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## The Heterogeneous Evolution of Multidrug-Resistant *Mycobacterium tuberculosis*

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### Abstract

Recent surveillance data of multidrug-resistant tuberculosis (MDR-TB) reported the highest rates of resistance ever documented. As further amplification of resistance in MDR strains of *Mycobacterium tuberculosis* occurs, extensively drug-resistant (XDR) and totally drug resistant (TDR) TB are beginning to emerge. Whilst for the most part, the epidemiological factors involved in the spread of MDR-TB are understood, insights into the bacterial drivers of MDR-TB have been gained only recently, largely owing to novel technologies and research in other organisms. Herein, we review recent findings on how bacterial factors such as persistence, hypermutation, the complex interrelationship between drug resistance and fitness, compensatory evolution, and epistasis affect the evolution of multidrug resistance in *M. tuberculosis*. Improved knowledge of these factors will help better predict the future trajectory of MDR-TB, and contribute to the development of new tools and strategies to combat this growing public health threat.

### Keywords

drug resistance; persistence; hypermutation; compensatory mutations; epistasis

### The rise of MDR-TB

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis*, is one of humankind's deadliest diseases. The World Health Organization (WHO) estimated 8.7 million new cases and 1.4 million deaths due to TB in 2011, including 430,000 deaths estimated to have occurred among HIV co-infected patients [1]. Although most recent models suggest that the absolute number of TB cases has decreased since 2006 [1], multidrug-resistant (MDR) TB is threatening disease control efforts throughout the globe [2]. MDR-TB is defined by the resistance to isoniazid and rifampicin, the two most potent anti-TB drugs (Box 1), and requires treatment with much more expensive second-line drugs, which, in addition to being costly, cause more adverse events and show lower cure rates [2, 3]. MDR-TB with additional resistance to any fluoroquinolone and one of the second-line injectable drugs

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kanamycin, amikacin, or capreomycin is referred to as extensively drug-resistant (XDR) TB [2, 3]. Recently, cases of vaguely defined totally drug-resistant (TDR) TB were reported [4, 5], suggesting that human TB is joining the growing list of bacterial diseases entering the post-antibiotic era [6].

Drug-resistant TB can be “acquired” *de novo* during treatment, or it can occur through the transmission of already resistant strains; the latter being referred to as “primary resistance” [2]. Primary resistance poses a particular challenge as less than 20% of the estimated MDR-TB cases in the world are believed to be properly diagnosed, largely due to the lack of appropriate laboratory infrastructure in low-income areas that bear the highest disease burden [1]. Disturbingly, while disease control has improved in many areas, MDR-TB incidence rates have increased considerably in other regions [7]. The prevalence of MDR-TB varies significantly across the globe (Figure 1) as a result of many contributing factors. These include variably effective control programs [7], the presence of co-morbidities, which may influence the emergence and spread of MDR-TB (e.g., HIV co-infection) [7, 8], numerous patient-related factors, including societal [2, 9], immunological [10] and genetic factors [11], and many less well understood bacterial properties such as bacterial physiology, genetics, and population biology (Figure 1 and 2) [12, 13]. Ecological theory predicts that bacterial fitness plays an important role in the emergence of drug-resistant bacteria [14]. Based on the dogma that drug-resistant strains are less fit than their drug-sensitive counterparts (see below) [15], early mathematical models suggested that MDR-TB would remain a localized problem [16, 17]. However, subsequent models allowed for heterogeneous fitness in drug-resistant strains and indicate that the former views were too optimistic [18, 19]. Importantly however, attempts to measure the relative fitness of drug-resistant strains of *M. tuberculosis* using epidemiological approaches have been highly inconclusive (reviewed in [20]). This highlights the need to better understand the underlying biology of *M. tuberculosis* and the evolutionary forces driving the emergence and spread of drug-resistant bacteria. For many years, the bacterial factors involved in the emergence of MDR-TB remained elusive, but recently, an increasing number of studies investigating how bacterial biology influences the evolution of MDR-TB have begun to provide new insights into this important facet of MDR-TB. The purpose of this review is to summarize these findings and place them in the broader context of our current understanding of MDR-TB. We then discuss how these new insights could be used to improve the treatment and prevention of this deadly disease.

## The development of drug resistance in *M. tuberculosis*

*M. tuberculosis* is intrinsically resistant to numerous antibiotics, and only a handful of drugs are effective for treatment [21]. This intrinsic resistance is partially due to the thick, lipid-rich cell wall, which is an important characteristic of mycobacteria, limiting the penetration of antibiotics. Moreover, mycobacteria possess various defence systems against the activity of antibiotics, including potent  $\beta$ -lactamases and other drug-neutralizing enzymes [21]. Herein, we will focus on the *de novo* development of resistance to the drugs that wild-type *M. tuberculosis* is usually susceptible to, i.e., the drugs used for standard TB treatment (Box 1). Clinically relevant drug resistance in TB is defined as the genetically encoded, increased capacity to tolerate the presence of a specific drug compared to drug-susceptible bacilli [2]. Unlike many other bacteria, *M. tuberculosis* harbours no resistance plasmids, and drug resistance arises through the acquisition of specific chromosomal mutations [22]. The population structure of *M. tuberculosis* is essentially clonal, suggesting a limited role for horizontal gene transfer (HGT) in the biology of *M. tuberculosis* [23]. A recent report has challenged this view [24], but a putative role of HGT in the emergence of drug resistance in *M. tuberculosis* remains to be demonstrated. Drug resistance-conferring mutations in *M. tuberculosis* have been described in genes encoding enzymes directly targeted by the

antibiotics, in regulatory regions of these genes, or in gene products involved in the activation of pro-drugs (Table 1) [25]. Classical Darwinian models suggest that resistance-conferring mutations appear stochastically within bacterial populations and independently of drug exposure [26]. The average rate of emergence of spontaneous resistance mutations to isoniazid and rifampicin in *M. tuberculosis* was estimated at  $10^{-8}$  and  $10^{-9}$  mutations/bacterium/cell division, respectively [27], suggesting that during mono-therapy, the appearance of resistant bacteria will occur almost inevitably, given an average number of approximately  $10^8$  bacilli present in individual TB lesions [28]. Consequently, TB has to be treated with a combination therapy including at least four drugs (Box 1) [2, 3]. Nevertheless, selection of drug-resistant bacteria can occur during intermittent exposure to sub-therapeutic drug levels, initially producing hetero-resistant and finally fully resistant bacterial populations. Hetero-resistance refers to the situation where patients harbour different subpopulations of bacteria, some drug-resistant, and others still drug-susceptible [29]. The *de novo* emergence of drug resistance in an individual patient can happen because of patient non-adherence [2], poor drug quality, and patient-dependent pharmacodynamic and -kinetic properties of the drugs administered [11, 30]. Additionally, treatment of co-morbidities (e.g., HIV) can influence pharmacodynamic properties of anti-TB drugs and increase the likelihood of resistance development [31]. Importantly, resistance-conferring mutations to a given drug may confer cross-resistance to other drugs targeting related metabolic pathways [32], and the anti-TB drugs themselves can interact in ways that could promote drug resistance [33].

Among the bacterial factors promoting drug resistance, recent findings highlight a possible role for “persisters” [34]. These are subpopulations of cells that can phenotypically tolerate increased concentrations of drugs, however, they are not genetically resistant (i.e., drug resistance will not be inherited by daughter cells). This phenomenon is also referred to as “phenotypic drug tolerance”. Following the classical understanding, persistence requires phenotypic differentiation into persister cells and is often linked to a state of slow growth or dormancy. This process can be triggered by various factors such as starvation, quorum sensing, intracellular signals, and antibiotic treatment itself [34, 35]. However, persistence is not strictly associated with dormancy; for example, active mycobacterial growth inside macrophages can induce efflux pumps and confer persistence to bacteria upon exposure to anti-TB drugs [36]. Moreover, phenotypic variation among genetically identical bacterial cells can also involve stochastic processes, including spontaneous phenotypic switches, which are thought to facilitate adaptation to fluctuating environments [37]. In addition, deterministic phenotypic variation among genetically identical bacteria can contribute to persistence. For example, it was recently shown that cell division occurs asymmetrically in mycobacteria, which results in an unusual, unipolar growth and a differential ability of the two daughter cells to tolerate anti-TB drugs [38]. Although the phenotypic resistance of persisters is not heritable, persistence prolongs the average lifetime of bacteria exposed to drugs and could therefore increase the likelihood for the subsequent acquisition of a high-level resistance mutation.

A recent study has shown that mutations in *M. tuberculosis* can occur during latent infections, when bacilli are thought to be replicating very slowly or not at all [39]. These mutations are presumably the result of oxidative DNA damage rather than replication errors, and indicate that *M. tuberculosis* might have the capacity to acquire drug resistance during latency [39]. This process could be further facilitated in bacteria with an intrinsically elevated mutation rate (mutator genotypes), such as ones with mutations in the mismatch repair system [40]. An association between the acquisition of drug resistance and heritable hypermutation has been described for various bacterial species, including *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (reviewed in [40]). In *M. tuberculosis*, a particular phylogenetic lineage known as the “Beijing family” has been

repeatedly associated with MDR-TB [reviewed in [20]]. It was hypothesized that “Beijing” strains might exhibit hypermutation as this strain family harbours various nonsynonymous substitutions in putative mutator genes [41]. However, subsequent attempts to measure differences in mutation rates across *M. tuberculosis* lineages have yielded contradictory results [42, 43].

In addition to heritable hypermutation, the emergence of transient mutators has been described. For example, in *M. tuberculosis*, increased expression of *dnaE2*, a gene encoding an additional copy of the major replicative DNA polymerase DnaE2, was linked to transient error-prone replication under stress and to the emergence of drug resistance *in vivo* [44]. Moreover, the use of fluoroquinolones and streptomycin, both important anti-TB drugs (Box 1, Table), was shown to induce mutator phenotypes in *E. coli* [45, 46]. Fluoroquinolones are being evaluated as new first-line antibiotics for TB [47]. Whether the wider use of these drugs could promote the development of drug resistance in the future is unknown, but it is a disturbing possibility that should be carefully considered. In summary, many bacterial factors contribute to the acquisition of drug resistance-conferring mutations in *M. tuberculosis*. Below we discuss the effect of these mutations on bacterial fitness, and the evolutionary forces that determine the fate of these mutations in bacterial populations.

## Fitness cost and compensatory mechanisms

Studies from the 1950s revealed a reduced pathogenicity of isoniazid-resistant tubercle bacilli compared to drug-sensitive bacteria when inoculated into guinea pigs [48]. These and other observations led to the dogma that drug-resistant *M. tuberculosis* show reduced “fitness”, as they are presumably less transmissible and therefore unlikely to spread successfully in immunocompetent human populations [15]. However, this rather simplistic model is contradicted by numerous recent reports describing community outbreaks of MDR-TB, and the finding that in some areas, MDR-TB is more frequently caused by patient-to-patient transmission of drug-resistant strains than through *de novo* acquisition of resistance during patient treatment [2, 7]. Experimental studies in various bacterial species have shown that not all resistance-conferring mutations inflict the same level of fitness-cost in absence of the drug, with some showing mild or no detrimental effects (reviewed in [49]). In *M. tuberculosis*, similar studies have identified such “low/no-cost mutations” conferring resistance to various anti-TB drugs (Figure 3) [50–55]. Intriguingly, these studies also found that these mutations account for the majority of the resistance detected in clinical isolates [50, 51, 53–55], suggesting that strains harbouring low/no-cost mutations are positively selected among drug-resistant strains, either within the bacterial population infecting a host and/or as a result of variable transmission efficiency. In support of the latter, several studies have shown that among isoniazid-resistant strains of *M. tuberculosis*, those carrying low/no-cost resistance-conferring mutations were more successfully transmitted than strains harbouring other mutations [56–58].

In addition to the occurrence of low/no-cost mutations, research in different bacterial species has shown that the deleterious effects of drug resistance mutations can be mitigated by compensatory mutations (reviewed in [59]). In *M. tuberculosis*, reversions of resistance mutations are rarely observed, indicating a higher likelihood for the occurrence of compensatory evolution, consistent with the view that many more mutational targets exist for compensation compared to reversion [60]. Compensatory mutations impact the disease epidemiology and treatment practices for bacterial infections by preventing drug-resistant bacteria from being out-competed by drug-susceptible strains, even if the respective antibiotics are withdrawn [59]. Many mechanisms of compensatory evolution have been described in other bacteria (reviewed in [61]). These involve intragenic or intergenic mutations, which may reduce the need for, or restore the functionality of an impaired

enzyme. Alternatively, the need of a particular enzyme may be by-passed by an alternative enzymatic pathway. Evidence for compensatory evolution has also been reported for *M. tuberculosis* (Figure 3). KatG, a catalase-peroxidase involved in the degradation of harmful reactive oxygen intermediates, is required to convert the pro-drug isoniazid into its bioactive form (Table 1). Whilst the most frequently detected resistance mutations in *katG* at amino-acid position 315 only confer a low fitness-cost and do not abolish the function of KatG [53], other, more deleterious mutations in this gene were shown to be associated with compensatory mutations in the regulatory region of *ahpC* (Figure 3) [56, 62]. The resulting over-expression of the *ahpC* gene product, another peroxidase, is believed to partly compensate for the loss of KatG activity [63]. Similarly, mycobacterial resistance to aminoglycoside is mediated by mutations in the 16S rRNA. Some of these mutations destabilize the rRNA secondary structure, which can be compensated by a secondary mutation in the same gene (Figure 3) [64]. Compensatory mutations in the *ahpC* promoter and in the 16S rRNA are rarely observed in clinical isolates of *M. tuberculosis* [22], indicating that these mutations might have only limited epidemiological relevance. By contrast, recently described compensatory mutations in the RNA polymerase of *M. tuberculosis* were detected in more than 30% of MDR clinical isolates from high-burden countries [65, 66]. These mutations are situated within *rpoA* and *rpoC*, encoding the  $\alpha$ - and  $\beta'$ -subunits of the RNA polymerase, respectively, and alleviate the fitness-cost associated with resistance mutations in *rpoB*, the  $\beta$ -subunit of the RNA polymerase and target of rifampicin (Figure 3) [65]. Recent genetic reconstructions in *Salmonella enterica* confirmed that similar compensatory mutations in *rpoA* and *rpoC* are necessary and sufficient to mitigate impaired fitness due to rifampicin resistance-conferring mutations in *rpoB* in that species as well [67]. Taken together, the available evidence strongly suggests that the notion of drug-resistance being invariably linked to a fitness burden in the absence of drugs is too simplistic. Because of the occurrence of low/no-cost drug resistance mutations and the potential for compensatory evolution, drug-resistant bacteria are unlikely to disappear, even if the drug pressure is removed. In fact, over time, more opportunity for compensatory evolution arises, and circulating MDR and XDR strains might gradually become more and more transmissible.

## The broader role of epistasis

The interaction between a drug resistance-conferring mutation and a compensatory mutation is an example of epistasis, which is defined as the effect of the interaction between two or more mutations on an organism's phenotype [68]. Similarly, different drug resistance-conferring mutations can also interact epistatically. Studies in *E. coli*, *P. aeruginosa* and *Streptococcus pneumoniae* found that generally, the observed cumulative fitness cost of carrying multiple drug resistance-conferring mutations was below the expected sum of the fitness cost associated with each individual mutation; an observation referred to as “positive” or “antagonistic” epistasis [69–72]. Moreover, in some cases, strains resistant to two drugs had a higher fitness than at least one of the corresponding single drug-resistant mutants. In other words, the acquisition of one or more additional resistance determinants may sometimes ameliorate the fitness-cost caused by a preceding resistance mutation; a phenomenon termed “sign epistasis” [71, 73]. Although the interaction between different drug resistance-conferring mutations has not yet been studied in *M. tuberculosis*, a similar phenomenon in MDR-TB would represent a worst case scenario, as MDR strains could improve their fitness by acquiring mutations conferring resistance to additional drugs.

Epistatic interactions may also determine the order in which drug resistance-conferring mutations and associated compensatory mutations are acquired over time [74]. The nonrandom nature of mutational pathways to drug resistance has been demonstrated in several bacterial species [75–77]. Whilst non-bacterial factors will play a key role in the

acquisition of the different drug resistance mutations (Figure 2), epistatic interactions may determine the optimal mutational pathway. Epistatic interactions may also occur between drug resistance mutations and pre-existing genomic differences, which characterize a given strain genetic background [78]. Phylogenetic analyses of human disease-associated strains of the *M. tuberculosis* complex identified six main phylogenetic lineages [23], and there is mounting evidence that the specific strain genetic background as defined by these lineages plays a role in the emergence and spread of MDR-TB [13]. For example, strains harbouring identical rifampicin resistance mutations but belonging to different lineages of *M. tuberculosis* showed different levels of fitness-cost [50]. Moreover, different phylogenetic lineages of *M. tuberculosis* have been associated with different drug resistance mutations [56, 79, 80], and the level of resistance conferred by specific isoniazid resistance-conferring mutations has been shown to differ depending on the *M. tuberculosis* lineage [80]. As mentioned above, the “Beijing family” of *M. tuberculosis* has often been associated with drug resistance [20]. The association between specific *M. tuberculosis* lineages and drug resistance could result from an increased ability to tolerate a larger range of resistance mutations, either due to a higher baseline fitness, or more favourable epistatic interactions between the strain genetic background and drug resistance mutations. This idea is supported by the presence of various lineage-specific non-synonymous nucleotide substitutions in genes associated with drug-resistance (Figure 4) [25, 81]. Thus it is possible that the pre-existence of non-resistance mutations in drug resistance-associated genes could modulate the fitness effects of subsequently acquired resistance-conferring mutations in these genes.

Taken together, current evidence suggests that epistatic interactions between the strain genetic background, drug resistance-conferring mutations, and compensatory mutations may play a role in defining evolutionary trajectories towards multidrug resistance in TB. The genetic landscape of an MDR strain differs from a drug-sensitive strain by at least the presence of resistance mutations. Being forced to manage the potential fitness-cost associated with drug resistance mutations (e.g., through the acquisition of compensatory mutations), certain selective pressures imposed onto MDR strain populations will differ from those acting on drug-sensitive strains. Moreover, treatment regimens for drug-sensitive and MDR-TB differ and are variably effective, and are therefore likely to differentially affect bacterial transmission. Thus, altogether, we expect evolutionary trajectories to differ considerably between drug-sensitive and MDR strains. This view is supported by the observation that the population structures of drug-sensitive and MDR strains of *M. tuberculosis* in the same geographic regions can differ substantially [82, 83].

## Clinical implications

Because in *M. tuberculosis* drug resistance is encoded on the chromosome [22, 25], rapid detection of resistance by molecular methods is possible. These techniques overcome some of the limitations of the classical techniques of phenotypic drug susceptibility testing (DST), which may require standard mycobacterial culture for up to several months [84]. Hence, developing improved methods of molecular DST in TB is currently high on the agenda, and recently, important progress has been made [85]. However, for many anti-TB drugs, at least a proportion of drug resistance-conferring mutations remain unknown. Hence, current molecular DST is still not 100% sensitive, making culture-based DST indispensable. With the advent of next-generation sequencing technologies, efforts are underway to identify these unknown resistance determinants in *M. tuberculosis*, which then could be incorporated into more sensitive diagnostic tools for drug-resistant TB. Once more sensitive molecular diagnostics become available, drug-resistant profiles could be determined at treatment onset, and drug regimens could be tailored to the individual patient needs. Ultimately, this would help curb the transmission of drug-resistant strains. However, in high-incidence areas that lack appropriate resources for DST, WHO recommends the use of standard treatment

regimens (Box 1) [3]. As a result, these standard regimens are often administered empirically, (i.e. without knowing whether a given patient might harbour an *M. tuberculosis* strain resistant to one or more of the drugs included in the standard regimen), raising the possibility of treating patients with drug-resistant TB with suboptimal or ineffective drugs (Box 1). Even though the standard WHO treatment protocols are highly efficacious for the treatment of drug-susceptible TB [3], in regions with a high burden of drug-resistant TB, empiric regimens without proper DST can contribute to the amplification of drug resistance [2]. The long prevailing dogma of drug-resistant strains being less transmissible than drug-sensitive strains created the belief that by preventing the acquisition of drug resistance by ensuring treatment adherence, drug resistance could be controlled [15, 16, 59]. However, the presence of low-cost resistance mutations, compensatory mutations, and the potential for positive epistasis between drug-resistance-conferring mutations suggests that MDR bacteria can evolve in a range of different ways to alleviate the detrimental effects of drug resistance mutations. Indeed, in the case of *M. tuberculosis*, the most recent global surveillance data revealed the highest rates of MDR-TB ever recorded, as well as increasing trends in MDR-TB rates in certain regions of Eastern Europe, Central Asia, and parts of Africa, although MDR-TB rates have remained constant or have decreased in many other areas [7]. Hence, preventing the acquisition of resistance through the use of standard regimens will not suffice to curb MDR-TB epidemics globally. Rather, increased efforts to directly target circulating MDR strains should be considered. Moreover, new drugs are urgently needed to control *M. tuberculosis* strains which are becoming resistant to an ever increasing number of compounds [86]. These new drugs will have to be deployed rationally to minimize the likelihood of resistance arising. An increased knowledge of the compensatory mechanisms and epistatic interactions among drug resistance-conferring mutations might be able to facilitate defining the ideal treatment regimens. One possibility would be to combine drugs in which the respective drug resistance-conferring mutations interact negatively, thereby lowering the fitness of strains carrying both drug resistance determinants. As discussed here, the evolution of MDR-TB is highly heterogeneous, which is likely to pose a major challenge to the elaboration of a universally effective anti-TB regimen in the future. For example, the high HIV prevalence among TB cases, such as observed in Southern Africa, complicates TB treatment because of the interactions between anti-TB drugs and antiretrovirals [86]. Finally, given the global phylogeography of *M. tuberculosis*, and the heterogeneous distribution of strains with different genetic backgrounds, ideal drug regimens might have to be tailored to different geographical settings as well as to individual patients.

## Concluding remarks

The emergence of MDR-TB is highly heterogeneous, involving a multitude of non-bacterial as well as bacterial factors (Figure 2). Epistatic interactions between drug resistance-conferring mutations, different strain genetic backgrounds, and compensatory mutations can influence the evolutionary trajectories of drug-resistant bacteria, including *M. tuberculosis*. These factors could impact the effectiveness of current as well as future treatment regimens against MDR-TB. However, as yet, chemotherapy of TB and MDR-TB has been based on globally standardized strategies (Box 1). Building on the evidence discussed here, future TB control might benefit from new strategies that target individual patients and the diverse populations of MDR strains circulating in different parts of the world. Moreover, much of our understanding of the importance of persistence, hypermutation, compensatory evolution, and epistasis in drug resistance evolution stems from research in other organisms. Hence more work is needed to explore these factors in *M. tuberculosis*, and determine whether they have any clinical relevance for the emergence and spread of MDR- and XDR-TB. Historically, actions against drug-resistant TB have been taken long after the damage was done [15]. We believe that understanding the bacterial factors driving the evolution of drug-

resistant *M. tuberculosis* will help anticipate its future trajectories and curb the progression of an ever deadlier disease.

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### Box 1. Treatment of tuberculosis

Treatment of TB consists of a standardized six or eight month chemotherapy for new or previously treated cases, respectively. In countries where DST is routinely performed, MDR-TB treatment is individualized. Otherwise, MDR-TB is often treated using standardized drug regimens; however, these may vary between countries.

#### New TB cases

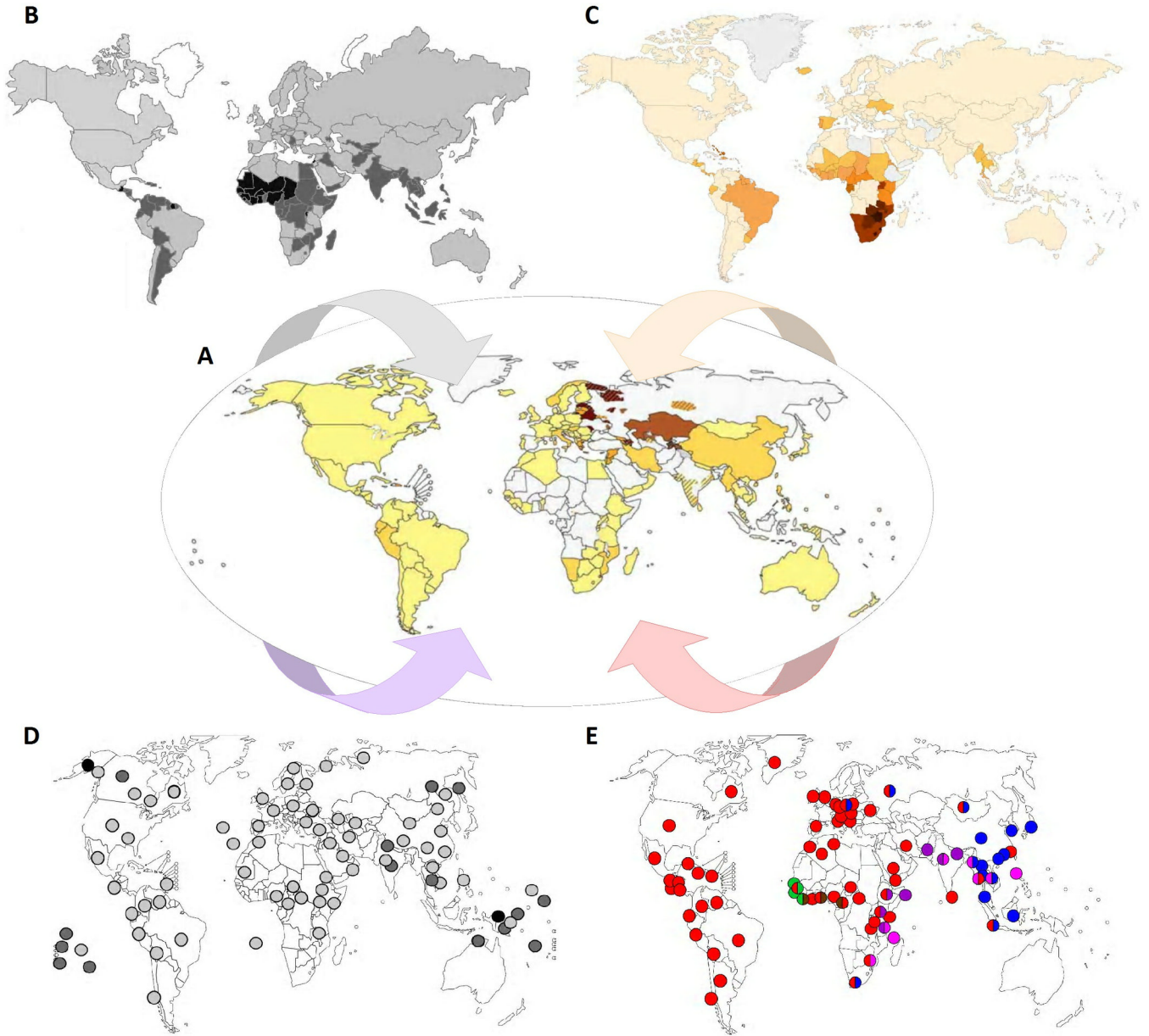
- Two months of isoniazid, rifampicin, pyrazinamide and ethambutol (intensive phase).
- Four months of isoniazid and rifampicin (continuation phase).

#### Previously treated TB cases

- Two months of isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin (intensive phase).
- One month of isoniazid, rifampicin, pyrazinamide and ethambutol (intensive phase).
- Five months of isoniazid, rifampicin and ethambutol (continuation phase).

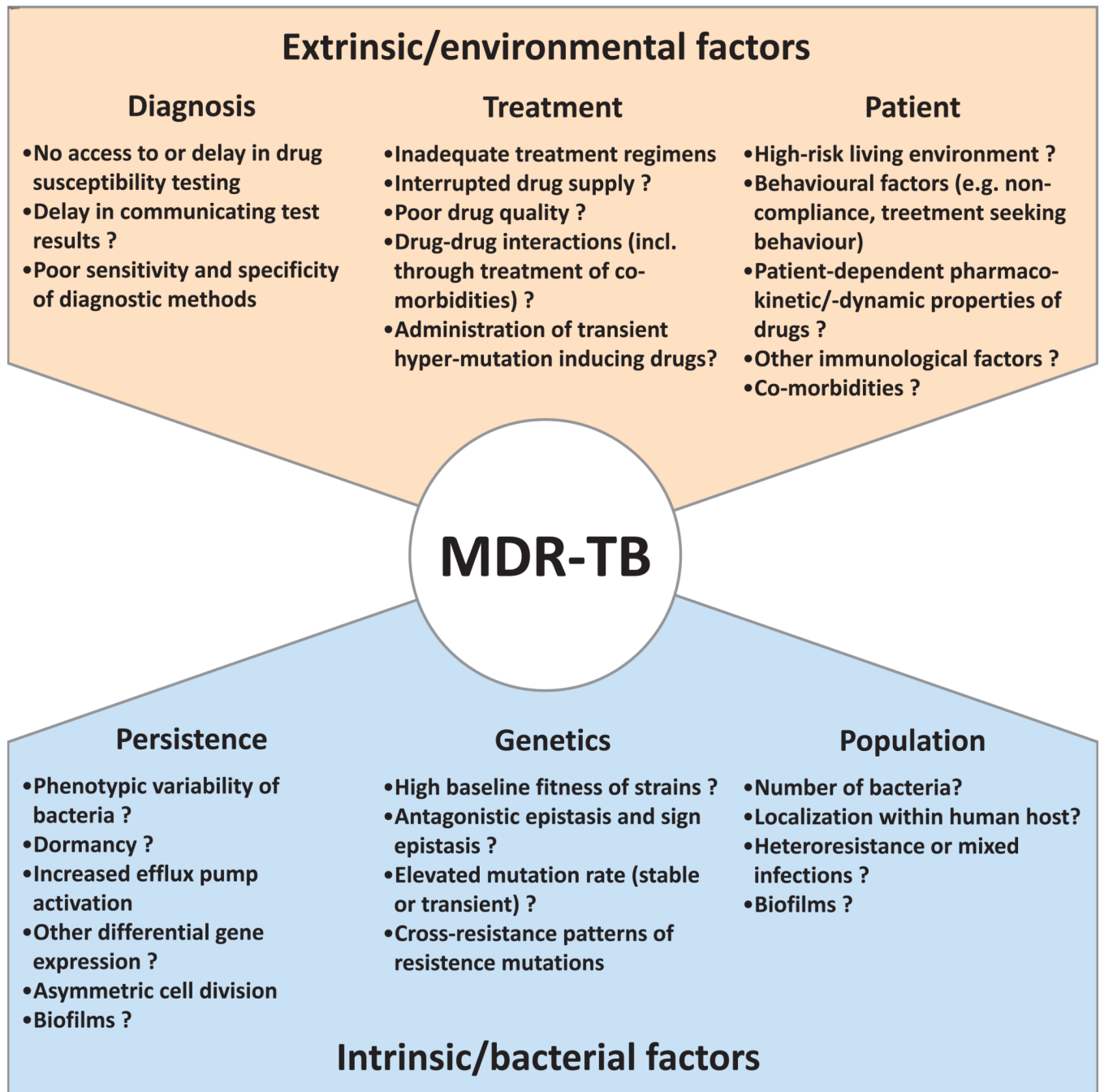
#### MDR-TB cases

- At least four drugs likely to be effective must be included.
- Any first-line oral agents likely to be effective should be included (e.g. pyrazinamide or ethambutol).
- One effective injectable aminoglycoside or polypeptide drug should be included (kanamycin, amikacin, capreomycin or streptomycin).
- One fluoroquinolone should be included.
- Intensive phase therapy including the injectable drug should last at least 6 months. Total duration of therapy should be at least 18 months.

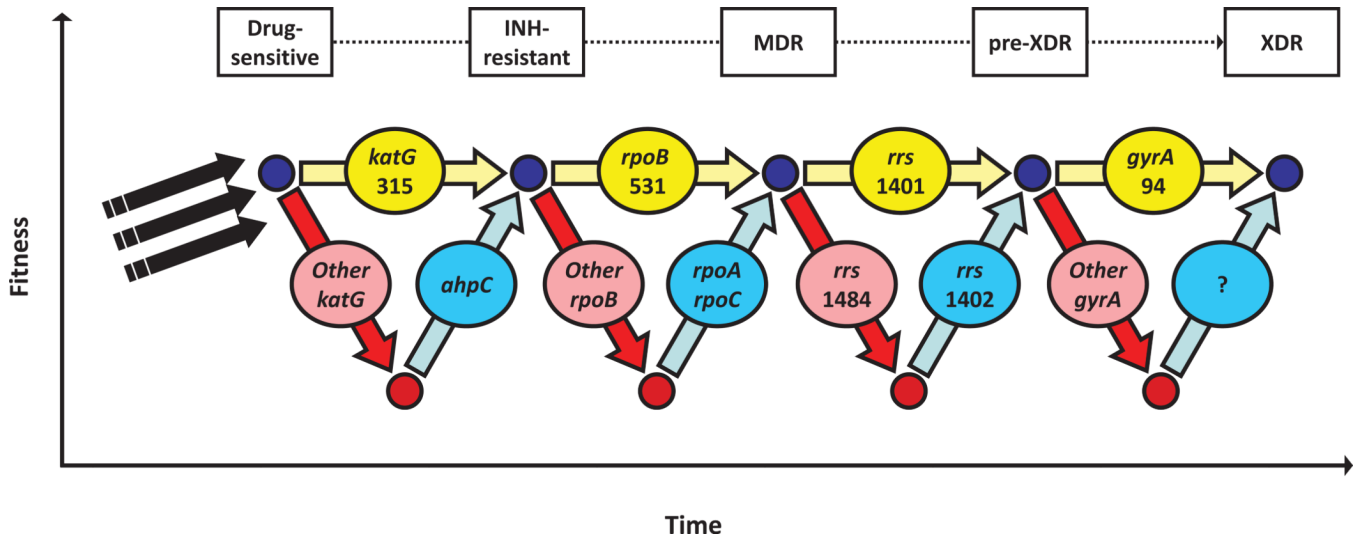


**Figure 1. The heterogeneity of MDR-TB**  
 A. The proportion of new TB cases with MDR-TB (centre; darker shadings indicate an increasing proportion) [7] is heterogeneously distributed and influenced by a multitude of factors including: B. Variably effective control programs (here, represented by the estimated global case detection rates; darker shadings indicate lower case detection rates) [87]; C. The variable presence of co-morbidities (here, represented by the HIV prevalence among incident TB cases; darker shadings indicate a higher prevalence) [1]; D. Different host-related factors (here, represented by the presence of the HLA II allele DQB1\*0503, which was the first HLA allele associated with increased TB risk; darker shading indicates a higher abundance) [88]; and E. The global distribution of different phylogenetic lineages of *M. tuberculosis* (the six main lineages of human TB-associated strains of *M. tuberculosis* known to date and their geographical distribution are represented by circles of different

colour; the colours correspond to the clades indicated in the phylogenetic tree shown in Figure 4). Figure adapted from [www.Globalhealthfacts.org](http://www.Globalhealthfacts.org) and [7, 89, 90].



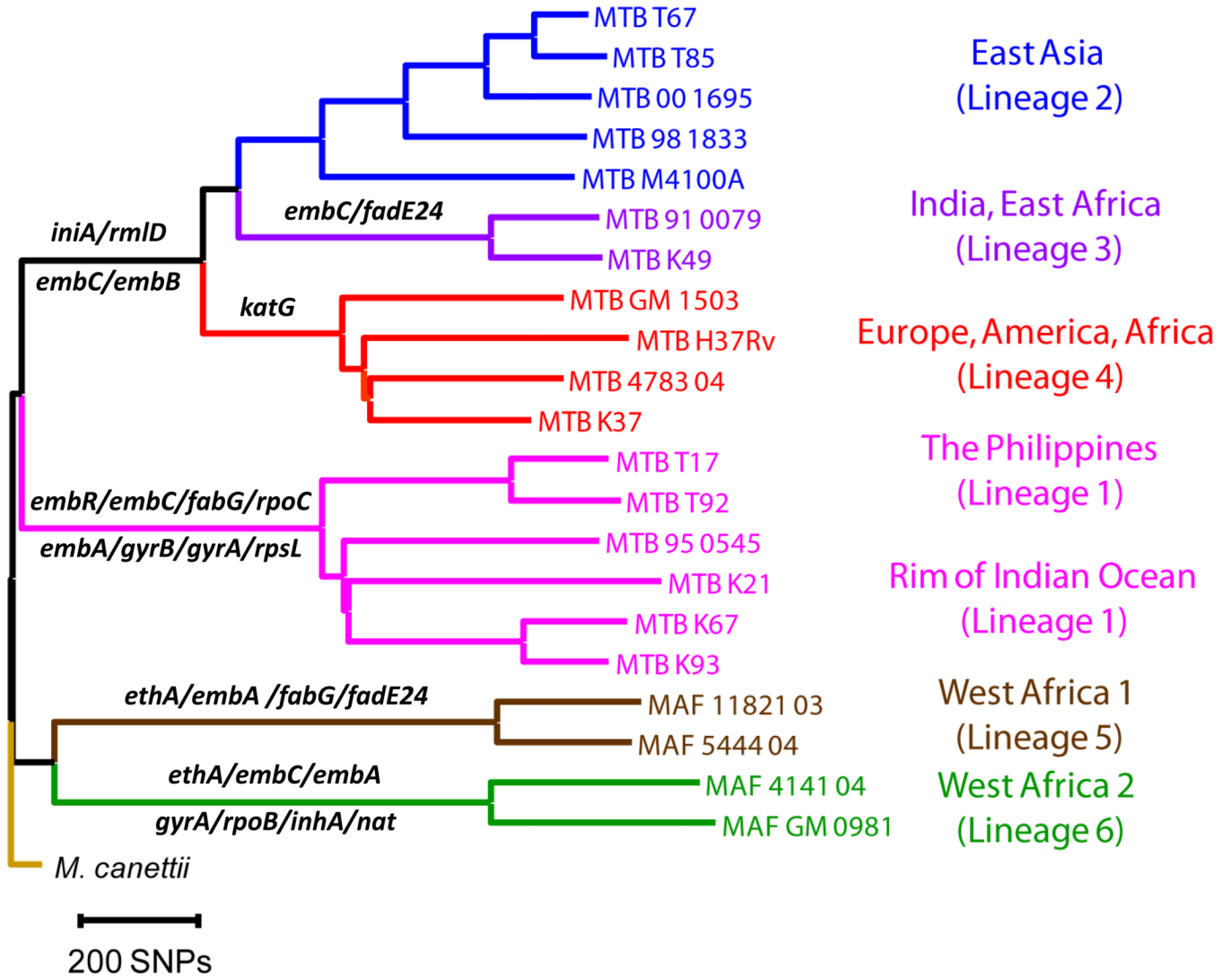
**Figure 2. A selection of extrinsic and intrinsic factors contributing to the emergence of MDR-TB**  
 A non-exhaustive list of extrinsic, non-bacterial, and intrinsic bacterial factors referred to in the text that modulate the evolution of MDR-TB. Question marks indicate factors that might be involved, but for which there is no direct evidence for in *M. tuberculosis*.



**Figure 3. The influence of Darwinian fitness on the emergence of MDR and XDR-TB**

In the absence of drugs, the acquisition of resistance mutations is often associated with a fitness-cost. The most prevalent resistance mutations in clinical isolates show low or no fitness cost. However, high fitness costs incurred by other generally less common mutations can be alleviated by compensatory mutations. Higher and lower fitness levels of bacteria are indicated by small blue or red circles, respectively. Yellow and red arrows and ovals indicate low-cost and high-cost resistance mutations, respectively. Blue arrows and ovals indicate compensatory mutations. Black arrows refer to baseline genomic characteristics of a particular strain genetic background. Figure based on data from [22, 25, 63–65].





**Figure 4. Drug resistance-associated genes containing *M. tuberculosis* lineage-specific mutations**  
 Phylogenetic tree of the six main lineages of *M. tuberculosis* associated with human TB, based on 21 whole genome sequences [81]. Genes indicated are associated with drug resistance (Table 1) and harbour lineage-defining, non-synonymous substitutions. This creates the potential for epistatic interactions between the genetic background of a given strain and specific drug resistance-conferring mutations.

Table 1

Anti-TB drugs and mechanisms of drug resistance.

Drug	Genetic region involved in resistance formation*	Natural function of gene	Role in resistance formation when mutated
Isoniazid	<i>ahpC</i>	Alkyl hyperperoxide reductase	Compensatory mutations
	<i>fabG</i>	3-oxoacyl-thioester reductases	Unknown
	<i>fadE24</i>	Involved in fatty acid $\beta$ -oxidation	Unknown
	<i>inhA</i>	Enoyl reductase	Alteration of drug target
	<i>inhA</i> promoter**	Regulation of expression of <i>InhA</i>	Overexpression of drug target
	<i>iniA</i>	Efflux pump associated	Altered efflux pump activity
	<i>katG</i> **	Catalase/peroxidase	Elimination of pro-drug conversion
Rifampicin	<i>rpoA</i>	$\alpha$ subunit of RNA polymerase	Compensatory mutations
	<i>rpoB</i> **	$\beta$ -subunit of RNA polymerase	Alteration of drug target
	<i>rpoC</i>	$\beta'$ -subunit of RNA polymerase	Compensatory mutations
Pyrazinimide	<i>pncA</i> **	Nicotinamidase	Elimination of pro-drug conversion
Streptomycin	<i>gidB</i>	7-methylguanosine methyltransferase	Alteration of drug target
	<i>rpsL</i> **	S12 ribosomal protein	Alteration of drug target
	<i>rrs</i> **	16S rRNA	Alteration of drug target
Ethambutol	<i>embA</i>	Arabinosyl transferase	Alteration of drug target
	<i>embB</i> **	Arabinosyl transferase	Alteration of drug target
	<i>embC</i>	Arabinosyl transferase	Alteration of drug target
	<i>embR</i>	Regulator of <i>embCAB</i> operon expression	Overexpression of drug target
	<i>iniA</i>	Efflux pump associated	Altered efflux pump activity
	<i>rmlD</i>	dTDP-4-dehydrohamnose reductase	Unknown
Fluoroquinolones	<i>gyrA</i> **	DNA gyrase	Alteration of drug target
	<i>gyrB</i> **	DNA gyrase	Alteration of drug target
Kanamycin/amikacin	<i>rrs</i> **	16S rRNA	Alteration of drug target
	<i>rrs</i>	16S rRNA	Compensatory mutations
Capreomycin/viomycin	<i>tyaA</i>	rRNA methyltransferase	Alteration of drug target
	<i>rrs</i> **	16S rRNA	Alteration of drug target
Ethionamide	<i>inhA</i>	Enoyl reductase	Alteration of drug target
	<i>inhA</i> promoter	Regulation of expression of <i>inhA</i>	Overexpression of drug target
para-amino salicylic acid	<i>thyA</i>	Thymidylate synthase A	Elimination of pro-drug conversion
PA-824 and OPC-67683	<i>Rv3547</i>	Hypothetical 16.4 kDa	Alteration of drug target
TMC207	<i>atpE</i>	ATP synthase	Alteration of drug target

\* A selection of genetic regions, previously associated with drug resistance according to [www.TBDreamDB.com](http://www.TBDreamDB.com) or previously identified as potential compensatory mutations (see text).

\*\* High-confidence drug resistance-associated genetic regions according to [www.TBDreamDB.com](http://www.TBDreamDB.com)