

REVIEW ARTICLE

Antimicrobial resistance in *Mycobacterium tuberculosis*: mechanistic and evolutionary perspectives

Sebastian M. Gygli^{1,2}, Sonia Borrell^{1,2}, Andrej Trauner^{1,2}
and Sebastien Gagneux^{1,2,*}

¹Swiss Tropical and Public Health Institute, Department of Medical Parasitology and Infection Biology, 4002 Basel, Switzerland and ²University of Basel, Basel, Switzerland

*Corresponding author: Swiss Tropical and Public Health Institute, Department of Medical Parasitology and Infection Biology, Socinstrasse 57, 4002 Basel, Switzerland. Tel: +41 61 284 8369; Fax: +41 61 284 8101; E-mail: sebastien.gagneux@unibas.ch

One sentence summary: This review summarises the current understanding of drug resistance mechanisms and their evolutionary trajectories in *Mycobacterium tuberculosis*.

Editor: Diarmaid Hughes

ABSTRACT

Antibiotic-resistant *Mycobacterium tuberculosis* strains are threatening progress in containing the global tuberculosis epidemic. *Mycobacterium tuberculosis* is intrinsically resistant to many antibiotics, limiting the number of compounds available for treatment. This intrinsic resistance is due to a number of mechanisms including a thick, waxy, hydrophobic cell envelope and the presence of drug degrading and modifying enzymes. Resistance to the drugs which are active against *M. tuberculosis* is, in the absence of horizontally transferred resistance determinants, conferred by chromosomal mutations. These chromosomal mutations may confer drug resistance via modification or overexpression of the drug target, as well as by prevention of prodrug activation. Drug resistance mutations may have pleiotropic effects leading to a reduction in the bacterium's fitness, quantifiable e.g. by a reduction in the *in vitro* growth rate. Secondary so-called compensatory mutations, not involved in conferring resistance, can ameliorate the fitness cost by interacting epistatically with the resistance mutation. Although the genetic diversity of *M. tuberculosis* is low compared to other pathogenic bacteria, the strain genetic background has been demonstrated to influence multiple aspects in the evolution of drug resistance. The rate of resistance evolution and the fitness costs of drug resistance mutations may vary as a function of the genetic background.

Keywords: *Mycobacterium tuberculosis*; drug resistance; mechanisms; fitness; epistasis; evolution

ABBREVIATIONS

TB: Human tuberculosis

MDR: Multidrug-resistant – resistance to isoniazid and rifampicin

XDR: Extensively drug-resistant – MDR + resistance to fluoroquinolones and any of the second-line injectable antibiotics (amikacin, kanamycin and capreomycin)

INTRODUCTION

Human tuberculosis (TB), a devastating disease caused by the gram-positive, acid-fast eubacterium *Mycobacterium tuberculosis*, was classified as a global health emergency by the World Health Organization in 1993. TB remains one of the deadliest infectious diseases with an estimated 1.8 million deaths occurring per year, mainly in the developing world (World Health

Organization 2016). Although the incidence of TB has declined over the past decades, there were an estimated 10.4 million new cases in 2015, of which 0.48 million were caused by *M. tuberculosis* strains classified as multidrug-resistant (MDR—resistant to the first-line drugs rifampicin and isoniazid). Drug-resistant *M. tuberculosis* strains are a major global health concern because treatment of these cases requires second-line drugs, which are less effective, more expensive and more toxic, as well as sophisticated infrastructure for drug susceptibility testing not readily available in resource-limited settings. TB treatment success rates of cases caused by MDR/XDR variants of *M. tuberculosis* are alarmingly low, with only 54% of MDR and 28% of extensively drug-resistant (XDR—MDR plus resistance to fluoroquinolones and any second-line injectable aminoglycoside/cyclic peptide) cases resulting in cure, compared to 83% of drug-susceptible cases (World Health Organization 2016). For clarity, we only refer to MDR/XDR *M. tuberculosis* variants when the defined resistance profiles are meant. Otherwise we use the term ‘drug resistance’ to refer to the topic in general, irrespective of specific drug resistance profiles.

The first effective antituberculous drug, streptomycin, was discovered in 1944 (Schatz, Bugle and Waksman 1944). The newly discovered drug was immediately used for treatment of TB patients. The condition of many individual TB patients receiving streptomycin improved during the first months of treatment, only to then deteriorate again as treatment continued. It was soon understood that this was due to the evolution of resistant *M. tuberculosis* strains, rendering streptomycin ineffective (Crofton and Mitchison 1948). To limit the evolution of resistance, the British Medical Research Council pioneered the first combination therapy for the treatment of a disease by using para-aminosalicylic acid (Lehmann 1946) together with streptomycin for treatment of pulmonary TB (Medical Research Council 1950). The subsequent years saw the introduction of an array of different antituberculous drugs. The discovery of rifampicin in 1965 (Sensi 1983) and the subsequent use of the drug in TB treatment was a game changer, allowing dramatically shortened treatment duration from 18 months or more to 9 months (British Thoracic and Tuberculosis Association 1975). During the 1990s, the current standard 6-month regimen known as Directly Observed Therapy Short Course (DOTS) was introduced by the World Health Organization (World Health Organization 1997). This regimen consists of 2 months treatment with isoniazid, rifampicin, ethambutol and pyrazinamide followed by 4 months of isoniazid and rifampicin (World Health Organization 1991), and is highly effective for drug-susceptible TB (Frieden et al. 1995; Feng-Zeng et al. 1996).

A short treatment duration and reduction of adverse drug effects are crucial for increasing patient treatment adherence, which is known to influence the evolution of drug resistance (Mahmoudi and Iseman 1993). However, despite the early establishment of TB combination therapies showing high cure and low relapse rates (British Thoracic and Tuberculosis Association 1975), drug-resistant *M. tuberculosis* strains continued to evolve in both high and low incidence settings. MDR *M. tuberculosis* variants evolved on multiple occasions in different parts of the world (Cohen et al. 2015; Eldholm et al. 2015). Furthermore, differences in the quality of public health systems contributed to the spread of drug-resistant *M. tuberculosis* variants leading to the unequal distribution of incidence rates of drug-resistant variants around the world we observe today (World Health Organization 2016). In the absence of an effective vaccine (Kaufmann et al. 2014), there is an urgent need for new treatment regimens, drugs and diagnostics to slow the evolution of drug resistance and limit

transmission of resistant variants, as well as to ameliorate the treatment outcome of patients infected with MDR/XDR *M. tuberculosis* strains. Understanding the molecular mechanisms and the evolutionary trajectory of drug resistance is important to limit the *de novo* evolution and subsequent spread of resistant *M. tuberculosis* strains. The first part of this review will summarise intrinsic and acquired mechanisms of drug resistance in *M. tuberculosis*; these are analysed in more detail in several recently published reviews (Smith, Wolff and Nguyen 2013; Zhang and Yew 2015; Nash 2016; Nguyen 2016). The second part of this review will focus on our current understanding of the evolutionary biology of drug resistance in *M. tuberculosis*.

MECHANISMS OF DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

Members of the genus *Mycobacterium* have long been noted for their intrinsic resistance to a wide array of antibiotics. This has mainly been attributed to the unusually thick, lipid-rich cell envelope (Jarlier and Nikaido 1994). After penetrating the cell envelope, certain antibiotics may be cleaved enzymatically or altered structurally to render them ineffective (Chambers et al. 1995; Quinting et al. 1997; Wang, Cassidy and Sacchetti 2006; Warrior et al. 2016). Furthermore, a number of efflux systems have been identified in *M. tuberculosis*, but their significance in conferring clinically relevant levels of drug resistance is a matter of debate. As efflux systems have been observed to be expressed under varying conditions (Gupta et al. 2010; Adams et al. 2011; Li et al. 2015), they might serve as a stepping stone for high-level drug resistance. A further peculiarity of *M. tuberculosis* is the apparent absence of ongoing horizontal gene transfer (Cole et al. 1998; Gagneux and Small 2007; Bolotin and Hershberg 2015). Although there have been reports of horizontal gene transfer between ‘species’ of the genus *Mycobacterium* (da Silva Rabello et al. 2012), horizontal gene transfer does not seem to be a driving factor in the acquisition of antimicrobial resistance in *M. tuberculosis*. The vast majority of drug resistance phenotypes in *M. tuberculosis* can be explained by chromosomal mutations and not by resistance plasmids or other mobile genetic elements. To study the mechanisms of drug resistance, many studies have been performed on a multitude of different mycobacterial species, due to the often lower pathogenicity/biosafety requirements and faster growth properties of these mycobacteria compared to *M. tuberculosis*. The most widely used model is *M. smegmatis*, an environmental mycobacterium with a genome roughly 1.5 times the size of that of *M. tuberculosis*. **We should therefore be cautious in applying the results of these studies directly to *M. tuberculosis*.**

INTRINSIC DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

The mycobacterial cell wall and drug penetration

The intrinsic resistance of mycobacteria against several classes of antibiotics has commonly been attributed to the unusual composition and structure of the mycobacterial cell envelope. Compared to other gram-positive bacteria, the cell wall of members of the genus *Mycobacterium* is much thicker and more hydrophobic, due to the presence of a wide array of different lipids that include mycolic acids. Many studies (reviewed in Jarlier and Nikaido 1994; Brennan and Nikaido 1995; Nguyen and Pieters 2009; Sarathy, Dartois and Lee 2012) performed in different mycobacterial species demonstrated that the composition of the cell envelope and the low numbers of porins

(Mailaender et al. 2004) contribute significantly to the cell envelope's low compound permeability. A major constituent of the cell wall is a layer of lipids, which are covalently linked to the peptidoglycan layer via arabinogalactan. Furthermore, the cell wall contains 'extractable' immunogenic glycolipids (Brennan and Nikaido 1995). The lipid-rich nature renders the cell wall extremely hydrophobic and prevents the permeation of hydrophilic compounds. It is thought that small hydrophilic compounds, including many antibiotics active against *M. tuberculosis*, can only traverse the cell wall via water-filled porins. Heterologous expression of the *M. smegmatis* porin MspA in *M. tuberculosis* did indeed decrease the minimal inhibitory concentration for several hydrophilic drugs (Mailaender et al. 2004), indicating that porins might play a role in the diffusion of hydrophilic antibiotics across the cell wall of *M. tuberculosis*. However, until recently reports on the presence of porins in *M. tuberculosis* were lacking. The outer membrane channel protein CpnT was demonstrated to be involved in nutrient uptake in *M. bovis* BCG and *M. tuberculosis* (Danilchanka et al. 2014) and in mediating susceptibility to nitric oxide and antibiotics in *M. bovis* BCG (Danilchanka et al. 2015). CpnT seems to be under positive selection in clinical *M. tuberculosis* isolates, demonstrated by the overrepresentation of non-synonymous mutations in the gene encoding CpnT (Rv3903c). However, the role of CpnT in mediating drug susceptibility to hydrophilic antibiotic compounds in *M. tuberculosis* needs further investigation, as CpnT deletion mutants do not demonstrate drug resistance phenotypes *in vitro*. However, the studies confirm the presence of porins in the outer membrane of *M. tuberculosis* and their role in uptake of small hydrophilic compounds. Furthermore, the physical organisation of the cell wall lipids is believed to limit the membrane's fluidity. A recent study (Rodriguez-Rivera et al. 2017) assessed the membrane fluidity in live cells of *M. smegmatis* and other actinobacteria by measuring the reorganisation of fluorescein-labelled therealose analogues by mycolyltransferases. The study demonstrated that, compared to other actinobacteria, *M. smegmatis* has the lowest membrane fluidity. This is thought to be a function of mycolic acid structure (length and presence of functional groups). Interestingly, exposure of *M. smegmatis* to subinhibitory concentrations of ethambutol increases the membrane's fluidity and diffusion of compounds across the cell envelope. This offers the possibility for novel drug combination therapies, as the reduction of the membrane fluidity using ethambutol can render *M. tuberculosis* susceptible against drug classes it is normally resistant against (Abate and Hoffner 1997; Bosne-David 2000).

The peculiar characteristics of the mycobacterial cell envelope hinder the diffusion of hydrophobic molecules including members of several antibiotics belonging to the classes of macrolides, rifamycins, tetracyclines and fluoroquinolones (Brennan and Nikaido 1995). However, it does appear that the rate of diffusion is a function of molecule hydrophobicity to a certain extent, with hydrophobic molecules diffusing more readily through the mycobacterial cell envelope (Rastogi and Goh 1990; Nikaido and Thanassi 1993). The hypothesis that the cell envelope lipids are a major factor in the intrinsic resistance of mycobacteria to many hydrophobic antibiotics is further substantiated by studies performed with mutants defective in lipid synthesis, which are susceptible to drugs that the corresponding wild-type strain is resistant against (Liu and Nikaido 1999). A recent study modelled the permeation of compounds through the mycobacterial cell wall and demonstrated that lipophilicity is an important but not exclusive factor of compound permeability (Janardhan, Ram Vivek and Narahari Sastry 2016).

Drug inactivation by *Mycobacterium tuberculosis*

After penetrating the cell wall as an initial defence layer, antibiotics may be cleaved enzymatically to render them ineffective. One of the most prominent examples is the enzymatic degradation of β -lactam antibiotics by β -lactamases, which hydrolyse the β -lactam ring of the antibiotics. Early studies involving penicillin demonstrated that *M. tuberculosis* is intrinsically resistant to this class of antibiotics (Abraham et al. 1941). The genome of *M. tuberculosis* encodes a single class A β -lactamase termed BlaC thought to localise to the periplasmic space, either anchored in the outer leaflet of the plasma membrane as a lipoprotein or unbound. The *M. tuberculosis* β -lactamase shows broad substrate specificity (including carbapenems), albeit with varying affinities, and is considered an extended-spectrum β -lactamase. BlaC is irreversibly inhibited by the β -lactamase inhibitor clavulanate (Wang, Cassidy and Sacchettini 2006; Hugonnet and Blanchard 2007). Due to the increasing numbers of cases caused by MDR/XDR *M. tuberculosis* strains, there has been a renewed interest in the use of β -lactam antibiotics in the treatment of TB. An early, small study reported no beneficial effect of including an amoxicillin/clavulanate combination in a salvage regimen (a regimen of last resort with unproven efficacy) to treat patients infected with MDR *M. tuberculosis* strains (Yew et al. 1995). Since then, several *in vitro* (Chambers et al. 1995; Hugonnet et al. 2009) and *in vivo* studies (Payen et al. 2012; De Lorenzo et al. 2013) reported encouraging results on treatment outcomes with various regimens by including β -lactam antibiotics with clavulanate. However, some MDR/XDR *M. tuberculosis* isolates still appear to be resistant to meropenem/clavulanate or amoxicillin/clavulanate without harbouring any mutations that could explain the observed variability in susceptibility to these drugs (Cohen et al. 2016). The true value of β -lactam antibiotics for the treatment of drug-resistant *M. tuberculosis* variants still needs further assessment. Given the positive results in diverse studies, the demonstrated safety profile of β -lactam antibiotics/ β -lactamase inhibitors and the limited treatment options for MDR/XDR TB warrants further investigation into treatment regimens including this class of antibiotics.

Apart from drug cleavage, antibiotics may be inactivated by modification, e.g. by methylation or acetylation. To date, the best described mechanism of drug inactivation by chemical modification in *M. tuberculosis* is the acetylation of various aminoglycoside/cyclic peptide antibiotics used for the treatment of MDR TB by the enhanced intracellular survival protein (Eis). Eis has been demonstrated to acetylate and inactivate the clinically relevant second-line injectable aminoglycoside antibiotic kanamycin A (Zaunbrecher et al. 2009), as well as the cyclic peptide antibiotic capreomycin (Houghton et al. 2013). Several promoter mutations identified in clinical *M. tuberculosis* isolates lead to overexpression of Eis, which in turn confers low-level resistance against kanamycin A but not amikacin (Zaunbrecher et al. 2009; Kamblí et al. 2016). It is not clear if Eis overexpression alone leads to clinically relevant levels of capreomycin resistance (Kamblí et al. 2016). Overexpression of Eis therefore might serve as a stepping stone for the evolution of high-level aminoglycoside/cyclic peptide resistance.

Recently, a novel mechanism of drug inactivation was discovered in *M. tuberculosis*. The pyrido-benzimidazole compound '14' was described as having potent bactericidal activity against aerobically growing *M. tuberculosis* (Warrier et al. 2015). Compound 14 may be N-methylated by a previously unknown methyltransferase encoded by the gene Rv0560c. The methylated compound 14 is unable to inhibit its target, the decaprenylphosphoryl- β -D-

ribose 2-oxidase (DprE1), which is involved in arabinogalactan synthesis (Warrier *et al.* 2016). Although this is a novel mechanism of drug resistance in *M. tuberculosis*, and in bacteria in general, it has no known clinical relevance to date.

Enzymatic drug target modification

Many antibiotics in use are natural products produced by bacteria, which requires the producing bacteria to be resistant to these compounds; some of the mechanisms used by these bacteria are conserved in mycobacteria. *Streptomyces* spp. produce diverse classes of antibiotics, e.g. macrolides, lincosamides and streptogramins. These antibiotics inhibit the bacterial ribosome by binding to the 50S ribosomal subunit. *Streptomyces* spp. are resistant to these antibiotics by expressing methyltransferases which mono- or dimethylate the adenosine residue 2058 (*Escherichia coli* notation) of the 23S rRNA, preventing the aforementioned drugs from binding to the ribosome and inhibiting translation. The *M. tuberculosis* genome encodes the methyltransferase Erm(37), a homologue of Erm methyltransferases found in many actinomycetes. However, the substrate specificity of Erm(37) differs from its homologues—Erm(37) is able to monomethylate residues 2057–2059 of the 23S rRNA, instead of only residue 2058. Monomethylation of positions 2057–2059 confers resistance to various macrolide antibiotics (Buriánková *et al.* 2004; Madsen *et al.* 2005).

Drug efflux in *Mycobacterium tuberculosis*

Efflux systems are important constituents of bacterial and eukaryotic physiology. Multiple reviews have been published (Louw *et al.* 2009, 2011; da Silva *et al.* 2011; Warrell 2012; Anthony Malinga and Stoltz 2016) focusing on efflux systems in *M. tuberculosis*; the main points are briefly summarised here. Early comparative studies revealed that the genome of *M. tuberculosis* encodes a multitude of different putative efflux systems, belonging to the classes of ATP-binding cassette, major facilitator superfamily, small multidrug resistance, multidrug and toxic-compound extrusion systems and resistance-nodulation-cell division (Paulsen *et al.* 2001).

The relevance of drug efflux for generating clinically relevant drug resistance in *M. tuberculosis* is controversial but has gained more attention in recent years. The observation that about 30% of isoniazid (Louw *et al.* 2009) and 3% of rifampicin (Telenti *et al.* 1993) resistant clinical *M. tuberculosis* isolates do not show any known resistance mutation might be explained by drug efflux. However, this unexplained resistance is potentially confounded by the fact that not all mutational targets of drug resistance are known. For certain antibiotics e.g. isoniazid, an array of different resistance mechanisms is already known (Vilchèze and Jacobs 2014). On the other hand, resistance to rifampicin is thought only to be conferred by mutations in the gene encoding one constituent of the drug target (further discussed below), making the contribution of efflux pumps to unexplained resistance phenotypes more likely.

Efflux pumps exhibit high levels of substrate promiscuity and are able to extrude a multitude of structurally unrelated compounds. Furthermore, efflux systems have been shown to be essential in *M. tuberculosis* for intracellular growth in macrophages (Lamichhane, Tyagi and Bishai 2005). Mycobacterial efflux pumps are able to extrude nearly all antituberculous drugs, including streptomycin, rifampicin, isoniazid, clofazimine, bedaquiline, fluoroquinolones and ethambutol (Anthony Malinga and Stoltz 2016). Expression of efflux pumps

can be viewed as a plastic trait, meaning that expression levels are modified via non-mutational processes upon changes in the environment. We can therefore say that efflux pumps are induced or upregulated when a specific environmental cue (e.g. antibiotics or the intracellular environment of a macrophage) is present. The term ‘overexpression’ should only be used for mutants where expression levels exceed the reaction norm (box 1) of the wild-type strains. To our knowledge, there have not been any studies systematically investigating the reaction norm of efflux pumps in *M. tuberculosis*.

Box 1. Definitions of commonly used terms in evolutionary biology.

Bottleneck

A bottleneck describes the stark reduction of the population size and therefore the genetic diversity of the population due to random sampling, i.e. genetic drift. In the context of *M. tuberculosis* infections, only a subset of the whole population of bacteria present in the lungs of a patient will gain access to the airways and may be aerosolised. Furthermore, presumably only a subset of that aerosolised population will survive the harsh environmental conditions found outside of the host, and is able to infect a new host—if one is present to inhale the aerosols.

Effective population size

In the context of bacterial (haploid), non-recombining, obligate pathogens like *M. tuberculosis*, the effective population size describes the proportion of the population e.g. in a patient, which has the possibility to transmit to a new host. *Mycobacterium tuberculosis* is able to establish infections in nearly all tissues—however, all of them are ‘evolutionary dead ends’, pulmonary (or in rare cases laryngeal) infections being the only exception. Consider the case of a patient with pulmonary and extrapulmonary TB. The effective population size could be defined as the total number of *M. tuberculosis* cells present in a patient minus the number of cells in the extrapulmonary location, *ergo* the pulmonary (laryngeal) *M. tuberculosis* population—as only this population will be able to transmit to a new host. Furthermore, transmission of *M. tuberculosis* requires substantial lung damage that ‘allows’ access to the bronchi, which is dependent on the location of the foci within the lung, *ergo* not all *M. tuberculosis* cells in the lung will have the potential to transmit, reducing the effective population size.

Epidemiological cutoff

The highest minimal inhibitory concentration of a drug observed in a wild-type strain. This is related to the reaction norm—see below.

Epistasis

Epistasis describes the phenomenon where the interaction of two or more genes/alleles produce an effect on the phenotype (e.g. on fitness) which is unequal to the sum each gene's/allele's effect on their own.

Fitness

The fitness of an organism can be defined as the ability to survive and reproduce in a given environment. In the case of an obligate parasite like *M. tuberculosis*, this entails establishing an infection in the human host, replicating and transmitting to a new host. Fitness can be parameterised by the effective reproductive number *R*, which quantifies how many secondary cases are produced on average by a single infected individual in a population of susceptible and resistant hosts. However, *R* is notoriously difficult to assess

in vivo. In absence of a better measure, fitness of bacteria is often approximated by measuring life history traits e.g. *in vitro* growth rates and/or growth yield in artificial growth media. However, there is some merit in measuring *in vitro* growth rates/yields e.g. of resistant *M. tuberculosis* variants as their growth rate/yields are often correlated with *in vivo* frequency of these variants.

Fitness cost

A fitness cost describes the reduction in the number of offspring produced by a genotype in a given environment caused e.g. by a drug resistance mutation, compared to a drug-susceptible variant. Drug resistance has long been assumed to be generally associated with a fitness cost in absence of the drug (i.e. a reduction in *R*, the number of secondary cases generated). Using *in vitro* methods, a fitness cost would manifest itself as a reduction in growth rate or yield.

Genetic background

The genetic background describes the genetic diversity present in a strain's genome resulting from mutations/insertions/deletions/rearrangements etc. when compared to other strains. The genetic background is an important factor influencing epistatic interactions.

Genetic drift

Genetic drift is an important mechanism of evolution whereby the genetic diversity of a population is reduced by random sampling error. The sampling error results from the differential probability of an organism to survive and reproduce based on chance. The effect of genetic drift on allele frequencies is strongest in populations with a small effective population sizes. Genetic drift can lead to the stochastic fixation or loss of alleles in a given population.

Natural selection

Natural selection is one of the major mechanisms of evolution and refers to the differential survival of organisms based on their phenotype. The phenotype results from the interaction of the organism's genotype with the environment. The differential survival results from competition (e.g. for resources) among organisms and is dependent on the degree of adaptation of the organism to its environment. The effect of natural selection is strongest in large populations and leads to changes in allele frequencies over time.

Mutational target size

The mutational target size in the narrow sense describes the number of different mutations which may confer resistance to a certain drug. Depending on the resistance mechanism, the mutational target size can vary greatly. The mutational target size in the broader sense describes the number of different mutations which confer resistance to a given drug and which do not result in lethality due to epistatic interactions with the strain genetic background.

Reaction norm

The phenotype of an organism results from the interaction of the organism's genotype with the environment. The range of different phenotypes of a given genotype in different environments is called the reaction norm (Griffiths et al. 2005).

Standing genetic diversity

The term standing genetic diversity describes the presence of multiple alleles at a locus which are segregating in the population. In other words, the sum of all genetic backgrounds (see above) constitutes the standing genetic diversity of a species.

Trait effect

Hall et al. describe the trait effect as e.g. the effect drug resistance mutations may have on life history traits like growth rate and/or yield of the strains carrying resistance mutations. The magnitude of the trait effect inflicted by drug resistance mutations is dependent on multiple factors including the genetic background, pre-existing drug resistance mutations, compensatory mutations and the environment (Hall et al. 2015).

It has been demonstrated in model systems of *M. tuberculosis* that efflux pumps are induced upon infection of macrophages, which coincides with increased minimal inhibitory concentrations for isoniazid (Adams et al. 2011). A subset of the strains was resistant to higher levels of isoniazid at the peak serum concentrations (Park et al. 2016). The expression of the efflux systems persists even after the mycobacterial cells have been released from the macrophages. However, in accordance with the concept of efflux pumps as a plastic trait, not all bacterial cells upregulate the expression of efflux systems (Gupta et al. 2010; Adams et al. 2011). Several antituberculous drugs have been demonstrated to induce the expression of efflux pump genes, but there is considerable variability between strains and no general pattern of efflux pump expression was recognisable (Gupta et al. 2010). Furthermore, MDR *M. tuberculosis* isolates have been shown to constitutively express genes involved in drug efflux (Li et al. 2015). Although there are reports on the upregulation of efflux systems generating minimal inhibitory concentrations slightly beyond the epidemiological cutoff (box 1), the clinical relevance of efflux pumps is not clear and warrants more investigation (Adams et al. 2011). The majority of drug-resistant strains harbour chromosomal mutations linked to drug resistance (further discussed below). However, there are examples of clinically relevant levels of resistance conferred by 'overexpression' of efflux pumps. Mutations in the transcriptional repressor *MmpR* lead to overexpression of the multisubstrate efflux pump *MmpL5* (Milano et al. 2009), which coincides with cross-resistance to clofazimine and the new antituberculous drug bedaquiline (Hartkoorn, Uplekar and Cole 2014; Bloemberg et al. 2015). As *MmpL5* is also involved in isoniazid extrusion, *MmpR* mutants are likely also to be resistant to isoniazid (Milano et al. 2009).

However, efflux systems may act as a stepping stone for the evolution of high-level resistance, as convincingly demonstrated by *in vitro* studies (Machado et al. 2012; Schmalstieg et al. 2012). As efflux pumps seem to be essential for macrophage infection (Lamichhane, Tyagi and Bishai 2005), efflux pump inhibitors might be used to inhibit bacterial growth and lower the MICs for certain drugs (Pule et al. 2016).

ACQUIRED DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

Apart from the intrinsic resistance mechanisms mentioned above, the majority of clinically relevant drug resistance in *M. tuberculosis* is conferred by chromosomal mutations. These chromosomal mutations confer drug resistance via a large array of different mechanisms and may confer different levels of resistance (Fig. 1). The most common targets of chromosomal mutations conferring drug resistance are summarised in Table 1. Depending on the antibiotic in question, there may be multiple mechanisms of resistance.

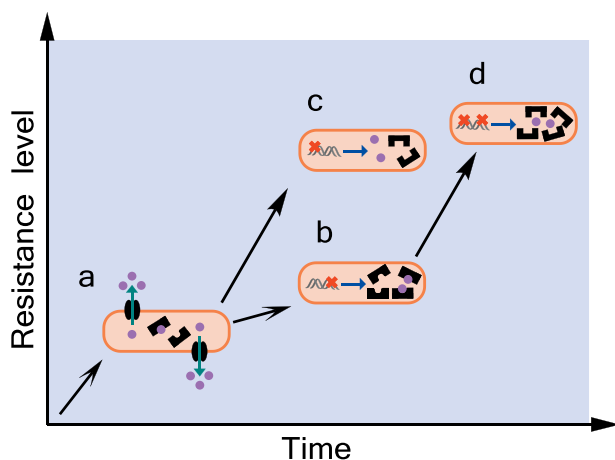


Figure 1. Levels of drug resistance conferred by different mechanisms. The red cross indicates a chromosomal mutation. (a) Low-level resistance due to induction of efflux pumps. Depiction of efflux pumps is omitted later stages for clarity. (b) Low-level resistance due to target overexpression caused by chromosomal mutation. (c) High-level resistance due to drug target modification conferred by chromosomal mutation. (d) High-level resistance due to overexpression and modification of drug target conferred by independent chromosomal mutations.

Drug target alteration

The most common mechanism of drug resistance in *M. tuberculosis* is drug target alteration. Interactions of drug and drug target moieties are highly specific. Changes in the drug–drug target interaction sites may reduce or completely abolish drug binding and therefore confer resistance to the drug in question. Non-synonymous mutations in drug target encoding gene(s) (Table 1) or nucleotide substitutions in the operon encoding the ribosomal RNA are frequently observed to confer drug resistance in *M. tuberculosis* as in the case of resistance against rifamycins, isoniazid, fluoroquinolones, aminoglycosides, cyclic peptides, para-aminosalicylic acid and oxazolidinones (e.g. linezolid). For example, mutations in the active site of the DNA-dependent RNA polymerase, corresponding to the 81 bp region known as the rifampicin resistance determining region, confer resistance to rifampicin, by decreasing the affinity of rifampicin for target (Campbell et al. 2001). Antibiotics target essential cellular functions and the drug targets performing these functions are highly conserved. The highly conserved nature of the drug targets limits the mutational target size (box 1) as the resistance mutation has to accomplish two things: first, it has to prevent the antibiotic from inhibiting the target and second it must ensure that the essential function of the drug target can still be performed. In many but not all cases this leads to a reduction in the bacterial cell's fitness in absence of the drug.

Abrogation of prodrug activation

Several antimycobacterial drugs are prodrugs, and abrogation of the drug-activating mechanisms leads to resistance as in the case of the first-line drugs isoniazid and pyrazinamide, the second-line drugs ethionamide and para-aminosalicylic acid, as well as the two new nitroimidazole drug candidates delamanid and pretomanid. In certain cases, the prodrug-activating enzyme is not essential for mycobacterial growth and survival (e.g. *pncA/ddn*; Table 1). The target size for drug resistance-conferring chromosomal mutations is therefore large—many point mutations, insertions/deletions, insertion of mobile genetic elements

etc. will cause disruption of the prodrug-activating gene product without compromising bacterial survival. Furthermore, mutations in the promoter of the gene might lead to lower transcript and therefore lower levels of the enzyme activating the prodrug. Lower levels of the prodrug-activating enzyme will then in turn lead to higher minimal inhibitory concentrations for the drug in question.

In pyrazinamide-resistant *M. tuberculosis* strains, we observe a wide array of different mutations in the gene *pncA* which encodes the enzyme metabolising pyrazinamide to its active form pyrazinoic acid. The mutational target size for delamanid/pretomanid resistance is considerably larger as multiple enzymes and cofactors are involved in the metabolism of prodrugs to their active forms. This suggests that resistance to the latter two drugs may evolve swiftly due to the large mutational target size (box 1). On the other hand, the gene *katG* encoding a catalase/peroxidase involved in the activation of isoniazid is required for robust replication of *M. tuberculosis* in macrophages (Manca et al. 1999). The mutational target size for isoniazid resistance is small, compared to pyrazinamide or delamanid/pretomanid. The resistance-conferring mutation in *katG* must retain the (*in vivo*) essential function of the enzyme (catalase/peroxidase—detoxification) as well as prevent the activation of isoniazid. Most clinical *M. tuberculosis* isolates harbour the point mutation *katG* S315T which retains most catalase/peroxidase functions as well as conferring high-level isoniazid resistance (Pym, Saint-Joanis and Cole 2002). On the other hand, *KatG* is not essential for *in vitro* replication—this greatly enlarges the mutational target size for *in vitro* resistance, as any mutation disrupting the function of *KatG* will lead to resistance (Bergval et al. 2009).

Overexpression of drug targets

Overexpression of the drug target may overcome the inhibition by the drug in question due to an overabundance of the target. Mutations in transcriptional repressors or the promoter of the drug target may cause the overexpression of the drug target as in the case of isoniazid, ethambutol and cycloserine. Drug target overexpression confers low-level resistance (e.g. to isoniazid or cycloserine), which usually can be overcome by increasing the dosing of drugs administered. Drugs are administered at fixed doses, often adjusted for patient weight/age. This is generally done to achieve the maximum effectiveness of the drug whilst minimising adverse effects of administered drugs. As certain antibiotics (e.g. cycloserine; Desjardins et al. 2016) show dramatic adverse effects, the dose given to patients is reduced as much as possible, which means there is little room for increasing the drug doses to overcome resistance due to drug target overexpression. Overexpression of drug targets may serve as a stepping stone to high-level resistance, which is conferred either by drug target alteration or abrogation of prodrug activation (Fig. 1).

EVOLUTION OF DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

The evolution of drug-resistant *M. tuberculosis* variants has generally been attributed to inadequate implementation of control measures, interrupted drug supply, low-quality drugs and patient non-adherence. However, it is increasingly evident that these factors alone are insufficient to explain the evolution of drug resistance in TB, as resistant *M. tuberculosis* strains evolve in well-functioning health systems and under strict treatment

Table 1. List of the most common targets of chromosomal mutation conferring drug resistance in *M. tuberculosis*.

Antibiotic	Target gene	Resistance mechanism	Reference
Rifampicin	<i>rpoB</i>	Drug target alteration	Telenti et al. (1993)
Isoniazid	<i>katG</i>	Abrogated prodrug activation	Heym et al. (1995)
	<i>inhA</i>	Drug target alteration	Banerjee et al. (1994); Morlock et al. (2003); Hazbón et al. (2006)
	<i>inhA</i> promotor	Drug target overexpression	Morlock et al. (2003); Hazbón et al. (2006)
Ethambutol	<i>embB</i>	Drug target alteration	Sreevatsan et al. (1997)
Pyrazinamide	<i>pncA</i>	Abrogated prodrug activation	Konno, Feldmann and McDermott (1967); Scorpio and Zhang (1996)
Ethionamide	<i>inhA</i>	Drug target alteration	Banerjee et al. (1994); Morlock et al. (2003); Hazbón et al. (2006)
	<i>inhA</i> promotor	Drug target overexpression	Morlock et al. (2003); Hazbón et al. (2006)
	<i>ethA</i>	Abrogated prodrug activation	Morlock et al. (2003)
Fluoroquinolones	<i>gyrA/B</i>	Drug target alteration	Takiff et al. (1994); Xu et al. (1996); Malik et al. (2012)
Streptomycin	<i>rrs</i>	Drug target alteration	Meier et al. (1994); Maus, Plikaytis and Shinnick (2005a)
	<i>rpsL</i>	Drug target alteration	Nair et al. (1993); Meier et al. (1994)
Amikacin	<i>rrs</i>	Drug target alteration	Alangaden et al. (1998); Maus, Plikaytis and Shinnick (2005a)
Kanamycin A	<i>rrs</i>	Drug target alteration	Alangaden et al. (1998)
	<i>eis</i> promotor	Overexpression of drug inactivating enzyme	Kambli et al. (2016)
Capreomycin	<i>rrs</i>	Drug target alteration	Maus, Plikaytis and Shinnick (2005a)
	<i>tlyA</i>	Abrogation of drug target methylation	Maus, Plikaytis and Shinnick (2005b); Monshupanee et al. (2012)
P-aminosalicylic acid	<i>thyA</i>	Drug target bypassing	Zhao et al. (2014); Minato et al. (2015)
	<i>folC</i>	Abrogation of prodrug activation	Zhao et al. (2014); Minato et al. (2015)
Cycloserine	<i>ald</i>	Overabundance of drug target substrate	Desjardins et al. (2016)
	<i>alr</i>	Drug target alteration	Desjardins et al. (2016)
	<i>alr</i> promotor	Drug target overexpression	Desjardins et al. (2016)
Bedaquiline	<i>atpE</i>	Drug target alteration	Huitric et al. (2010)
	Promotor/ <i>mmpR</i>	Overexpression of efflux pump MmpL5	Hartkoorn, Uplekar and Cole (2014); Bloemberg et al. (2015)
Linezolid	<i>rplC</i>	Drug target alteration	Beckert et al. (2012)
	<i>rrl</i>	Drug target alteration	Hillemann, Rusch-Gerdes and Richter (2008)
Delamanid/pretomanid	<i>ddn</i>	Abrogation of prodrug activation	Manjunatha et al. (2006); Bloemberg et al. (2015); Haver et al. (2015)
	<i>fgd1</i>	Abrogation of prodrug activation	
	<i>fbiA/B/C</i>	Abrogation of prodrug activation	
Clofazimine	Promotor/ <i>mmpR</i>	Overexpression of efflux pump MmpL5	Hartkoorn, Uplekar and Cole (2014); Bloemberg et al. (2015)

adherence (Caminero 2008; Calver et al. 2010). Pathogen and host determinants are increasingly recognised to influence the evolution of drug resistance. For instance, the emerging field of pharmacogenomics has demonstrated that the current dosage regimen for TB treatment fails to generate sterilising concentrations of certain antituberculous drugs in all patients and may contribute to treatment failure, as well as facilitate the evolution of drug resistance (Gumbo 2010; Swaminathan and Ramachandran 2012). Furthermore, recent studies have demonstrated that there is considerable variability in drug penetration into TB lesions, generating spatial and temporal variation in drug concentrations within the infected lung (Prideaux et al. 2015). It is well established that subinhibitory drug concentrations facilitate the evolution of drug resistance (Gillespie et al. 2005; Gullberg et al. 2011; Andersson and Hughes 2014), and heterogeneity between and within patients may mean that some TB cases are being unwittingly exposed to subinhibitory treatment regimes.

Although the genus *Mycobacterium* in general and *M. tuberculosis* in particular show low genetic diversity compared to other bacteria (Achtman 2008), the standing genetic diversity (box 1) exhibited by *M. tuberculosis* translates into phenotypic diversity.

There are seven extant *M. tuberculosis* lineages, which demonstrate specific phylogeographic patterns. Lineages 5 and 6 are restricted to West Africa and lineage 7 to the Horn of Africa. Lineage 1 is found along the rim of the Indian Ocean and lineage 3 is predominantly found in East Africa and South Asia. Lineage 4 and to a lesser extent lineage 2 are globally distributed (Borrell and Gagneux 2011; Coscolla and Gagneux 2014). Lineage 2 and lineage 4 have been frequently associated with drug resistance (Fenner et al. 2012; Cohen et al. 2015).

To better understand the *de novo* evolution of drug resistance, it is helpful to separate the different processes involved. The rate of evolution of resistance (Ford et al. 2013) and the effect of drug resistance on bacterial life history traits like growth rate/yield may differ as a function of the strain genetic background. Drug resistance is often associated with reduction in bacterial fitness (box 1) in the absence of the drug. This reduction is, however, not universal and can vary as a function of the genetic background (Gagneux et al. 2006c). The fitness cost of drug resistance may be ameliorated by secondary, so-called compensatory mutations, which do not contribute to resistance on their own (Comas et al. 2011; Casali et al. 2012; de Vos et al. 2013; Hughes and

Brandis 2013). Furthermore, several drug resistance-conferring mutations present in a single strain might interact epistatically (box 1) and influence bacterial fitness (Gagneux et al. 2006c; Borrell et al. 2013). This means that the strain genetic background, compensatory mutations and the presence of multiple resistance mutations may interact to influence the fitness of drug-resistant *M. tuberculosis* strains (Fig. 3).

The spread and maintenance of resistant variants in the population not only depends on the effect of drug resistance on the bacterial life history traits, but is also strongly dependent on the pathogen's population structure and effective population size, as well as genetic drift (box 1). In the case of *M. tuberculosis*, genetic drift is thought to strongly influence the genetic diversity of the organism, as the effective population size is believed to be small and subject to large bottlenecks during patient-to-patient transmission (box 1) (Hershberg et al. 2008).

DE NOVO EVOLUTION OF DRUG RESISTANCE

There are three important factors influencing the *de novo* evolution of drug resistance: the population size, as it relates to the number of binary fission events the population has undergone; the mutation rate; and the mutational target size (box 1). Together, these factors determine the rate of resistance acquisition. The strain genetic background may influence any of these parameters. It is, however, not trivial to determine the contribution of each of the factors, as they are either difficult to study *in vivo* or are not independent of each other.

Population size

In a series of iconic experiments, Luria and Delbrück demonstrated that, for simple traits (e.g. most bacteriophage/antibiotic resistance), bacterial populations which undergo a sufficient number of doubling events inevitably harbour resistant variants, following what is now called a Luria-Delbrück distribution (Luria and Delbrück 1943). The larger the population, the more cell division events the population experienced, and therefore the larger probability for a drug resistance mutation to arise. Furthermore, if a resistance-conferring mutation evolves early during population expansion, the vast majority of the population will be resistant to a given drug even before treatment onset. We unfortunately do not have good estimates of the number of *Mycobacterium tuberculosis* cells present in human lungs during infection. The number of bacteria present in a single lesion is estimated to be in the order of 10^8 bacterial cells per lesion (Shimao 1987), although it is not clear what the basis of this estimate is. The best estimates of viable *M. tuberculosis* cells were obtained from the lungs of cynomolgus macaques (*Macaca fascicularis*) infected with *M. tuberculosis*. The disease presentation in macaques resembles that observed in humans. The study demonstrated that a macaque lung contains anything between $\approx 10^5$ and 5×10^8 cells, depending on the individual macaque (Lin et al. 2009). However, most monkeys did not develop cavitary disease. It is believed that *M. tuberculosis* may reach very high cell densities when replicating on the interior surface of open (i.e. with access to the airways) lung cavities, but the exact cell numbers have, to our knowledge, never been assessed in humans. There is evidence that drug-resistant *M. tuberculosis* variants are predominantly found where cell densities are high, i.e. at the interior of cavitating granulomas (Kaplan et al. 2003). It is exceedingly difficult to obtain reliable estimates of bacterial cell numbers in the lungs of TB patients. Recent studies have highlighted that the disease presentation in the lung is far more dynamic as previously

believed (reviewed in Lenaerts, Barry and Dartois 2015). The number of granuloma cannot be directly used to estimate the number of bacterial cells present in a patient lung, as there is a large diversity of microenvironments within granuloma and not all environments allow bacterial replication. However, if we solely focus on cavitating granuloma, we might be able to use disease severity, i.e. the number and extent of cavitations as a very rough proxy for population size. The fewer the cavities present in a patient's lung, the smaller the population size of tubercle bacilli. There are reports of differences between lineages in terms of disease severity (reviewed in Coscolla and Gagneux 2010, Coscolla and Gagneux 2014). Lineages 2 and 4, especially the so-called Beijing sublineage of lineage 2, have been associated with more severe disease presentation compared to other lineages. The *M. tuberculosis* lineages 2 and 4 are also associated with drug resistance (Pardini et al. 2009; Niemann et al. 2010; Mokrousov et al. 2012; Ford et al. 2013; Casali et al. 2014; Cohen et al. 2015; Merker et al. 2015). Differences in bacterial population sizes between lineages might therefore contribute to the differential association of specific lineages with drug resistance.

In general, we can say that the probability of evolving resistance is dependent on the number of binary fission events. *M. tuberculosis* is an intracellular pathogen and its primary niche is the macrophage. Although *M. tuberculosis* is able to survive and replicate inside macrophages, the tubercle bacteria seem to be inhibited in growth when the bacterial numbers are small (Welin et al. 2011). It is not clear if this is due to killing or growth inhibition by the macrophage. However, there is evidence that the innate immune system is able to clear *M. tuberculosis* infections before the onset of adaptive immunity (Verrall et al. 2014). It is therefore likely that a proportion of the tubercle bacilli are killed by the macrophage. This means that a *M. tuberculosis* population of a given size has likely undergone more binary fission events than expected from exponential growth, as the immune system continuously removes bacterial cells from the population. As killing of tubercle bacilli by macrophages is likely to occur stochastically, one can view this as a form of genetic drift (box 1), potentially slowing the rate of drug resistance evolution. A recent study demonstrated that there are differences in the replication potential between different *M. tuberculosis* lineages—lineage 2 and 4 being proficient in replicating in macrophages, and strains belonging to lineage 3 and *Mycobacterium africanum* less so (Reiling et al. 2013). However, it is not clear if the high cell densities observed in macrophages infected with *M. tuberculosis* strains belonging to lineage 2 and 4 are due to better survival in the macrophage or faster growth or both. In conclusion, it is not entirely clear if there is substantial variability in terms of number of binary fission events between lineages of *M. tuberculosis*. The recent adaptation of a fluorescence dilution assay to *M. tuberculosis* offers the exciting possibility to assess the number of doubling events and the extent of cell death experienced by mycobacterial bacilli during macrophage infection (Helaine et al. 2016). Measuring growth properties of different *M. tuberculosis* lineages in an *ex vivo* system will help to clarify if there are differences between lineages concerning the number of binary fission events and therefore the rate of drug resistance evolution.

Mutation rates

The mutation rate is thought to be largely defined by the replication fidelity of the bacterial DNA polymerases. The basal mutation rate of *M. tuberculosis* is difficult to study as the long generation time makes mutation accumulation experiments

unfeasible. A workaround is expressing the *M. tuberculosis* DnaE1 DNA polymerase heterologously in *M. smegmatis*. The generation time of *M. smegmatis* is much lower compared to *M. tuberculosis*, but it has the drawback that the effect of the genetic background (box 1) on mutation rates cannot be taken into account. Using the heterologous DnaE1 expression approach, the mutation rate has been determined to be 4.52×10^{-10} ($2.95\text{--}7.35 \times 10^{-10}$ 95% confidence interval) per bp and generation, which is on the lower end of the spectrum compared to other bacteria (Rock et al. 2015). Other studies have assessed the mutation rate using whole genome sequencing (Ford et al. 2011). However, there are potentially many more factors influencing the mutation rate, including the intracellular environment of macrophages which are rich in reactive oxygen and nitrogen species, expression of error-prone DNA polymerases (DnaE2) due to the stressful macrophage environment, enzymes involved in repair–replication–recombination, existing drug resistance mutations, exposure to UV radiation and/or desiccation during aerosolisation (McGrath et al. 2014).

Mutational target size

Given a mutation rate, the number of potential sites which may be mutated to confer drug resistance is an important factor involved in determining the rate of drug resistance evolution. As mentioned earlier, the mutational target size varies depending on resistance mechanism (box 1). The mutational target size for prodrugs activated by non-essential enzymes is much larger compared to the target sizes for mutational drug target alteration. The mutational target size for drug target alteration is in the range of 81 bp in the gene *rpoB* for rifampicin resistance (Musser 1995) and 117 bp in the gene *gyrA* for fluoroquinolone resistance (Maruri et al. 2012). The majority of resistance-conferring mutations are found in these regions in *clinico* (Sandgren et al. 2009). Furthermore, the number of mutations conferring drug resistance is dependent on the resistance level in question. Drug resistance mutations differ in the level of resistance they confer. There is an inverse relationship between the drug resistance level that mutations confer and the mutational target size—the higher the selective concentration, the smaller the target size, i.e. there are fewer mutations conferring high-level than low-level resistance (Ford et al. 2013). Drug resistance mutations interact epistatically with the genetic background of a strain—a given drug resistance mutation may have different trait effects (box 1) in different genetic backgrounds. This may lead to a reduction in the mutational target size in the broader sense when the interaction of a resistance mutation with the strain genetic background results in a detrimental trait effect in absence of the selective agent.

Rate of drug resistance acquisition

The rate of drug resistance acquisition is defined by the mutation rate and the mutational target size in the broader sense and may be calculated by Luria–Delbrück fluctuation assays (Luria and Delbrück 1943). In the past, there has been considerable debate on the influence of the genetic background on the rate of resistance acquisition. As mentioned previously, lineage 2 and lineage 4 have been disproportionately associated with drug resistance. It was found previously that *M. tuberculosis* strains belonging to lineage 2 carry mutations in genes involved in DNA replication, repair and recombination (Mestre et al. 2011), potentially elevating mutation rates. However, this would imply that lineage 2 in general should demonstrate greater average genetic

diversity compared to other lineages, which does not appear to be the case (Coscolla and Gagneux 2014). Multiple studies have focused on the rate of resistance acquisition in *M. tuberculosis*. The rate of resistance acquisition is influenced by the basal mutation rate and the mutational target size in the broader sense. Some studies report differences in resistance acquisition rates between lineages, where strains from the Beijing sublineage of lineage 2 show higher rates of resistance acquisition compared to other lineages (de Steenwinkel et al. 2012; Ford et al. 2013), others reported similar rates between lineages (Werngren and Hoffner 2003). If there are differences in the rate of resistance acquisition between lineages, this could indicate that the mutational target size in the broader sense is different between lineages. Even if the results are discrepant, we are still missing a large part of the picture. Due to the labour-intensive nature of conducting Luria–Delbrück fluctuation assays with *M. tuberculosis*, most studies have so far focused on the rate of acquisition of resistance to rifampicin; there are few data available on differences in drug resistance acquisition rates between lineages for other drugs commonly in use to treat TB.

De novo evolution of drug resistance and treatment with drug combinations

TB cases caused by drug-susceptible *M. tuberculosis* strains are treated with a combination of four drugs to limit the evolution of drug resistance, as a strain would need to acquire at least four independent resistance mutations in order to achieve high-level resistance, which is theoretically unlikely to happen if the *M. tuberculosis* bacilli carry no pre-existing resistance mutations and are exposed to all drugs at the same time. However, in some patients the tubercle bacilli evolve drug resistance despite strict treatment adherence (Calver et al. 2010). This may be caused by functional monotherapy and/or subinhibitory drug concentrations as discussed above, known to facilitate the evolution of drug resistance (Gillespie et al. 2005; Andersson and Hughes 2014). Suboptimal drug concentrations can arise due to the differential potential of certain drugs to penetrate bacteria containing lesions in the human lung. For instance, pyrazinamide and rifampicin have been demonstrated to be able to diffuse and accumulate in the hypoxic and acidic granuloma, whereas the other first-line drugs do not accumulate in these structures (Prideaux et al. 2015). These processes are likely to generate spatial and temporal variation in drug concentrations, facilitating the evolution of drug resistance (Moreno-Gamez et al. 2015). Drug penetration into TB lesions is dependent on multiple factors including lipophilicity and solubility. Detailed knowledge of the chemical properties needed for effective distribution throughout all *M. tuberculosis* containing lesions will aid the design of novel, more effective antituberculous drug regimens (Dartois 2014).

Drug resistance levels and the strain genetic background

Apart from the rate of resistance evolution, the genetic background may influence the level of resistance conferred by a drug resistance mutation. In the case of isoniazid resistance, it has been shown that the level of resistance conferred by different mutations varies with the genetic background of *M. tuberculosis*, whereby the isoniazid resistance mutation *katG* S315T conferred lower levels of resistance in strains belonging to lineage 1 compared to lineages 2, 3 and 4 (Fenner et al. 2012). This phenomenon

could also contribute to different mutational target sizes in the broader sense in different genetic backgrounds (Ford et al. 2013). Depending on the concentration of the drug, fewer resistance mutations might be available to certain lineages, which in turn would lower the rate of resistance evolution.

FITNESS OF DRUG-RESISTANT MYCOBACTERIUM TUBERCULOSIS STRAINS

Drug resistance was long believed to be universally associated with a reduction in the drug-resistant organism's fitness in the absence of antibiotics (box 1). In the case of mycobacteria, this dogma was established by early studies performed with isoniazid resistant *M. tuberculosis* variants. These studies demonstrated a marked reduction in virulence of certain, but not all, isoniazid resistant strains in guinea pigs and mice (Barnett, Busby and Mitchison 1953; Middlebrook and Cohn 1953). The observation that most drug resistance is associated with a reduction in fitness is further corroborated by studies performed in other pathogenic microorganisms (Melnik, Wong and Kassen 2015). Early mathematical models predicted that MDR-TB would remain a localised public health problem (Dye and Espinal 2001; Dye 2002). Hence, initially, the World Health Organization recommended focusing on DOTS for patients with drug-susceptible TB and against treating patients infected with MDR *M. tuberculosis* strains, as treating these patients is exceedingly expensive and the MDR strains were not believed to transmit enough to establish a sustainable infection chain (Espinal and Dye 2005).

Mathematical models predict that the probability of a drug-resistant strain spreading in the absence of antibiotic pressure is largely dependent on the resistant variant's reproductive fitness (Blower and Chou 2004; Cohen and Murray 2004; Luciani et al. 2009; Knight et al. 2015). The ultimate measure of fitness would be the effective reproductive number R (box 1), which measures the average number of secondary cases generated per infected individual in a population of susceptible and resistant hosts. The effective reproductive number R is a high-level composite measure comprising all aspects concerning transmission, including pathogen, host and environmental factors. Changes in R due to drug resistance mutations can lead to changes in allele frequencies over time. However, assessing R *in vivo* requires prospective cohort studies which are labour intensive and expensive to conduct. Although cohort studies allow assessment of epidemiological factors associated with transmission, they often suffer from small sample sizes and are restricted to a single setting relevant for transmission (e.g. households).

To better study the impact of drug resistance on the fitness (i.e. R) of *M. tuberculosis*, it is helpful to separate the effect of drug resistance on bacterial life history traits—the trait effect (box 1), from effects of drug resistance on allele frequencies in the population—the selective effect (Hall et al. 2015). Trait effects of drug resistance mutations may be assessed by measuring life history traits like growth rate/yield of drug-resistant *M. tuberculosis* variants *in vitro*. Drug resistance mutations may have diverse pleiotropic effect on bacterial physiology resulting in a reduced *in vitro* growth (Fig. 2). However, this reduction in growth yield/rates is not universal; it is strongly dependent on the mechanism of resistance, as well as the specific mutation in question. Furthermore, there is a strong effect of the strain genetic background on the fitness costs. Fitness costs may be ameliorated by secondary, so-called compensatory mutations, which do not contribute to drug resistance on their own. Compensatory mutations may lead to the retention of drug-resistant

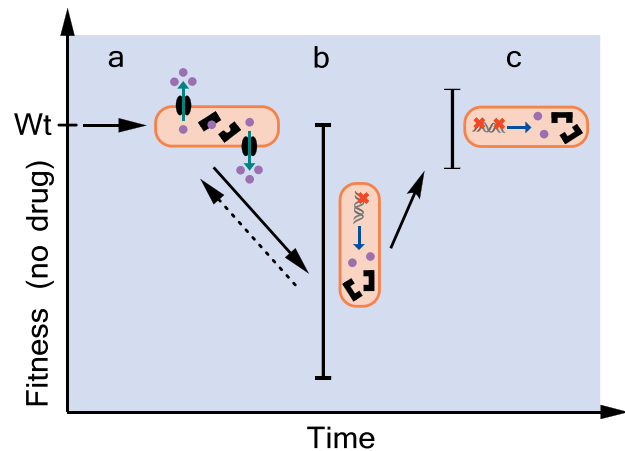


Figure 2. Fitness of drug resistant *M. tuberculosis* strains in absence of the drug. Solid arrows indicate the trajectories of drug resistance. Dashed arrow indicates the unlikely possibility of reversion. The red cross indicates a chromosomal mutation. (a) Induction of efflux pumps at no or minimal fitness cost. (b) Large variation in fitness costs due to resistance via chromosomal mutations—ranging from very low/no to lethal. The observed variation may arise due to epistatic interactions of the strain genetic background and the resistance mutation(s). (c) Variation in fitness levels of strains harbouring secondary compensatory mutations—ranging from comparable to wild-type levels to slightly below.

M. tuberculosis variants in the population in absence of selective pressure due to the drug. Epistatic interactions between the genetic background and the resistance-conferring mutations seem to be pervasive and an important determinant in shaping the population biology of drug-resistant *M. tuberculosis* variants. Measuring *in vitro* fitness of drug resistance mutations as a proxy for *in vivo* fitness has been criticised for not capturing the complex dynamics involving nutrient limitation, activity of the immune system, host genetics and comorbidities (Björkman et al. 2000). However, the frequency of resistance alleles in the population correlates well with the *in vitro* fitness of the strains carrying mutations for rifampicin, fluoroquinolone and aminoglycoside resistance in laboratory adapted, as well as in clinical *M. tuberculosis* isolates (Böttger et al. 1998; Sander et al. 2002; Gagneux et al. 2006; Böttger and Springer 2008; Borrell et al. 2013). However, all *in vitro* fitness cost assessments are based on the ability of the *M. tuberculosis* strain being able to readily grow in artificial growth media. It is well established that not all bacilli present in patient sputum will demonstrate *in vitro* growth. This is problematic as our frequency estimates for drug resistance alleles are based on culturing *M. tuberculosis* bacilli from patient sputum, isolating genetic material and subsequent sequencing. However, there is no *a priori* reason to assume that the frequencies of drug resistance mutations are different in the populations of culturable and unculturable bacilli. Culture-free metagenomic approaches will offer a less biased view on the frequencies of drug resistance mutations present in the lungs (sputa) of TB patients (Koch, Mizrahi and Warner 2014).

In vitro fitness of drug-resistant Mycobacterium tuberculosis strains

There are two methods for assessing *in vitro* fitness of resistant variants: growth rate measurements and competition assays. Growth rate assessment focuses on the replication rate of a strain during exponential growth in absence of any drug pressure. In competition assays, the resistant variant is co-cultured

with the susceptible wild-type strain for one or several bacterial growth cycles in absence of the drug. At the endpoint of the experiment, the ratio of resistant to susceptible variants is assessed. Both methods allow calculation of selection coefficients. The advantage of competition assays is that they take growth yield as well as growth rate into account. Selection on growth rate occurs when there is no population structure and resources are freely available to all individuals. On the other hand, growth yield is favoured in structured populations where resources are restricted and efficiency in nutrient utilisation is favoured (Frank 2010). Populations of *M. tuberculosis* in human lungs have been thought of as highly structured, as observed by the concurrent presence of strains carrying different resistance mutations in separate parts of the infected lung (Kaplan et al. 2003; Post et al. 2004; Lieberman et al. 2016).

Several studies have investigated the effect of drug resistance mutations on *in vitro* fitness of *M. tuberculosis* or *M. smegmatis* either using competition assays or growth rate assessments. These studies have focused on assessing the impact of rifampicin (Gagneux et al. 2006c; Song et al. 2014), aminoglycoside (Sander et al. 2002; Freihofer et al. 2016) and fluoroquinolone (Borrell et al. 2013) resistance on the bacterium's fitness compared to the susceptible ancestor in absence of the drug. We do not have reliable data on isoniazid resistance as the *in vitro* resistance mechanism differs from that observed *in vivo* (Bergval et al. 2009). However, there is reason to assume that there are no or very low fitness costs of the most frequent isoniazid resistance mutation (*katG* S315T), as *M. tuberculosis* variants carrying this mutation transmit efficiently and are virulent in mice (van Soolingen et al. 2000; Pym, Saint-Joanis and Cole 2002; van Doorn et al. 2006; Gagneux et al. 2006a). For the same reasons we do not have reliable data on the fitness of clinical MDR/XDR strains. We are restricted to measuring the fitness of such strains relative to each other, rather than to their cognate drug-susceptible ancestor (Spies et al. 2013; Naidoo and Pillay 2014).

Most rifampicin resistance mutations carry a significant fitness cost in *M. tuberculosis* (Billington, McHugh and Gillespie 1999; Mariam et al. 2004; Gagneux et al. 2006b; Koch, Mizrahi and Warner 2014) (Fig. 2). However, there are some mutations (e.g. *rpoB* S450L—*M. tuberculosis* notation; Andre et al. 2017) which only cause a small defect in fitness. There seems to be some influence of the genetic background on the cost of rifampicin resistance, whereby lineage 2 experiences smaller reductions in fitness compared to lineage 4 (Gagneux et al. 2006c). Interestingly, the fitness costs of rifampicin resistance are elevated under nutrient-restricted conditions (Song et al. 2014). Nutrient limitation is pervasive in the human host, indicating that there will be strong selection against costly drug resistance mutations. Fluoroquinolone resistance mutations show varying degrees of costs in *M. smegmatis* (Borrell et al. 2013). Some mutations conferring fluoroquinolone resistance even show a fitness benefit, but this could be an artefact of the *in vitro* assessment conditions. Most aminoglycoside resistance is also costly. There are, however, also resistance mutations which do not demonstrate any discernable fitness cost (Sander et al. 2002; Freihofer et al. 2016). The effect of the strain genetic background on the fitness cost of aminoglycoside resistance is yet to be determined. The *in vitro* fitness of MDR and especially XDR *M. tuberculosis* strains is variable. Some strains demonstrate very low replication rates, whilst others replicate at rates similar to drug-susceptible strains (Spies et al. 2013; Naidoo and Pillay 2014). However, it is striking that combinations of low-cost resistance mutations rifampicin (*rpoB* S450L—*M. tuberculosis* notation) and isoniazid (*katG* S315T) in MDR/XDR strains are frequently observed in clinico. TB caused

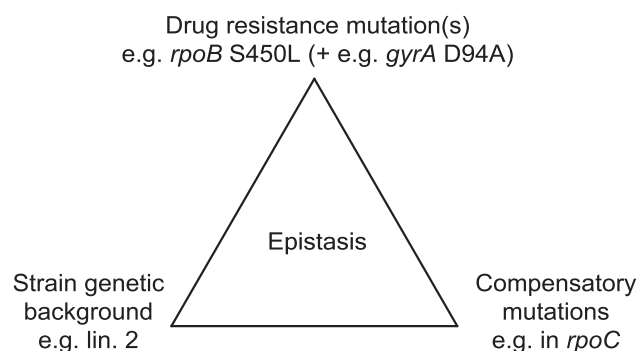


Figure 3. Pervasive epistatic interactions influencing the physiology and population biology of drug-resistant *M. tuberculosis* strains. The effect of drug resistance mutations alone or in combination as well as the presence of compensatory mutations affects the fitness of drug-resistant *M. tuberculosis* strains as a function of the strain genetic background.

by XDR *M. tuberculosis* strains is a rather new phenomenon. It is thought that most XDR *M. tuberculosis* isolates arise due to *de novo* evolution rather than transmission (Gandhi et al. 2006; Casali et al. 2014). The isolation of XDR *M. tuberculosis* strains with very low replication rates could reflect the fact that selection has not yet removed the most unfit XDR strains from the population. However, it is remarkable that there is a strong association of MDR/XDR strains with lineages 2 and 4 (Pardini et al. 2009; Niemann et al. 2010; Mokrousov et al. 2012; Ford et al. 2013; Casali et al. 2014; Cohen et al. 2015; Merker et al. 2015). This might indicate that strains belonging to these lineages experience lower fitness costs due to drug resistance. On the other hand, lineages 2 and 4 are also the most widespread. The association of these lineages with drug resistance could just be due to the large numbers of TB patients infected with strains from these lineages. To elucidate if the frequent association of lineages 2 and 4 with drug resistance is due to lower fitness costs of drug resistance experienced by these strains, we need detailed assessments of the population structures of drug-resistant and susceptible *M. tuberculosis* strains over time.

Compensation of fitness costs

Fitness costs caused by drug resistance mutations may be ameliorated by secondary so-called compensatory mutations (Fig. 2). These mutations do not contribute to drug resistance directly. Fitness cost compensation results from the epistatic interaction of the compensatory mutation with the drug resistance mutation and the genetic background of the strain (Fig. 3, box 1). Fitness cost compensation is poorly understood. Whole genome sequencing studies have demonstrated that MDR and especially XDR strains tend to harbour a multitude of mutations (Zhang et al. 2013; Merker et al. 2015). A high proportion of these mutations are thought to have functional consequences. As these strains are resistant to many antibiotics, the reported mutations can be speculated to be involved in compensation of fitness costs. To date, we know of three distinct mechanisms involved in the compensation of fitness costs (Andersson and Hughes 2010). First, secondary mutations in the genes encoding the drug target might restore or improve the mutated enzyme's function to levels comparable to the wild-type enzyme (Comas et al. 2011; Casali et al. 2012; Hughes and Brandis 2013). To be classified as a putative compensatory mutation, these mutations should only be found in resistant strains and never in susceptible strains. However, only fitness measurements can confirm or refute the

compensatory role of the mutation. Second, overexpression of an enzyme which performs a similar function to the drug target may compensate for the diminished or abolished function of the drug target (Sherman et al. 1996; Heym et al. 1997). Third, non-mutational gene regulatory responses may compensate for the fitness defect inflicted by the resistance mutation (Freihofer et al. 2016). However, the latter case is a form of phenotypic plasticity and the term 'buffering' of fitness costs would be more appropriate than compensation.

Compensatory evolution has been demonstrated to occur very frequently for rifampicin resistance (Comas et al. 2011; Casali et al. 2012; Hughes and Brandis 2013). Fitness costs of rifampicin resistance due to mutations in the β' -subunit of DNA-dependent RNA polymerase encoded by *rpoB* can be compensated by mutations in *rpoA/B/C*. The molecular basis of fitness costs and their compensation in rifampicin-resistant *M. tuberculosis* strains is not well understood. However, studies performed with rifampicin-resistant *Pseudomonas aeruginosa* have indicated that rifampicin resistance mutations in *rpoB* reduce the transcriptional efficiency of the DNA-dependent RNA polymerase and may explain the observed fitness costs. Compensatory mutations restored the transcriptional efficiency to wild-type levels in rifampicin-resistant strains (Qi, Preston and MacLean 2014). The connection between transcriptional efficiency and *in vitro* growth rate (fitness cost) seems plausible in fast-growing bacteria like *P. aeruginosa*. However, it remains to be demonstrated that fitness costs inflicted by mutations in *rpoB* are caused by reduced transcriptional efficiency in slow-growing bacteria like *M. tuberculosis*. Interestingly, the compensatory mutations are often found at the interface between the different RNA polymerase subunits. More specifically, the compensatory mutations home to positions in the vicinity of the active site and the RNA exit tunnel (Song et al. 2014). Compiling data from several studies (Comas et al. 2011; de Vos et al. 2013; Farhat et al. 2013; Lanzas et al. 2013; Müller et al. 2013; Casali et al. 2014; Song et al. 2014; Bloemberg et al. 2015; Eldholm et al. 2015; Merker et al. 2015), rifampicin resistance mutations in *rpoB* and putative compensatory mutations in *rpoA/B/C* offer some striking insights. In *M. tuberculosis*, most compensatory mutations in *rpoA/C* (Fig. 4b and c) are associated with the most clinically frequent rifampicin resistance mutation *rpoB* S450L (Fig. 4a and d). The rifampicin resistance-conferring mutation *rpoB* S450L consistently shows the lowest fitness cost *in vitro* (de Vos et al. 2013; Casali et al. 2014; Cohen et al. 2015; Meftahi et al. 2016). There are two non-exclusive explanations for this observation. First, the low average fitness cost of *rpoB* S450L might offer a large mutational spectrum for compensation, i.e. there are a multitude of different mutations in *rpoA/B/C* which compensate for the fitness cost of *rpoB* S450L. More costly rifampicin resistance mutations might have a more restricted mutational target for compensation. An alternative explanation would be a sequential nature of resistance and compensatory mutation acquisition. *Mycobacterium tuberculosis* strains carrying *rpoB* S450L are able to outcompete other rifampicin resistance mutations due to its low cost. This will lead to an overabundance of *M. tuberculosis* strains carrying the *rpoB* S450L mutation in the population, making it more likely for these strains to acquire compensatory mutations. Loss-of-function mutations in the gene *katG* are known to confer resistance to isoniazid (Table 1). However, the catalase/peroxidase activity of KatG is essential for efficient *in vivo* growth of *M. tuberculosis*. Besides KatG, the genome of *M. tuberculosis* encodes the peroxidase AhpC. Certain clinical isoniazid-resistant *M. tuberculosis* strains have been noticed to harbour mutations in the regulatory regions of the gene *ahpC*, which leads to overexpression of

AhpC. However, it was noticed that overexpression of *ahpC* does not compensate for the reduction in virulence observed in *katG* deletion mutants. Overexpression of *ahpC* might therefore only partially restore the fitness costs of *katG* deletions by enhancing detoxification of detrimental reactive oxygen species (Sherman et al. 1996; Heym et al. 1997). Recently, a non-mutational mechanism of fitness cost compensation was described. Upregulation of *tlyA* expression in capreomycin-resistant *M. tuberculosis* strains was found to partially compensate (buffer) the fitness cost inflicted by the mutation conferring resistance to capreomycin (Freihofer et al. 2016). Resistance to capreomycin can be conferred by mutations in the ribosomal RNA (Table 1). The gene *tlyA* encodes a methyltransferase which methylates nucleotides of the ribosomal RNA. Methylation of the ribosomal RNA nucleotide directly adjacent to the resistance conferring mutated nucleotide seems to partially restore fitness levels (Freihofer et al. 2016). Mitigation of fitness costs via non-mutational processes can be viewed as a plastic trait.

In vivo fitness of drug-resistant *Mycobacterium tuberculosis* strains

Although *in vitro* fitness measurements have provided us with valuable estimates of the fitness costs inflicted by drug resistance, the ultimate measure incorporating all steps from establishing an infection to transmission of *M. tuberculosis* is captured by the effective reproductive number *R* (box 1). Several studies have attempted to approximate *R* by conducting prospective cohort studies. These have mainly focused on the number of secondary cases generated in households of TB patients infected with drug-susceptible or drug-resistant *M. tuberculosis* strains. These studies (reviewed in Fox et al. 2013) have revealed conflicting results, with some showing considerably lower transmission rates among MDR vs susceptible *M. tuberculosis* strains and others showing comparable transmission rates. The heterogeneity in the determined transmission rates might reflect differences in study design.

Household contact studies are expensive and labour intensive to conduct. A more convenient and powerful way of assessing the *in vivo* fitness of drug-resistant *M. tuberculosis* strains is a population-based high-resolution genotyping study. The combination of whole genome sequencing together with high-quality epidemiological data will allow assessment of transmission rates at high spatial and temporal resolution. Several studies have already demonstrated the value of whole genome sequencing for inferring transmission networks (Walker et al. 2013; Casali et al. 2014; Luo et al. 2014; Hatherell et al. 2016; Yang et al. 2016).

Epistatic interactions between drug resistance mutations

TB infections are always treated with a combination of drugs. There is therefore strong selection for *M. tuberculosis* strains which are concomitantly resistant to several drugs used for treatment. The effect of multiple drug resistance mutations on the organism's phenotype is dependent on the epistatic interactions of the different resistance mutations with each other and with the genetic background of the strain. Certain combinations of resistance mutations may therefore be especially favoured if they do not confer a fitness deficit. Due to the vast number of different combinations of different drug resistance mutations, it is difficult to assess the contribution of different combinations

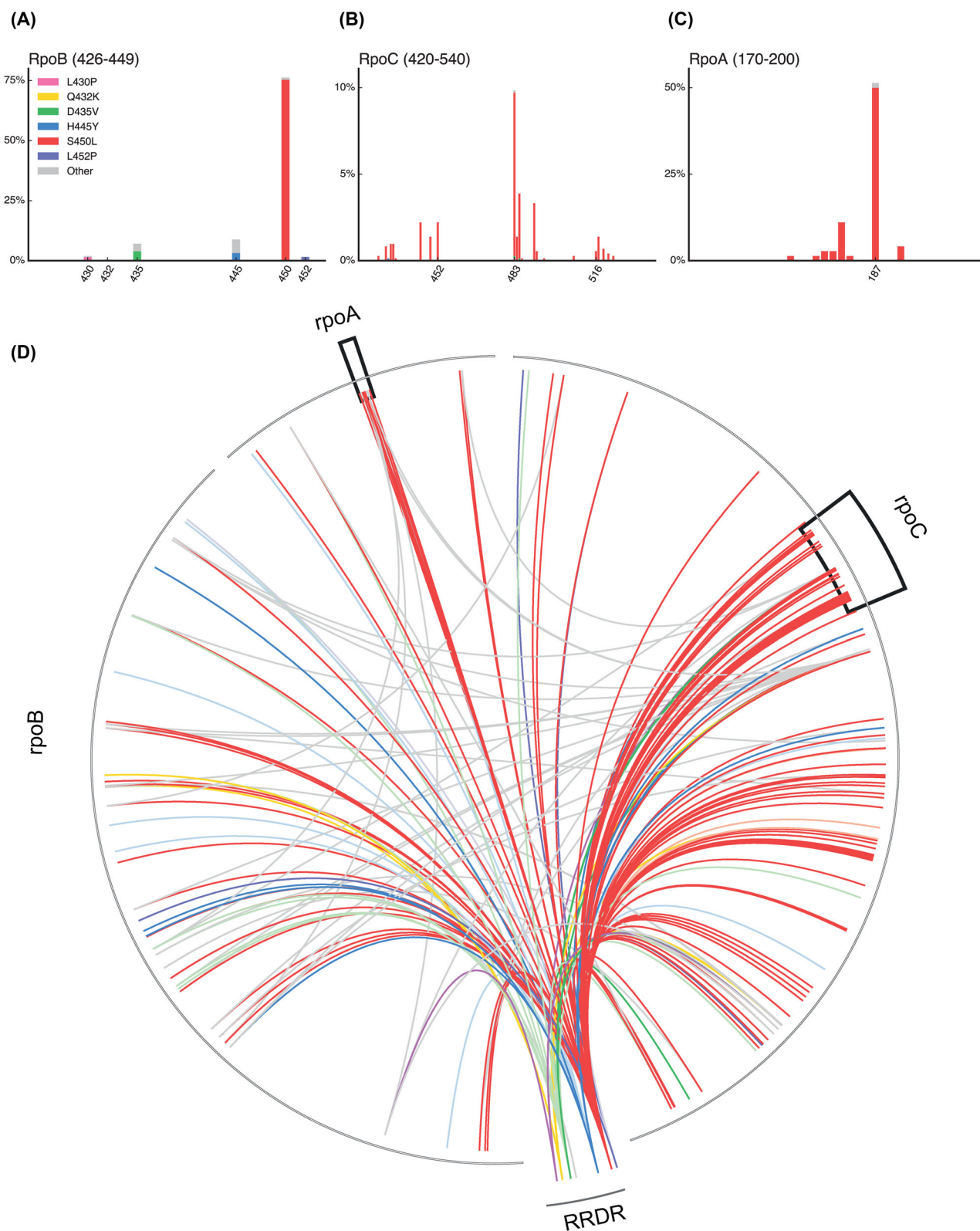


Figure 4. Summary of rifampicin resistance and fitness cost compensatory mutations in *rpoB* and *rpoA/C* respectively. (a) Frequency of rifampicin resistance mutations in *rpoB*. (b) Frequency of putative compensatory mutations in *rpoC* in codons 420–540. (c) Frequency of putative compensatory mutations in *rpoA* in codons 170–200. (d) Association of rifampicin resistance mutations in *rpoB* with putative compensatory mutations in *rpoA/C*.

of resistance mutations to the phenotype experimentally, especially in *M. tuberculosis*. However, assessing allele frequencies from clinical isolates may inform us about successful vs detrimental combinations of drug resistance alleles.

Several drugs target the information pathway from DNA to mRNA to proteins by inhibiting key enzymes involved in these processes. Mutations in these enzymes alone or in combination have pleiotropic effects on the fitness of *M. tuberculosis*. Infections with MDR *M. tuberculosis* strains are treated with drug regimens containing fluoroquinolones. Per definition these strains are resistant to isoniazid and rifampicin. Fluoroquinolone resistance mutations will therefore mostly evolve in rifampicin-resistant *M. tuberculosis* strains. Rifampicin targets the DNA-dependent RNA polymerase and fluoroquinolones target the DNA-gyrase and these two enzymes work in concert. Studies performed with *M. smegmatis* demonstrated that depending on the combination of rifampicin and fluoroquinolone resistance mutation, the effects on the *in vitro* growth rate range from detrimental to even beneficial. This goes so far, that fitness cost of a resistance mutation is fully compensated by the presence of a second resistance mutation. In certain cases, the double-resistant strain had an even faster growth rate than the drug-susceptible wild type. Combinations of the most beneficial resistance alleles are also the most frequent in clinical *M. tuberculosis* isolates (Borrell et al. 2013). *In vitro* studies have demonstrated that pre-existing isoniazid resistance mutations influence which rifampicin resistance mutations are subsequently acquired. This is probably due to a reduction in the mutational target size in the broader sense (box 1) due to epistatic interactions between the two resistance-conferring mutations, resulting in a dramatic reduction in fitness of specific combinations (Bergval et al. 2012). Streptomycin inhibits the bacterial ribosome, and resistance is often conferred by mutations in the gene *rpsL*. Streptomycin was the first active antituberculous drug in use and resistance to this drug is widespread. Household contact studies have determined that *M. tuberculosis* strains harbouring the streptomycin resistance-conferring mutation *rpsL* K43R together with the isoniazid resistance mutation *katG* S315T generated significantly fewer secondary cases indicating an epistatic interaction between the streptomycin and isoniazid resistance mutations resulting in a fitness cost (Salvatore et al. 2016). However, this is in contrast to other studies, which have found that the combination of *rpsL* K43R with *katG* S315T does not reduce fitness of *M. tuberculosis* harbouring these mutations (Spies et al. 2013). These discrepancies demonstrate the large variability in these interactions, highlighting the influence of the strain genetic background on all these interactions. In turn, drawing general conclusions from rather small datasets is not possible or at best challenging.

POPULATION GENETICS OF DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

The forces of evolution may determine the fate of drug-resistant *M. tuberculosis* variants at multiple stages during infection. During transmission, *M. tuberculosis* populations undergo stark bottlenecks (box 1) as new infections are thought to be established only by a few bacilli (Jacobs 1941). This means that in most cases, the bacterial populations in the lungs of TB patients will demonstrate only very low levels of genetic diversity. Only about 12% of all infections with *M. tuberculosis* will result in active disease within the life time of a patient (Dheda, Barry and Maartens 2016). After the formation of the primary complex, *M. tubercu-*

losis bacilli are disseminated throughout the lung where they form new foci. The bacteria present in these foci are thought to form isolated subpopulations with little migration between the populations, at least in the early stages of the infection. Structured populations are thought to select for growth yield optimisation, as they do not have to compete for resources with other, faster growing variants due to the private nature of the resources (Frank 2010). Indeed, sampling bacteria from different lesions revealed the presence of distinct subpopulations throughout the lungs of TB patients (Lieberman et al. 2016). However, sampling *M. tuberculosis* bacteria from patient sputum during treatment at different time points revealed a dynamic picture of the bacterial population. Multiple different resistance alleles e.g. conferring resistance to fluoroquinolones were present concomitantly—a phenomenon known as heteroresistance. Furthermore, certain drug resistance alleles were increasing over time whilst others were disappearing. Moreover, there was selection for strains harbouring less costly resistance and/or known compensatory mutations (Merker et al. 2013; Eldholm et al. 2014; Bloemberg et al. 2015; Bernard et al. 2016). Selection for *M. tuberculosis* variants with higher growth rates might indicate that resources available in a patient's lung are not private and that the bacterial populations are less structured than we had anticipated. Indeed, studies performed in cynomolgus macaques and TB patients (Lin et al. 2013; Coleman et al. 2014) demonstrate a much more dynamic picture. During the course of *M. tuberculosis* infection, existing foci have been observed to be eliminated by the immune system, whereas new foci seem to appear, without any discernable pattern. The immune system is apparently able to eliminate foci and presumably kill the bacteria present in these lesions. The removal of bacteria from these lesions by the immune system can be viewed as a form of genetic drift. This has implications for the *de novo* evolution of drug resistance. Populations of *de novo* evolved resistant *M. tuberculosis* variants inevitably start out with low numbers. Populations with small effective population sizes are vulnerable to extinction by genetic drift. This means that the action of the immune system might slow the *de novo* evolution of drug resistance, by stochastically removing resistant variants from the population. Host-directed therapies (Zumla et al. 2015) boosting the action of the immune system in clearing TB infections will inevitably also reduce the rate at which drug-resistant variants evolve *de novo*.

CONCLUSION AND OUTLOOK

The intrinsic resistance of *Mycobacterium tuberculosis* against many classes of antibiotics, the ever rising numbers of drug-resistant strains as well as the scarcity of novel antituberculous compounds is threatening the progress in containing the disease. The remarkable capability of *M. tuberculosis* to evolve drug resistance against all efficacious drugs at low or no cost underlines the necessity of a multipronged strategy to reduce the incidence of both drug-susceptible and drug-resistant *M. tuberculosis* variants. Aside from improving the quality of public health systems in resource-limited settings, novel, cost-effective point-of-care diagnostic tools, drugs and an effective vaccine are urgently needed. Furthermore, the influence of the strain genetic background on virtually all aspects of drug resistance evolution highlights that it is not sufficient to focus research on laboratory strains. It is crucial to include *M. tuberculosis* strains which ideally represent the whole phylogenetic space of the species in order to assess the impact of a novel drug, or for that matter, any

planned intervention on the population biology of this extraordinarily successful pathogen.

ACKNOWLEDGEMENTS

We would like to thank the members of the group and Alexandra Mushegian for critically reading the manuscript and providing helpful input.

FUNDING

This work was supported by the European Research Council [grant number 309540-EVODRTB], the Swiss National Science Foundation [grant number 310030.166687] and SystemsX.ch.

Conflict of interest. None declared.

REFERENCES

- Abate G, Hoffner SE. Synergistic antimycobacterial activity between ethambutol and the beta-lactam drug cefepime. *Diagn Microb Infect Dis* 1997;**28**:119–22.
- Abraham EP, Chain E, Fletcher CM et al. Further observations on penicillin. *Lancet* 1941;**238**:177–89.
- Achtman M. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. *Annu Rev Microbiol* 2008;**62**:53–70.
- Adams KN, Takaki K, Connolly LE et al. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 2011;**145**:39–53.
- Alangaden GJ, Kreiswirth BN, Aouad A et al. Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* 1998;**42**:1295–7.
- Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol* 2010;**8**:260–71.
- Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol* 2014;**12**:465–78.
- Andre E, Goeminne L, Cabibbe A et al. Consensus numbering system for the rifampicin resistance-associated *rpoB* gene mutations in pathogenic mycobacteria. *Clin Microbiol Infect* 2017;**23**:167–72.
- Anthony Malinga L, Stoltz A. Efflux pump mediated second-line tuberculosis drug resistance. *Mycobacterial Dis* 2016;**6**, DOI: 10.4172/2161-1068.1000222.
- Banerjee A, Dubnau E, Quemard A et al. *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* (80-) 1994;**263**:227–30.
- Barnett M, Busby SR, Mitchison DA. Tubercle bacilli resistant to isoniazid: virulence and response to treatment with isoniazid in guinea-pigs and mice. *Br J Exp Pathol* 1953;**34**:568–81.
- Beckert P, Hillemann D, Kohl TA et al. *rplC* T460C Identified as a dominant mutation in linezolid-resistant *Mycobacterium tuberculosis* strains. *Antimicrob Agents Ch* 2012;**56**:2743–5.
- Bergval I, Kwok B, Schuitema A et al. Pre-existing isoniazid resistance, but not the genotype of *Mycobacterium tuberculosis* drives rifampicin resistance codon preference in vitro. *PLoS One* 2012;**7**:e29108.
- Bergval IL, Schuitema ARJ, Klatser PR et al. Resistant mutants of *Mycobacterium tuberculosis* selected in vitro do not reflect the in vivo mechanism of isoniazid resistance. *J Antimicrob Chemoth* 2009;**64**:515–23.
- Bernard C, Aubry A, Chauffour A et al. In vivo *Mycobacterium tuberculosis* fluoroquinolone resistance emergence: a complex phenomenon poorly detected by current diagnostic tests. *J Antimicrob Chemoth* 2016;**71**:3465–72.
- Billington OJ, McHugh TD, Gillespie SH. Physiological cost of rifampin resistance induced in vitro in *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* 1999;**43**:1866–9.
- Björkman J, Nagaev I, Berg OG et al. Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. *Science* 2000;**287**:1479–82.
- Bloemberg GV, Keller PM, Stucki D et al. Acquired resistance to bedaquiline and delamanid in therapy for tuberculosis. *N Engl J Med* 2015;**373**:1986–8.
- Blower SM, Chou T. Modeling the emergence of the “hot zones”: tuberculosis and the amplification dynamics of drug resistance. *Nat Med* 2004;**10**:1111–6.
- Bolotin E, Hershberg R. Gene loss dominates as a source of genetic variation within clonal pathogenic bacterial species. *Genome Biol Evol* 2015;**7**:2173–87.
- Borrell S, Gagneux S. Strain diversity, epistasis and the evolution of drug resistance in *Mycobacterium tuberculosis*. *Clin Microbiol Infect* 2011;**17**:815–20.
- Borrell S, Teo Y, Giardina F et al. Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. *Evol Med Public Heal* 2013;**2013**:65–74.
- Bosne-David S. Intrinsic resistance of *Mycobacterium tuberculosis* to clarithromycin is effectively reversed by subinhibitory concentrations of cell wall inhibitors. *J Antimicrob Chemoth* 2000;**46**:391–5.
- Böttger EC, Springer B. Tuberculosis: drug resistance, fitness, and strategies for global control. *Eur J Pediatr* 2008;**167**:141–8.
- Böttger EC, Springer B, Pletschette M et al. Fitness of antibiotic-resistant microorganisms and compensatory mutations. *Nat Med* 1998;**4**:1343–4.
- Brennan PJ, Nikaido H. The envelope of mycobacteria. *Annu Rev Biochem* 1995;**64**:29–63.
- British Thoracic and Tuberculosis Association. Short-course chemotherapy in pulmonary tuberculosis. *Lancet* 1975;**305**:119–24.
- Buriánková K, Doucet-Populaire F, Dorson O et al. Molecular basis of intrinsic macrolide resistance in the *Mycobacterium tuberculosis* complex. *Antimicrob Agents Ch* 2004;**48**:143–50.
- Calver AD, Falmer AA, Murray M et al. Emergence of increased resistance and extensively drug-resistant tuberculosis despite treatment adherence, South Africa. *Emerg Infect Dis* 2010;**16**:264–71.
- Caminero JA. Likelihood of generating MDR-TB and XDR-TB under adequate National Tuberculosis Control Programme implementation. *Int J Tuberc Lung D* 2008;**12**:869–77.
- Campbell EA, Korzhova N, Mustaev A et al. Structural mechanism for rifampicin inhibition of bacterial rna polymerase. *Cell* 2001;**104**:901–12.
- Casali N, Nikolayevskyy V, Balabanova Y et al. Microevolution of extensively drug-resistant tuberculosis in Russia. *Genome Res* 2012;**22**:735–45.
- Casali N, Nikolayevskyy V, Balabanova Y et al. Evolution and transmission of drug-resistant tuberculosis in a Russian population. *Nat Genet* 2014;**46**:279–86.
- Chambers HF, Moreau D, Yajko D et al. Can penicillins and other beta-lactam antibiotics be used to treat tuberculosis? *Antimicrob Agents Ch* 1995;**39**:2620–4.
- Cohen KA, Abeel T, Manson McGuire A et al. Evolution of extensively drug-resistant tuberculosis over four decades: whole genome sequencing and dating analysis of *Mycobacterium tuberculosis* isolates from KwaZulu-Natal. *PLoS Med* 2015;**12**:e1001880.

- Cohen KA, El-Hay T, Wyres KL et al. Paradoxical hypersusceptibility of drug-resistant *Mycobacterium tuberculosis* to β -lactam antibiotics. *EBioMedicine* 2016;9:170–9.
- Cohen T, Murray M. Modeling epidemics of multidrug-resistant *M. tuberculosis* of heterogeneous fitness. *Nat Med* 2004;10:1117–21.
- Cole ST, Brosch R, Parkhill J et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 1998;393:537–44.
- Coleman MT, Chen RY, Lee M et al. PET/CT imaging reveals a therapeutic response to oxazolidinones in macaques and humans with tuberculosis. *Sci Transl Med* 2014;6:265ra167.
- Comas I, Borrell S, Roetzer A et al. Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. *Nat Genet* 2011;44:106–10.
- Coscolla M, Gagneux S. Does *M. tuberculosis* genomic diversity explain disease diversity? *Drug Discov Today Dis Mech* 2010;7:e43–59.
- Coscolla M, Gagneux S. Consequences of genomic diversity in *Mycobacterium tuberculosis*. *Semin Immunol* 2014;26:431–44.
- Crofton J, Mitchison D. Streptomycin resistance in pulmonary tuberculosis. *Br Med J* 1948;2:1009–15.
- da Silva PEA, von Groll A, Martin A et al. Efflux as a mechanism for drug resistance in *Mycobacterium tuberculosis*. *FEMS Immunol Med Mic* 2011;63:1–9.
- da Silva Rabello MC, Matsumoto CK, de Almeida LGP et al. First description of natural and experimental conjugation between mycobacteria mediated by a linear plasmid. *PLoS One* 2012;7:1–8.
- Danilchanka O, Pires D, Anes E et al. The *Mycobacterium tuberculosis* outer membrane channel protein CpnT confers susceptibility to toxic molecules. *Antimicrob Agents Ch* 2015;59:2328–36.
- Danilchanka O, Sun J, Pavlenok M et al. An outer membrane channel protein of *Mycobacterium tuberculosis* with exotoxin activity. *P Natl Acad Sci USA* 2014;111:6750–5.
- Dartois V. The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. *Nat Rev Microbiol* 2014;12:159–67.
- De Lorenzo S, Alffenaar JW, Sotgiu G et al. Efficacy and safety of meropenem-clavulanate added to linezolid-containing regimens in the treatment of MDR/XDR-TB. *Eur Respir J* 2013;41:1386–92.
- de Steenwinkel JEM, ten Kate MT, de Knecht GJ et al. Drug susceptibility of *Mycobacterium tuberculosis* Beijing genotype and association with MDR TB. *Emerg Infect Dis* 2012;18:660–3.
- de Vos M, Müller B, Borrell S et al. Putative compensatory mutations in the *rpoC* gene of rifampin-resistant *Mycobacterium tuberculosis* are associated with ongoing transmission. *Antimicrob Agents Ch* 2013;57:827–32.
- Desjardins CA, Cohen KA, Munsamy V et al. Genomic and functional analyses of *Mycobacterium tuberculosis* strains implicate *ald* in D-cycloserine resistance. *Nat Genet* 2016;48:544–51.
- Dheda K, Barry CE, Maartens G. Tuberculosis. *Lancet* 2016;387:1211–26.
- Dye C. Erasing the World's Slow Stain: Strategies to beat multidrug-resistant tuberculosis. *Science* (80-) 2002;295:2042–6.
- Dye C, Espinal MA. Will tuberculosis become resistant to all antibiotics? *P Roy Soc B-Biol Sci* 2001;268:45–52.
- Eldholm V, Monteserin J, Rieux A et al. Four decades of transmission of a multidrug-resistant *Mycobacterium tuberculosis* outbreak strain. *Nat Commun* 2015;6:7119.
- Eldholm V, Norheim G, von der Lippe B et al. Evolution of extensively drug-resistant *Mycobacterium tuberculosis* from a susceptible ancestor in a single patient. *Genome Biol* 2014;15:490.
- Espinal MA, Dye C. Can DOTS control multidrug-resistant tuberculosis? *Lancet* 2005;365:1206–9.
- Farhat MR, Shapiro BJ, Kieser KJ et al. Genomic analysis identifies targets of convergent positive selection in drug-resistant *Mycobacterium tuberculosis*. *Nat Genet* 2013;45:1183–9.
- Feng-Zeng Z, Murray C, Spinaci S et al. Results of directly observed short-course chemotherapy in 112 842 Chinese patients with smear-positive tuberculosis. *Lancet* 1996;347:358–62.
- Fenner L, Egger M, Bodmer T et al. Effect of mutation and genetic Background on drug Resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* 2012;56:3047–53.
- Ford CB, Lin PL, Chase MR et al. Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. *Nat Genet* 2011;43:482–6.
- Ford CB, Shah RR, Maeda MK et al. *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat Genet* 2013;45:784–90.
- Fox GJ, Barry SE, Britton WJ et al. Contact investigation for tuberculosis: a systematic review and meta-analysis. *Eur Respir J* 2013;41:140–56.
- Frank SA. The trade-off between rate and yield in the design of microbial metabolism. *J Evol Biol* 2010;23:609–13.
- Freihofer P, Akbergenov R, Teo Y et al. Nonmutational compensation of the fitness cost of antibiotic resistance in mycobacteria by overexpression of *tlyA* rRNA methylase. *RNA* 2016;22:1836–43.
- Frieden TR, Fujiwara PI, Washko RM et al. Tuberculosis in New York City—turning the tide. *N Engl J Med* 1995;333:229–33.
- Gagneux S, Burgos M V, DeRiemer K et al. Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis*. *PLoS Pathog* 2006a;2:e61.
- Gagneux S, DeRiemer K, Van T et al. Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. *P Natl Acad Sci USA* 2006b;103:2869–73.
- Gagneux S, Long CD, Small PM et al. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. *Science* 2006c;312:1944–6.
- Gagneux S, Small PM. Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. *Lancet Infect Dis* 2007;7:328–37.
- Gandhi NR, Moll A, Sturm AW et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* 2006;368:1575–80.
- Gillespie SH, Basu S, Dickens AL et al. Effect of subinhibitory concentrations of ciprofloxacin on *Mycobacterium fortuitum* mutation rates. *J Antimicrob Chemoth* 2005;56:344–8.
- Griffiths AJF, Wessler SR, Lewontin RC et al. *An Introduction to Genetic Analysis*, 8th edn. New York: W H Freeman & Co, 2005.
- Gullberg E, Cao S, Berg OG et al. Selection of resistant *Bacteria* at very low antibiotic concentrations. *PLoS Pathog* 2011;7:e1002158.
- Gumbo T. New Susceptibility breakpoints for first-line antituberculosis drugs based on antimicrobial pharmacokinetic/pharmacodynamic science and population pharmacokinetic variability. *Antimicrob Agents Ch* 2010;54:1484–91.
- Gupta AK, Katoch VM, Chauhan DS et al. Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacterium*

- tuberculosis during stress induced by common anti-tuberculous drugs. *Microb Drug Resist* 2010;16:21–8.
- Hall AR, Angst DC, Schiessl KT et al. Costs of antibiotic resistance - separating trait effects and selective effects. *Evol Appl* 2015;8:261–72.
- Hartkoorn RC, Uplekar S, Cole ST. Cross-resistance between clofazimine and bedaquiline through upregulation of MmpL5 in *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* 2014;58:2979–81.
- Hatherell H-A, Colijn C, Stagg HR et al. Interpreting whole genome sequencing for investigating tuberculosis transmission: a systematic review. *BMC Med* 2016;14:21.
- Haver HL, Chua A, Ghode P et al. Mutations in genes for the F420 biosynthetic pathway and a nitroreductase enzyme are the primary resistance determinants in spontaneous in vitro-selected PA-824-resistant mutants of *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* 2015;59:5316–23.
- Hazbón MH, Brimacombe M, Bobadilla del Valle M et al. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* 2006;50:2640–9.
- Helaine S, Holden DW, Mouton JM et al. Elucidating population-wide mycobacterial replication dynamics at the single-cell level. *Microbiology* 2016;162:966–78.
- Hershberg R, Lipatov M, Small PM et al. High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. *PLoS Biol* 2008;6:e311.
- Heym B, Alzari PM, Honore N et al. Missense mutations in the catalase-peroxidase gene, *katG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Mol Microbiol* 1995;15:235–45.
- Heym B, Stavropoulos E, Honoré N et al. Effects of overexpression of the alkyl hydroperoxide reductase *AhpC* on the virulence and isoniazid resistance of *Mycobacterium tuberculosis*. *Infect Immun* 1997;65:1395–401.
- Hillemann D, Rusch-Gerdes S, Richter E. In Vitro-Selected linezolid-resistant *Mycobacterium tuberculosis* mutants. *Antimicrob Agents Ch* 2008;52:800–1.
- Houghton JL, Green KD, Pricer RE et al. Unexpected N-acetylation of capreomycin by mycobacterial *Eis* enzymes. *J Antimicrob Chemother* 2013;68:800–5.
- Hughes D, Brandis G. Rifampicin Resistance: Fitness costs and the significance of compensatory evolution. *Antibiotics* 2013;2:206–16.
- Hugonnet J-E, Blanchard JS. Irreversible inhibition of the *Mycobacterium tuberculosis* beta-lactamase by clavulanate. *Biochemistry* 2007;46:11998–2004.
- Hugonnet J-E, Tremblay LW, Boshoff HI et al. Meropenem-clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. *Science* (80-) 2009;323:1215–8.
- Huitric E, Verhasselt P, Koul A et al. Rates and mechanisms of resistance development in *Mycobacterium tuberculosis* to a novel diarylquinoline ATP synthase inhibitor. *Antimicrob Agents Ch* 2010;54:1022–8.
- Jacobs AL. Infective dose in pulmonary tuberculosis. *Tubercle* 1941;22:266–71.
- Janardhan S, Ram Vivek M, Narahari Sastry G. Modeling the permeability of drug-like molecules through the cell wall of *Mycobacterium tuberculosis*: an analogue based approach. *Mol Biosyst* 2016;12:3377–84.
- Jarlier V, Nikaido H. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. *FEMS Microbiol Lett* 1994;123:11–8.
- Kambli P, Ajbani K, Nikam C et al. Correlating *rrs* and *eis* promoter mutations in clinical isolates of *Mycobacterium tuberculosis* with phenotypic susceptibility levels to the second-line injectables. *Int J Mycobacteriol* 2016;5:1–6.
- Kaplan G, Post FA, Moreira AL et al. *Mycobacterium tuberculosis* growth at the cavity surface: a microenvironment with failed immunity. *Infect Immun* 2003;71:7099–108.
- Kaufmann SHE, Lange C, Rao M et al. Progress in tuberculosis vaccine development and host-directed therapies—a state of the art review. *Lancet Respir Med* 2014;2:301–20.
- Knight GM, Colijn C, Shrestha S et al. The distribution of fitness costs of resistance-conferring mutations is a key determinant for the future burden of drug-resistant tuberculosis: A Model-Based Analysis. *Clin Infect Dis* 2015;61 (suppl 3):S147–54.
- Koch A, Mizrahi V, Warner DF. The impact of drug resistance on *Mycobacterium tuberculosis* physiology: what can we learn from rifampicin? *Emerg Microbes Infect* 2014;3:e17.
- Konno K, Feldmann FM, McDermott W. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. *Am Rev Respir Dis* 1967;95:461–9.
- Lamichhane G, Tyagi S, Bishai WR. Designer arrays for defined mutant analysis to detect genes essential for survival of *Mycobacterium tuberculosis* in mouse lungs. *Infect Immun* 2005;73:2533–40.
- Lanzas F, Karakousis PC, Sacchetti JC et al. Multidrug-resistant tuberculosis in panama is driven by clonal expansion of a multidrug-resistant *Mycobacterium tuberculosis* strain related to the KZN extensively drug-resistant *M. tuberculosis* strain from South Africa. *J Clin Microbiol* 2013;51:3277–85.
- Lehmann J. Para-aminosalicylic acid in the treatment of tuberculosis. *Lancet* 1946;247:15–6.
- Lenaerts A, Barry CE, Dartois V. Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunol Rev* 2015;264:288–307.
- Li G, Zhang J, Guo Q et al. Efflux pump gene expression in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates. *PLoS One* 2015;10:e0119013.
- Lieberman TD, Wilson D, Misra R et al. Genomic diversity in autopsy samples reveals within-host dissemination of HIV-associated *Mycobacterium tuberculosis*. *Nat Med* 2016;22:1470–4.
- Lin PL, Ford CB, Coleman MT et al. Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. *Nat Med* 2013;20:75–9.
- Lin PL, Rodgers M, Smith L et al. Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. *Infect Immun* 2009;77:4631–42.
- Liu J, Nikaido H. A mutant of *Mycobacterium smegmatis* defective in the biosynthesis of mycolic acids accumulates meromycolates. *P Natl Acad Sci USA* 1999;96:4011–6.
- Louw GE, Warren RM, Gey van Pittius NC et al. A balancing act: efflux/influx in mycobacterial drug resistance. *Antimicrob Agents Ch* 2009;53:3181–9.
- Louw GE, Warren RM, Gey van Pittius NC et al. Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant *Mycobacterium tuberculosis* through efflux. *Am J Resp Crit Care* 2011;184:269–76.
- Luciani F, Sisson, SA, Jiang H et al. The epidemiological fitness cost of drug resistance in *Mycobacterium tuberculosis*. *P Natl Acad Sci USA* 2009;106:14711–5.
- Luo T, Yang C, Peng Y et al. Whole-genome sequencing to detect recent transmission of *Mycobacterium tuberculosis* in settings

- with a high burden of tuberculosis. *Tuberculosis* 2014;**94**:434–40.
- Luria SE, Delbrück M. Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 1943;**28**:491–511.
- Machado D, Couto I, Perdigão J et al. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. *PLoS One* 2012;**7**:e34538.
- McGrath M, Gey van Pittius NC, van Helden PD et al. Mutation rate and the emergence of drug resistance in *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2014;**69**:292–302.
- Madsen CT, Jakobsen L, Buriánková K et al. Methyltransferase Erm(37) slips on rRNA to confer atypical resistance in *Mycobacterium tuberculosis*. *J Biol Chem* 2005;**280**:38942–7.
- Mahmoudi A, Iseman MD. Pitfalls in the care of patients with tuberculosis. Common errors and their association with the acquisition of drug resistance. *JAMA* 1993;**270**:65–8.
- Mailaender C, Reiling N, Engelhardt H et al. The MspA porin promotes growth and increases antibiotic susceptibility of both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. *Microbiology* 2004;**150**:853–64.
- Malik S, Willby M, Sikes D et al. New insights into fluoroquinolone resistance in *Mycobacterium tuberculosis*: functional genetic analysis of *gyrA* and *gyrB* Mutations. *PLoS One* 2012;**7**:e39754.
- Manca C, Paul S, Barry CE et al. *Mycobacterium tuberculosis* catalase and peroxidase activities and resistance to oxidative killing in human monocytes in vitro. *Infect Immun* 1999;**67**:74–9.
- Manjunatha UH, Boshoff H, Dowd CS et al. Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in *Mycobacterium tuberculosis*. *P Natl Acad Sci USA* 2006;**103**:431–6.
- Mariam DH, Mengistu Y, Hoffner SE et al. Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* 2004;**48**:1289–94.
- Maruri F, Sterling TR, Kaiga AW et al. A systematic review of gyrase mutations associated with fluoroquinolone-resistant *Mycobacterium tuberculosis* and a proposed gyrase numbering system. *J Antimicrob Chemother* 2012;**67**:819–31.
- Maus CE, Plikaytis BB, Shinnick TM. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* 2005a;**49**:3192–7.
- Maus CE, Plikaytis BB, Shinnick TM. Mutation of *tlyA* confers capreomycin resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* 2005b;**49**:571–7.
- Medical Research Council. Treatment of pulmonary tuberculosis with streptomycin and para-amino-salicylic acid: a Medical Research Council Investigation. *BMJ* 1950;**2**:1073–85.
- Meftahi N, Namouchi A, Mhenni B et al. Evidence for the critical role of a secondary site *rpoB* mutation in the compensatory evolution and successful transmission of an MDR tuberculosis outbreak strain. *J Antimicrob Chemother* 2016;**71**:324–32.
- Meier A, Kirschner P, Bange FC et al. Genetic alterations in streptomycin-resistant *Mycobacterium tuberculosis*: mapping of mutations conferring resistance. *Antimicrob Agents Ch* 1994;**38**:228–33.
- Melnyk AH, Wong A, Kassen R. The fitness costs of antibiotic resistance mutations. *Evol Appl* 2015;**8**:273–83.
- Merker M, Blin C, Mona S et al. Evolutionary history and global spread of the *Mycobacterium tuberculosis* Beijing lineage. *Nat Genet* 2015;**47**:242–9.
- Merker M, Kohl TA, Roetzer A et al. Whole genome sequencing reveals complex evolution patterns of multidrug-resistant *Mycobacterium tuberculosis* Beijing strains in patients. *PLoS One* 2013;**8**:e82551.
- Mestre O, Luo T, Dos Vultos T et al. Phylogeny of *Mycobacterium tuberculosis* Beijing strains constructed from polymorphisms in genes involved in DNA replication, recombination and repair. *PLoS One* 2011;**6**:e16020.
- Middlebrook G, Cohn ML. Some observations on the pathogenicity of isoniazid-resistant variants of tubercle bacilli. *Science* 1953;**118**:297–9.
- Milano A, Pasca MR, Provvedi R et al. Azole resistance in *Mycobacterium tuberculosis* is mediated by the MmpS5–MmpL5 efflux system. *Tuberculosis* 2009;**89**:84–90.
- Minato Y, Thiede JM, Kordus SL et al. *Mycobacterium tuberculosis* folate metabolism and the mechanistic basis for para-aminosalicylic acid susceptibility and resistance. *Antimicrob Agents Ch* 2015;**59**:5097–106.
- Mokrousov I, Vyazovaya A, Otten T et al. *Mycobacterium tuberculosis* population in Northwestern Russia: an update from Russian-EU/Latvian border region. *PLoS One* 2012;**7**:e41318.
- Monshupanee T, Johansen SK, Dahlberg AE et al. Capreomycin susceptibility is increased by TlyA-directed 2'-O-methylation on both ribosomal subunits. *Mol Microbiol* 2012;**85**:1194–203.
- Moreno-Gamez S, Hill AL, Rosenbloom DIS et al. Imperfect drug penetration leads to spatial monotherapy and rapid evolution of multidrug resistance. *P Natl Acad Sci USA* 2015;**112**:E2874–83.
- Morlock GP, Metchock B, Sikes D et al. *ethA*, *inhA*, and *katG* loci of ethionamide-resistant clinical *Mycobacterium tuberculosis* isolates. *Antimicrob Agents Ch* 2003;**47**:3799–805.
- Müller B, Chihota VN, Pillay M et al. Programmatically selected multidrug-resistant strains drive the emergence of extensively drug-resistant tuberculosis in South Africa. *PLoS One* 2013;**8**:e70919.
- Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. *Clin Microbiol Rev* 1995;**8**:496–514.
- Naidoo CC, Pillay M. Increased in vitro fitness of multi- and extensively drug-resistant F15/LAM4/KZN strains of *Mycobacterium tuberculosis*. *Clin Microbiol Infect* 2014;**20**:O361–9.
- Nair J, Rouse DA, Bai G-H et al. The *rpsL* gene and streptomycin resistance in single and multiple drug-resistant strains of *Mycobacterium tuberculosis*. *Mol Microbiol* 1993;**10**:521–7.
- Nash KA. Multidrug resistance in mycobacteria. *Curr Clin Microbiol Rep* 2016;**3**:53–61.
- Nguyen L. Antibiotic resistance mechanisms in *M. tuberculosis*: an update. *Arch Toxicol* 2016;**90**:1585–604.
- Nguyen L, Pieters J. Mycobacterial subversion of chemotherapeutic reagents and host defense tactics: challenges in tuberculosis drug development. *Annu Rev Pharmacol* 2009;**49**:427–53.
- Niemann S, Diel R, Khechinashvili G et al. *Mycobacterium tuberculosis* Beijing lineage favors the spread of multidrug-resistant tuberculosis in the Republic of Georgia. *J Clin Microbiol* 2010;**48**:3544–50.
- Nikaido H, Thanassi DG. Penetration of lipophilic agents with multiple protonation sites into bacterial cells: tetracyclines and fluoroquinolones as examples. *Antimicrob Agents Ch* 1993;**37**:1393–9.
- Pardini M, Niemann S, Varaine F et al. Characteristics of drug-resistant tuberculosis in Abkhazia (Georgia), a high-prevalence area in Eastern Europe. *Tuberculosis* 2009;**89**:317–24.

- Park J, Lee J, Lee Y et al. Serum levels of antituberculosis drugs and their effect on tuberculosis treatment outcome. *Antimicrob Agents Ch* 2016;**60**:92–8.
- Paulsen IT, Chen J, Nelson KE et al. Comparative genomics of microbial drug efflux systems. *J Mol Microb Biotech* 2001;**3**:145–50.
- Payen MC, De Wit S, Martin C et al. Clinical use of the meropenem-clavulanate combination for extensively drug-resistant tuberculosis. *Int J Tuberc Lung D* 2012;**16**:558–60.
- Post FA, Willcox PA, Mathema B et al. Genetic polymorphism in *Mycobacterium tuberculosis* isolates from patients with chronic multidrug-resistant tuberculosis. *J Infect Dis* 2004;**190**:99–106.
- Prideaux B, Via LE, Zimmerman MD et al. The association between sterilizing activity and drug distribution into tuberculosis lesions. *Nat Med* 2015;**21**:1223–7.
- Pule CM, Sampson SL, Warren RM et al. Efflux pump inhibitors: targeting mycobacterial efflux systems to enhance TB therapy. *J Antimicrob Chemoth* 2016;**71**:17–26.
- Pym AS, Saint-Joanis B, Cole ST. Effect of *katG* mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans. *Infect Immun* 2002;**70**:4955–60.
- Qi Q, Preston GM, MacLean RC. Linking system-wide impacts of RNA polymerase mutations to the fitness cost of rifampin resistance in *Pseudomonas aeruginosa*. *MBio* 2014;**5**:e01562–14.
- Quinting B, Reyrat JM, Monnaie D et al. Contribution of β -lactamase production to the resistance of mycobacteria to β -lactam antibiotics. *FEBS Lett* 1997;**406**:275–8.
- Rastogi N, Goh KS. Action of 1-isonicotinyl-2-palmitoyl hydrazine against the *Mycobacterium avium* complex and enhancement of its activity by m-fluorophenylalanine. *Antimicrob Agents Ch* 1990;**34**:2061–4.
- Reiling N, Homolka S, Walter K et al. Clade-specific virulence patterns of *Mycobacterium tuberculosis* complex strains in human primary macrophages and aerogenically infected mice. *MBio* 2013;**4**:e00250–13.
- Rock JM, Lang UF, Chase MR et al. DNA replication fidelity in *Mycobacterium tuberculosis* is mediated by an ancestral prokaryotic proofreader. *Nat Genet* 2015;**47**:677–81.
- Rodriguez-Rivera FP, Zhou X, Theriot JA et al. Visualization of mycobacterial membrane dynamics in live cells. *J Am Chem Soc* 2017;**139**:3488–95.
- Salvatore PP, Becerra MC, Abel zur Wiesch P et al. Fitness costs of drug resistance mutations in multidrug-resistant *Mycobacterium tuberculosis*: a household-based case-control study. *J Infect Dis* 2016;**213**:149–55.
- Sander P, Springer B, Prammananan T et al. Fitness cost of chromosomal drug resistance-conferring mutations. *Antimicrob Agents Ch* 2002;**46**:1204–11.
- Sandgren A, Strong M, Muthukrishnan P et al. Tuberculosis drug resistance mutation database. *PLoS Med* 2009;**6**:e1000002.
- Sarathy JP, Dartois V, Lee EJD. The role of transport mechanisms in *Mycobacterium tuberculosis* drug resistance and tolerance. *Pharmaceuticals* 2012;**5**:1210–35.
- Schatz A, Bugle E, Waksman SA. Streptomycin, a substance exhibiting antibiotic activity against Gram-positive and Gram-negative bacteria. *Exp Biol Med* 1944;**55**:66–9.
- Schmalstieg AM, Srivastava S, Belkaya S et al. The antibiotic resistance arrow of time: efflux pump induction is a general first step in the evolution of mycobacterial drug resistance. *Antimicrob Agents Ch* 2012;**56**:4806–15.
- Scorpio A, Zhang Y. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat Med* 1996;**2**:662–7.
- Sensi P. History of the development of rifampin. *Rev Infect Dis* 1983;**5** (suppl 3):S402–6.
- Sherman DR, Mdluli K, Hickey MJ et al. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science* (80-) 1996;**272**:1641–3.
- Shimao T. Drug resistance in tuberculosis control. *Tubercle* 1987;**68**:5–18.
- Smith T, Wolff KA, Nguyen L. Molecular biology of drug resistance in *Mycobacterium tuberculosis*. *Curr Top Microbiol* 2013;**374**:53–80.
- Song T, Park Y, Shamputa IC et al. Fitness costs of rifampicin resistance in *Mycobacterium tuberculosis* are amplified under conditions of nutrient starvation and compensated by mutation in the β' subunit of RNA polymerase. *Mol Microbiol* 2014;**91**:1106–19.
- Spies FS, von Groll A, Ribeiro AW et al. Biological cost in *Mycobacterium tuberculosis* with mutations in the *rpsL*, *rrs*, *rpoB*, and *katG* genes. *Tuberculosis* 2013;**93**:150–4.
- Sreevatsan S, Stockbauer KE, Pan X et al. Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of *embB* mutations. *Antimicrob Agents Ch* 1997;**41**:1677–81.
- Swaminathan S, Ramachandran G. Role of pharmacogenomics in the treatment of tuberculosis: a review. *Pharmacogenomics Pers Med* 2012;**5**:89.
- Takiff HE, Salazar L, Guerrero C et al. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. *Antimicrob Agents Ch* 1994;**38**:773–80.
- Telenti A, Imboden P, Marchesi F et al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 1993;**341**:647–51.
- van Doorn HR, de Haas PEW, Kremer K et al. Public health impact of isoniazid-resistant *Mycobacterium tuberculosis* strains with a mutation at amino-acid position 315 of *katG*: a decade of experience in The Netherlands. *Clin Microbiol Infect* 2006;**12**:769–75.
- van Soolingen D, de Haas PE, van Doorn HR et al. Mutations at amino acid position 315 of the *katG* gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of *Mycobacterium tuberculosis* in the Netherlands. *J Infect Dis* 2000;**182**:1788–90.
- Verrall AJ, G. Netea M, Alisjahbana B et al. Early clearance of *Mycobacterium tuberculosis*: a new frontier in prevention. *Immunology* 2014;**141**:506–13.
- Vilchèze C, Jacobs WR. Resistance to isoniazid and ethionamide in *Mycobacterium tuberculosis*: genes, mutations, and causalities. *Microbiol Spectr* 2014;**2**:MGM2-0014-2013.
- Walker TM, Ip CL, Harrell RH et al. Whole-genome sequencing to delineate *Mycobacterium tuberculosis* outbreaks: a retrospective observational study. *Lancet Infect Dis* 2013;**13**:137–46.
- Wang F, Cassidy C, Sacchetti JC. Crystal structure and activity studies of the *Mycobacterium tuberculosis* -lactamase reveal its critical role in resistance to -lactam antibiotics. *Antimicrob Agents Ch* 2006;**50**:2762–71.
- Warrell MJ. Intradermal rabies vaccination: the evolution and future of pre- and post-exposure prophylaxis. *Curr Top Microbiol* 2012;**351**:139–57.
- Warrier T, Kapilashrami K, Argyrou A et al. N-methylation of a bactericidal compound as a resistance mechanism in *Mycobacterium tuberculosis*. *P Natl Acad Sci USA* 2016;**113**:E4523–30.
- Warrier T, Martinez-Hoyos M, Marin-Amieva M et al. Identification of novel anti-mycobacterial compounds by screening a

- pharmaceutical small-molecule library against nonreplicating *Mycobacterium tuberculosis*. *ACS Infect Dis* 2015;1:580–5.
- Welin A, Raffetseder J, Eklund D et al. Importance of phagosomal functionality for growth restriction of *Mycobacterium tuberculosis* in primary human macrophages. *J Innate Immun* 2011;3:508–18.
- Werngren J, Hoffner SE. Drug-susceptible *Mycobacterium tuberculosis* Beijing genotype does not develop mutation-conferred resistance to rifampin at an elevated rate. *J Clin Microbiol* 2003;41:1520–4.
- World Health Organization. *Guidelines for TB Treatment in Adults and Children in National TB Programmes*. Geneva, 1991.
- World Health Organization. *TB: WHO Report on the Tuberculosis Epidemic 1997*. Geneva, 1997.
- World Health Organization. *Global Tuberculosis Report 2016*. Geneva, 2016.
- Xu C, Kreiswirth BN, Sreevatsan S et al. Fluoroquinolone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*. *J Infect Dis* 1996;174:1127–30.
- Yang C, Luo T, Shen X et al. Transmission of multidrug-resistant *Mycobacterium tuberculosis* in Shanghai, China: a retrospective observational study using whole-genome sequencing and epidemiological investigation. *Lancet Infect Dis* 2016;3099:1–10.
- Yew WW, Wong CF, Lee J et al. Do β -lactam- β -lactamase inhibitor combinations have a place in the treatment of multidrug-resistant pulmonary tuberculosis? *Tuber Lung Dis* 1995;76:90–2.
- Zaunbrecher MA, Sikes RD, Metchock B et al. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*. *P Natl Acad Sci USA* 2009;106:20004–9.
- Zhang H, Li D, Zhao L et al. Genome sequencing of 161 *Mycobacterium tuberculosis* isolates from China identifies genes and intergenic regions associated with drug resistance. *Nat Genet* 2013;45:1255–60.
- Zhang Y, Yew W-W. Mechanisms of drug resistance in *Mycobacterium tuberculosis*: update 2015. *Int J Tuberc Lung D* 2015;19:1276–89.
- Zhao F, Wang XDE, Erber LN et al. Binding pocket alterations in dihydrofolate synthase confer resistance to para-aminosalicylic acid in clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* 2014;58:1479–87.
- Zumla A, Chakaya J, Hoelscher M et al. Towards host-directed therapies for tuberculosis. *Nat Rev Drug Discov* 2015;14:511–2.