBC and differentiating between various gene classes [22].

The studies reviewed here show that strain genetic diversity in MTBC includes genomic insertions/deletions and duplications, as well as non-synonymous single nucleotide polymorphisms, many of which are predicted to have functional effects. An important question is how this genetic diversity translates into phenotypic diversity. A recent review compiled the results of 100 published studies that evaluated the effect of MTBC strain diversity on experimental and clinical phenotypes [25]. Based on this review, one concludes that clinical strains of MTBC differ in immunogenicity and virulence in infection models. However, the role of strain diversity in clinical settings is less clear. One of the difficulties when studying the impact of strain diversity in patient populations is that many additional variables need to be taken into account [3]. Furthermore, strain classification for MTBC remains an issue as no universally accepted standard has been defined. Such a new standard is urgently needed and should be based on whole genome sequencing [26].

In addition to the possible impact of strain variation on the outcome of TB infection and disease, this diversity is relevant for our understanding of drug resistance. For example, the so-called 'Beijing' family of strains has been associated with drug resistance in multiple reports (reviewed in [27]). However, the underlying cause of this association remains unknown, even though multiple hypotheses have been put forward. One thought is that the genetic background of Beijing strains might predispose to the acquisition of mutations that confer drug resistance [28]. However, evidence for an increased mutation rate in Beijing strains is still lacking [29]. Before discussing other possible effects of MTBC diversity on drug resistance, let us briefly review some of the most important features of the current epidemic of drug-resistant TB.

Factors Driving MDR/XDR-TB

The past 20 years have seen the worldwide appearance of MDR-TB, followed by XDR-TB [2], and, most recently, strains that are resistant to all antituberculosis drugs, the so-called 'totally drug-resistant' or TDR strains [30]. MDR-TB is caused by MTBC that is resistant to at least isoniazid and rifampicin, the most important first-line drugs against TB [2]. XDR-TB is caused by MDR strains with additional resistance to any fluoroquinolone and one of the three injectable drugs, capreomycin, kanamycin and amikacin. MTBC acquires MDR and XDR through a stepwise accumulation of chromosomal mutations. In most cases, each of these mutations confers resistance to an individual drug [31].

Drug resistance in MTBC can be acquired *de novo* in individual patients undergoing TB treatment, either because of lack of patient adherence or through an interrupted drug supply. Alternatively, drug-resistant strains can spread through direct transmission of MTBC strains that are already drug-resistant. In addition, treatment for other diseases can also contribute to acquired resistance in TB. For instance, widespread use of fluoroquinolones for respiratory tract and other infections might drive resistance to fluoroquinolones in TB [32]. Given the financial burden and logistical difficulties associated with treating MDR-TB and XDR-TB [2], it is important to understand the evolutionary mechanisms that promote the emergence of highly drug-resistant MTBC.

Mathematical models predict that one of the most important factors influencing the future of MDR/XDR-TB is the relative fitness of drug-resistant strains compared with drug-susceptible strains [33–35]. As in other bacteria [36], resis-

tance-conferring mutations are often associated with a fitness 'cost' in MTBC [37–39]. However, this fitness cost varies depending on the specific drug-resistance-conferring mutations, and mutations associated with no fitness cost have also been described [37–41]. Furthermore, the strain genetic background in which a specific resistance-conferring mutation occurs can modulate the fitness impact of this mutation [37]. In clinical settings, there seems to be a strong selection for low- or no-fitness cost mutations [37,38,40–42]. However, data on the relative transmissibility of MDR strains compared with drug-susceptible strains are inconsistent (reviewed in [27]), suggesting that the fitness of drug-resistant strains is heterogeneous [33]. Both MDR-TB and XDR-TB are often associated with human immunodeficiency virus (HIV) co-infection, indicating that drug-resistant strains might be less fit than fully susceptible strains [27]. However, in some areas of the world, such as the countries of the former Soviet Union where the prevalence of MDR is particularly high, MDR strains of MTBC are highly successful, despite low rates of HIV [1]. One possibility is that because MDR strains have been circulating for a long time in these areas, compensatory evolution has occurred [43], which alleviated initial fitness deficits. Interestingly, these regions are also where the Beijing family of strains has been associated most frequently with drug resistance (reviewed in [27]), suggesting that the strain genetic background may also play a role.

Another question is how the effects of different drug-resistance-conferring mutations influence each other. Is it that with each additional mutation a strain will become less and less fit, and we therefore need not worry about the transmission of highly drug-resistant strains? Despite the importance of fitness cost in the evolution of drug resistance, no study has addressed this question in MTBC.

In summary, the emergence of drug resistance in MTBC depends primarily on the initial acquisition of drug-resistance-conferring mutations. However, the fitness effects of these mutations could be modulated by additional genetic factors, including pre-existing differences in strain genetic background, additional drug-resistance-conferring mutations, and compensatory adaptations. The interactions between these types of genetic changes are generally referred to as epistasis (Fig. 1).

Epistasis in Drug-resistant Bacteria

Epistasis occurs when the phenotypic effect of a mutation changes depending on the presence or absence of other mutations in the same genome [44]. Interactions among

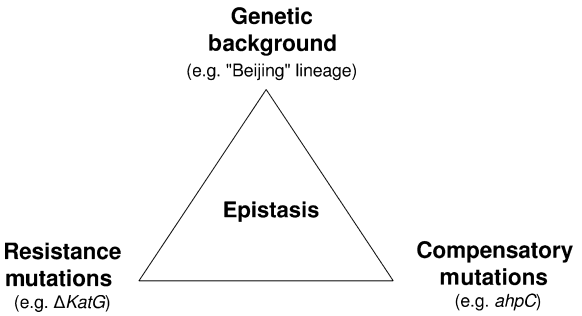

FIG. 1. Proposed epistatic interactions in drug-resistant *Mycobacterium tuberculosis* complex (MTBC). Human-adapted MTBC consists of six main phylogenetic lineages. The genetic background of these strain lineages could interact differently with drug-resistance-conferring mutations. Similar interactions could occur between different drug-resistance-conferring mutations and compensatory mutations.

mutations can result in positive epistasis in the case of beneficial mutations and negative epistasis in the case of deleterious mutations. Although epistasis can impact any phenotype, in this review, we will focus on the role of epistasis in drug-resistant bacteria. Here, epistasis becomes manifest when a particular drug-resistance-conferring mutation has a different fitness effect depending on the strain genetic background. Several studies have observed this phenomenon in drug-resistant bacteria [37,45,46]. Alternatively, when bacteria carry multiple drug-resistance-conferring mutations, the overall fitness cost of resistance will depend on the cost associated with each individual resistance mutation and on the epistatic interactions between these resistance mutations [47]. Positive epistasis occurs when the combined cost of carrying multiple resistance mutations is less than what would be expected if the mutations had independent and simple additive effects on fitness. Whereas positive epistasis promotes the evolution of MDR by minimizing its cost, negative epistasis constrains the evolution of MDR by aggravating its cost [47].

Epistatic interactions can also affect compensatory adaptation to the fitness costs of resistance. Positive epistasis can be caused by a compensatory mutation, when the mutation mitigates the fitness costs of a particular resistance-conferring mutation but does not in itself contribute to drug resistance [48]. Compensatory evolution has been described in many drug-resistant bacteria (reviewed in [43]). Importantly, several studies have found that when compensatory mutations were placed into their respective wild-type drug-susceptible strain backgrounds, they became deleterious to strain fitness, thereby demonstrating the effect of epistasis [47,49,50]. These observations have important implications for public health because once drug-

resistant bacteria have acquired a compensatory mutation, positive epistasis between the drug-resistance-conferring mutation and the compensatory mutation will prevent the loss of resistance, even if the corresponding antibiotic is withdrawn [43].

Few studies have looked at epistatic interactions between multiple drug-resistance-conferring mutations in bacteria. A study in *Pseudomonas aeruginosa* showed that the cost of streptomycin resistance was lower when the streptomycin-resistance-conferring mutations were in a rifampicin-resistant genetic background as opposed to an isogenic rifampicin-susceptible control background [51]. Similarly, Trindade *et al.* [47] showed that in *Escherichia coli*, the cost of streptomycin resistance could be compensated by various mutations conferring resistance to rifampicin. In the remaining part of this review, we will discuss some observations that might hint towards possible epistatic interactions in drug-resistant MTBC.

Signs of Epistasis in Drug-resistant MTBC

As mentioned above, the Beijing family of strains has repeatedly been associated with drug resistance [27]. This association could reflect positive epistasis between the Beijing genetic background and one or several drug-resistance-conferring mutations. A study from San Francisco reported an association between different phylogenetic lineages of MTBC and particular isoniazid-resistance-conferring mutations [52]. Interestingly, some of these associations were independently reported in a study from London [14]. Similarly, a study from Ghana found differences between the frequencies of particular isoniazid-resistance-conferring mutations in *M. africanum* compared with other MTBC lineages [53]. Taken together, these data are consistent with possible epistatic interactions between different isoniazid-resistance-conferring mutations and pre-existing differences in the genetic backgrounds of the different lineages of MTBC [19].

Little is known with respect to the interactions between different drug-resistance-conferring mutations in MTBC. One intriguing observation though is that the isoniazid-resistance-conferring mutation *katG* S315T is associated with MDR-TB [54]. *KatG* encodes a catalase–peroxidase that converts isoniazid into its bioactive form. However, this protein also protects MTBC against oxidative stress [55]. Clinical strains with an inactivated *KatG* are highly resistant to isoniazid but are also attenuated in virulence [56,57]. In contrast, *katG* S315T has only a minimal effect on virulence and strain fitness [58]. If MDR clinical strains are selected to minimize overall fitness deficits, low- or no-cost mutations like *katG* S315T are expected to be over-represented in clinical settings [59]. An alternative but not mutually exclusive explanation is that the *katG* S315T mutation might interact in a positive epistatic way with other drug-resistance-conferring mutations. A study by Hazbon *et al.* [60] showed that laboratory-generated mutants with the *embB306* mutation, usually associated with resistance to ethambutol, had a growth advantage under subminimal inhibitory concentrations of isoniazid and rifampicin. Therefore, the authors postulated that *embB306* might be involved in the acquisition of resistance to isoniazid and rifampicin.

To date, only two papers have reported compensatory mechanisms in drug-resistant MTBC. Sherman *et al.* [61] described a putative compensatory mutation related to isoniazid resistance. They showed that MTBC strains with an inactivated *katG* acquired promoter mutations of the alkyl hydroperoxide reductase *ahpC*, leading to the over-expression of this protein. The authors concluded that over-expression of *ahpC* might compensate for the lack of detoxification through an inactivated *katG*. Another study used the model organism *Mycobacterium smegmatis* to investigate compensatory evolution in aminoglycoside resistance in MTBC [50]. Clinically acquired resistance to kanamycin and amikacin in MTBC is mainly the result of point mutations in the 16S rRNA. The change G1491U confers an intermediate-level of resistance but is associated with a significant fitness cost in *M. smegmatis*. Introduction of C1409A into the G1491U mutant restored fitness to wild-type levels while maintaining resistance. Interestingly, C1409A/G1491U double mutants are also found in MTBC clinical isolates, albeit at low frequencies [50].

Conclusions

In summary, the extent of strain genetic diversity in MTBC is more pronounced than was traditionally believed, which has implications for the development of new TB diagnostics, drugs and vaccines [5]. Although there is clear evidence that this diversity impacts the host immune response and virulence in infection models, more work is needed to demonstrate consistent effects in clinical settings [25]. Furthermore, there are reasons to believe that strain diversity also plays a role in the global emergence of MDR-TB and XDR-TB. In particular, epistatic interactions between different drug-resistance-conferring mutations, the strain genetic background, and compensatory mutations need to be studied in more detail. Such studies will be necessary to make more accurate predictions about the future trajectory of the global epidemics of MDR-TB and XDR-TB.

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Transparency Declaration

Conflicts of interest: nothing to declare.

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