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Types and functions of heterogeneity in mycobacteria

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Abstract

The remarkable ability of *Mycobacterium tuberculosis* to survive attacks from the host immune response and drug treatment is due to the resilience of a few bacilli rather than a result of survival of the entire population. Maintenance of mycobacterial subpopulations with distinct phenotypic characteristics is key for survival in the face of dynamic and variable stressors encountered during infection. Mycobacterial populations develop a wide range of phenotypes through an innate asymmetric growth pattern and adaptation to fluctuating microenvironments during infection that point to heterogeneity being a vital survival strategy. In this Review, we describe different types of mycobacterial heterogeneity and discuss how heterogeneity is generated and regulated in response to environmental cues. We discuss how this heterogeneity may have a key role in recording memory of their environment at both the single-cell and population level to give mycobacterial populations plasticity to withstand complex stressors.

Graphical Abstract

In this Review, Aldridge and colleagues describe different types of mycobacterial heterogeneity and discuss how cell-to-cell and environmental heterogeneity is generated and regulated in response to environmental cues.

Introduction

Tuberculosis (TB) is caused by infection with *Mycobacterium tuberculosis* and remains a major cause of death. Every year, there are 10 million new cases of TB and 1.5 million deaths globally [1]. TB requires a lengthy antibiotic treatment of a minimum of

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Competing interests

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6 months[1]. The primary reason for this long treatment duration is that *M. tuberculosis* generates phenotypically diverse subpopulations during pathogenesis, and some of these subpopulations are drug-tolerant. This variation in drug response is due to two predominant sources of heterogeneity that develop during the disease. One is the ability of the pathogen to adapt to different microenvironments within granulomas **[G]**. *M. tuberculosis* bacteria adapt their metabolic and physiological states to these different lesions, resulting in altered growth properties and antimicrobial susceptibilities[2]. Therefore, heterogeneity among lesions is a major cause of variation wherein distinct *M. tuberculosis* subpopulations are compartmentalized into different microenvironments. The other primary source of this variation in drug susceptibility is inherent bacterial heterogeneity that arises among closely related bacteria in the same environment, in part, through an asymmetric growth and division pattern[3-9]. By overlaying the sources of phenotypic variation, mycobacteria amplify bacterial heterogeneity within just a few generations. Recently, a strong link between this bacterial heterogeneity and drug tolerance in stressful host microenvironments has been demonstrated by several studies on clinical strains[10-14].

Technological and analytical advancements have provided researchers with the tools to uncover the numerous facets of heterogeneity in TB in the past few decades. Fluorescence-activated cell sorting coupled with innovative use of live and dead bacterial reporters or a replication reporter and RNA-sequencing have revealed the immense variation of macrophage activation **[G]** and its effect on *M. tuberculosis* growth[15-17]. Use of microfluidics and the development of staining and rapid imaging technologies have enabled high-definition time-lapse studies on mycobacteria at the single-cell level under precisely controlled environments[18-20]. Recent technological improvements have also expanded our understanding of the host response to *M. tuberculosis* infection. Heterogeneity in TB lesion structure, metabolic activity and drug penetrance is being interrogated through laser-capture microdissection, immunofluorescence and mass spectrometry[21-23]. In the clinic, positron emission tomography and computed tomography are now often used to evaluate patients' disease progression and response to anti-TB treatment[24]. Images of mycobacteria, especially *M. tuberculosis*, are more challenging to automatically segment and track compared to other model organisms due to their irregular shape and tendency to form aggregates[25,26]. The application of machine learning has led to substantial improvements in image analysis software for bacteria in recent years; these advances may enable high-throughput analysis of microscopy experiments for mycobacteria[23,25-27]. These technologies have the potential to reveal the immense diversity in *M. tuberculosis* phenotypes and how this variation affects treatment and disease outcomes.

In this Review, we describe how heterogeneous *M. tuberculosis* subpopulations form from cell-to-cell and environmental variation (that is; the microenvironments that *M. tuberculosis* faces during infection, such as acidic phagosomes or hypoxic necrotic caseum **[G]**). Although heterogeneity refers to the variation between single cells, each microenvironment affects heterogeneity differently by causing *M. tuberculosis* populations to adapt within a compartment in the host tissue. Therefore, in response to changes in the environment, both the average (central part of the population distribution) and distribution (variation and density) shift. Despite the crucial role of the microenvironment on *M. tuberculosis*

heterogeneity in virulence and treatment outcome, studies on mycobacterial phenotypes have concentrated on either population-level changes in different growth environments[28-36] or single-cell analyses in nutrient-rich conditions[3,4,42,5,7,8,37-41]. Understanding how variation is controlled at both the population and single-cell levels in the host niche environments is critical to rationally designing improved TB interventions that mitigate relapse. This Review frames the most recent research around the idea that these layers of heterogeneity function as a survival mechanism against the host immune response and drug treatment.

Bacterial heterogeneity

Cell-to-cell variation in a clonal population, which occurs even within the same microenvironment, has been observed in various cell types, including mammalian and bacterial cells. The etiology of this heterogeneity has been studied extensively in model organisms and attributed to stochastic gene expression, mistranslations and random partitioning of cell components[43-46]. As a bet-hedging strategy, model bacterial organisms, such as *Escherichia coli* and *Bacillus subtilis*, rapidly expand the cellular population to survive. By contrast, *M. tuberculosis* is a slow-growing organism so that a population-level strategy for survival may be to rapidly expand variation in cellular state rather than to achieve a large population size. Below, we describe mechanisms whereby *M. tuberculosis* controls multiple aspects of cell physiology to generate and compound bacterial heterogeneity (Fig. 1).

Asymmetric growth and division.

Mycobacterial growth and division patterns are unlike the model species *E. coli* and *B. subtilis*, and mycobacteria elongate from the cell poles, which provides a mechanism to grow and divide asymmetrically. Asymmetric growth and division generate uneven partitions of cell components, and different growth behaviors and drug susceptibilities between sister cells[3-5].

Through cell-wall labeling experiments with time-lapse and fixed-cell imaging, the old pole was observed to grow more than the new pole in *Mycobacterium smegmatis*[3,7,8,38,42] (FIG. 1a). Additionally, the abundance of cell wall elongation machinery at the old pole relative to the new pole suggests that regulation of protein localization may have an essential role in growth asymmetry[37-40]. For example, key proteins in cell wall synthesis, such as MurG, GlfT2 and Pks13, are at a higher concentration at the old pole relative to the new pole[38]. This may confer asymmetry of the mycobacterial cell wall under nutrient-rich conditions[38]. However, more studies are needed to understand the relationship between the concentrations of cell wall elongation machinery at each pole and polar growth asymmetry. Recent studies suggest that the new pole of mycobacteria may grow less over the course of one cell cycle period because there is a lag before the new pole is licensed to elongate[8,20]. Using a creative genetic screen, a group identified a key mediator of asymmetric growth, LamA, which is a member of the mycobacterial divisome **[G]** complex that actively inhibits growth at the new pole[8] (FIG. 1b). Interestingly, *lamA*-deleted *M. tuberculosis* mutants were killed more rapidly compared to the wild type by rifampicin and vancomycin, which suggests that

asymmetric growth may contribute to mycobacterial survival against antibiotic treatment[8]. Additionally, it was demonstrated that in nutrient-rich growth conditions, the new pole (but not the old pole) has a lag phase before growth, which accounts for an apparent slower elongation rate throughout the division cycle[20]. The authors suggested that the ratio between the lag time and inter-division time may affect the heterogeneity in cell wall age distribution[20]. However, some studies performed in nutrient-rich conditions suggest that *M. tuberculosis* growth is less asymmetric compared to that of *M. smegmatis*[41]. By using fluorescent D-alanine analog (FDAA) to visualize the elongation of the new cell wall, the study found that the FDAA is incorporated at both poles in larger cells[41]. Further studies are necessary to determine the asymmetry of cell growth in *M. tuberculosis* by time-lapse imaging and in the context of host-relevant growth environments.

Mycobacteria do not divide perfectly mid-cell. This asymmetric division creates sister cells that are born at varying sizes and with different growth properties[3-5,7,8,42]. In *M. smegmatis*, the septal site is localized at a 45:55 position from the new:old pole such that the larger daughter inherits the old growth pole from the mother cell (accelerator cell **[G]**) and the smaller sister (alternator cell) inherits the slower growing new pole from the mother cell. Cell size is correlated with linear growth rate, so cells born larger (such as the accelerator cells **[G]**) elongate more per hour and before division than cells born small[5,7,37,42,47,48]. Asymmetry of the septal site is mirrored by an asymmetry in sub-cellular organization, including the origin of replication (ori) and the chromosomal terminus (ter). The chromosome is in a mid-cell position closer to the new pole and organized with the ori near the old pole in young cells and an ori-ter-ter-ori orientation in cells at later cell cycle stages (FIG. 1c). The asymmetric positioning of the chromosome is proportional to cell size, which is consistent regardless of different cell age or growth pole[7]. This proportional 'ruler' of cellular organization may be established for a daughter cell by the grandmother cell. For example, it was demonstrated with time-lapse atomic-force microscopy that waveform troughs on the surface of *M. smegmatis* cells are at the future cell division sites[6] (FIG. 1c). Additionally, the results suggest that the coordination of wave troughs and chromosome partitioning restricts cell division at the mid-cell position, causing asymmetric cell division[6].

Genetic and epigenetic bacterial heterogeneity.

Whereas asymmetric growth and division generate heterogeneity on a rapid time scale (approximately the duration of one cell cycle), phenotypic heterogeneity also involves heritable (inheritable genetic mutations) and non-heritable (epigenetic changes which occur temporally within a generation) mechanisms (Table 1).

One of the heritable phenotypes in mycobacteria is the switch of colony morphology between smooth (S) and rough (R) forms. Colony morphology is highly correlated with disease severity in some mycobacterial species including *M. tuberculosis*, *Mycobacterium avium* and *Mycobacterium abscessus*, with R morphotypes being associated with more severe and persistent infections relative to the S morphotypes[49-52]. Colony morphological changes can be mediated by genetic variations. For example, small insertions and deletions and single nucleotide polymorphisms (SNPs) within the gene cluster *mps1-mps2-gap* or *mmpl4b*, which are involved in the synthesis and transport of glycopeptidolipids **[G]**

(GPLs), converts the S form into the R forms in *M. abscessus*[52]. Clinically relevant SNPs in mycobacteria are not only related to changes in morphology and virulence; several mutations in clinical isolates have an impact on drug tolerance and thus treatment outcomes[12,13,53,54]. Through whole-genome sequencing, SNPs in *prpR*, which encodes a transcriptional regulator involved in propionate metabolism, were detected among clinical strains[12]. These genetic differences conferred multi-drug tolerance to isoniazid, rifampicin and ofloxacin under propionate-rich conditions within the macrophage[12]. This drug tolerance could not be detected in standard *in vitro* assays[12], highlighting the importance of mimicking host microenvironment conditions to unveil drug-tolerant mutations. It was also found that clinical strains with genetic mutations in *Rv0565c*, which functions as an activator of ethionamide and prothionamide, were substantially more tolerant to ethionamide and prothionamide treatment[13]. High-frequency mutations in carbon metabolic pathways, such as frameshift mutations in *glpK*, which encodes glycerol kinase, led to slower growth and reduced sensitivity to drug regimens containing pyrazinamide in clinical strains[53,54].

Single amino-acid variants, which can be caused by nonsynonymous single nucleotide variants and insertions and deletions, may also contribute to genetic heterogeneity among strains[55]. For example, an analysis of clinical isolates with integrated whole-genome sequencing and mass spectrometry-based proteomics identified that proteome characteristics vary from strain-to-strain[55]. It was shown that two clinical Latin American-Mediterranean isolates contained 59 peptides with single amino-acid variants, along with 29 distinct peptides that induce phenotypic variation in clinical *M. tuberculosis* strains[55].

Mycobacterial variation, and the resulting drug tolerance, are also generated at the transcriptional, translational and post-translational levels. Differences in gene expression within a population can result in semi-heritable drug tolerance in mycobacteria[56,57]. For example, rifampicin treatment inhibited *rpoB* promoter I expression but induced expression from *rpoB* promoter II, leading to a maximal production of RpoB in a subpopulation of *M. smegmatis*[57]. This differential response within the population to RNA polymerase-targeting drugs, even among isogenic sister cells **[G]**, can permit tolerance to first-line antimicrobials. Using time-lapse imaging, it was found that individual cells expressed different levels of catalase-peroxidase (KatG), which activates isoniazid. The expression level of KatG was negatively correlated with cell survival, which suggests that drug tolerance is influenced by epigenetic heterogeneity at the single-cell level[56].

Reversible phenotypic errors can generate drug tolerance in subpopulations during translation, such as the substitution of amino acids[58]. Substitution of glutamate for glutamine and aspartate for asparagine of the β-subunit of RNA polymerase occurs in mycobacteria under nutrient-rich conditions, which induces phenotypic resistance to rifampicin[58]. In addition, the rate of these errors increases under host-relevant stress conditions such as in stationary-phase, which suggests that this is an intentional strategy for adaptation to stressors[58]. KatG can be modified by the substitution of serine to threonine at codon 315 (S315T), which is most frequently found in isoniazid-resistant mutants[59]. This alteration enables the mutant to retain KatG activity but decreased capacity to activate isoniazid, possibly due to subtle alterations in the binding of isoniazid to KatG [59-62].

Post-translational modifications involved in cell growth, drug activation and signal transduction systems can also regulate phenotypic resistance to drugs in mycobacteria. The mycobacterial histone-like protein, HupB, is post-translationally modified by lysine acetylation and methylation, altering mycobacterial chromatin structure[63,64]. This change in chromatin structure can cause phenotypic resistance to isoniazid due to modified genome organization [64,65]. It was also suggested that chemical modifications after translation may induce reversible phenotypic resistance to antibiotics[65]. For example, two-component regulatory signal transduction system MtrAB, which enables mycobacteria to respond to stressors caused by toxic radicals through chemical modifications, can control tolerance to antimicrobials in mycobacteria[66]. Phosphotransfer to the MtrA response regulator at Y102 causes changes in cell division and cell wall synthesis, and increases tolerance to isoniazid and vancomycin[66]. Phosphorylation of another enzyme involved in *M. tuberculosis* growth, peptidoglycan synthase (PonA1) increases the cell length as well as the minimum inhibitory concentration **[G]** (MIC) of rifampicin[39].

Environmental heterogeneity

M. tuberculosis readily adapt to their local environment through metabolic flexibility, entering growth arrest, or changing physical attributes including the thickness of the cell wall[67]. During pulmonary infection, *M. tuberculosis* resides within granulomas, which consist primarily of various immune cells and caseum. The composition of this granulomatous tissue can vary substantially among patients, within a single patient, and within a granuloma[68-70] (FIG. 2). Moreover, these compartments change over time so that the composition and abundance of nutrients and stressors are dynamic. Because *M. tuberculosis* adapts into distinct states (and population structures) in different granulomas, compartmentalization of the *M. tuberculosis* population into physically separated granulomas imposes an additional layer of variation[21,28,71-78].

Because adaptation to different types of lesions induces major phenotypic changes in *M. tuberculosis*, studies of *M. tuberculosis* pathogenesis and drug response that include many *in vitro* and animal models can better capture the range of key states[79-83]. For example, integrating studies of two mouse models (for example, C3HeB/FeJ mice may have caseating lesions and BALB/c mouse lesions are primarily cellular) broadens the types of *M. tuberculosis* populations that are being evaluated[68,81]. Using panels of diverse mice for TB studies (through collaborative crosses **[G]**) expands our ability to interrogate a range of lesion types[84]. In this Review, we do not thoroughly discuss lesion types in different animal models, as this is well described in other reviews[68,85]. Instead, we focus on aspects of the microenvironment in TB lesions, such as nutrient shifts, acidic pH, hypoxia and oxidative stress, and describe how these conditions alter *M. tuberculosis* subpopulations and their behaviors[21,68,76,86]. We highlight these well-characterized host environments and their contributions in establishing and maintaining mycobacterial heterogeneity.

Nutrient shift.

M. tuberculosis can derive energy from many different carbon sources but, during the course of infection, the bacteria mainly encounter lipids. The type of lipid environment *M.*

tuberculosis experiences can vary substantially, ranging from cholesterol, cholesterol esters, triacylglycerols (TAGs) and lactosylceramide[87]. Remarkably, the pathogen can readily use or co-metabolize these various lipids as carbon sources which differentially affect growth state, morphologies and drug susceptibility patterns[28,29,88-90]. When *M. tuberculosis* encounters a fatty acid-rich environment, for example, in a propionate-rich medium, it induces enzymes such as isocitrate lyase to sustain citric acid cycle activity[30,91,92]. Infected macrophages accumulate lipid droplets induced by *M. tuberculosis*, gradually converting into foamy macrophages **[G]** [31,32,88,93] (FIG. 2). As *M. tuberculosis* adapts to utilize these lipids they develop a remarkable nongrowing dormant state, a phenotype consistently observed in hypoxic, lipid-loaded macrophages[31,88]. Nearly half of all *M. tuberculosis* inside lipid-rich macrophages developed phenotypic tolerance to isoniazid and rifampicin by accumulating lipid droplets containing host TAG[31,32]. A group sought to target this subpopulation by treating infected macrophages with vitamin D, which reduces the accumulation of lipid droplets[94]. Bacterial burden was reduced, which suggests that obstruction of lipid metabolism in the macrophage may be an essential determinant of *M. tuberculosis* killing[94]. Nutrient shifts that result in altered bacterial metabolism can enable *M. tuberculosis* to endure immune-mediated killing as well[88]. Activation of interferon-γ in the macrophages induces a cholesterol-rich intracellular environment which in turn enables mycobacteria to utilize this lipid as a carbon source and persist[88].

Acidic pH.

M. tuberculosis encounters a range of pH levels during infection. Caseum pH ranges from pH ~6-8, whereas macrophages impose acidic conditions in phagolysosomal compartments[77,95-98] (FIG. 2). Differences in pH not only affect the metabolic state of *M. tuberculosis*, but also determine drug treatment responses, as has been well described for pyrazinamide, ethambutol, isoniazid and linezolid[99-101].

The acidic microenvironment within the macrophage influences growth rate, metabolism, and induction of virulence factors **[G]** [33,102,103]. *M. tuberculosis* slows its growth at a pH below 6.4 and arrests growth at pH 5.0 in a nutrient-rich medium, but this trend can change depending on the carbon source that *M. tuberculosis* Mtb utilizes inside macrophages[33]. Recently, it was found that *M. tuberculosis* can grow in an acidic environment, as low as pH 4.5, if it alters its carbon metabolism to utilize lipids, such as oleic acid, instead of carbohydrates[104]. In addition, the *M. tuberculosis* PhoPR two-component system is induced as a response to low pH, reducing growth[33,34,105] and modulating virulence factor and lipid anabolism[106,107].

Production of virulence factors, such as cell surface lipids and secretion systems **[G]** , protects *M. tuberculosis* from acidic stress in macrophages by inhibiting the formation of phagosomes and preventing the acidification of phagolysosomes[108-111]. Trehalose-6,6'-dimycolate, also known as cord factor, is a toxic glycolipid that delays maturation of phagosomes by preventing acidification and reducing hydrolytic activity[109,110]. ESX-1 and ESX-3, the type VII secretion systems, and lipoarabinomannan also inhibit phagosomal maturation by phagosomal rupture and/or blocking the delivery of lysosomal constituents to the phagosome and interfering with endosomal sorting, respectively[108,111-114].

Adaptation to the acidic environment and expression of virulence factors can have a profound impact on the fitness of *M. tuberculosis* in the host and affect drug sensitivity[107,115]. For example, ethoxzolamide, a carbonic anhydrase inhibitor, downregulates the PhoPR regulon, which impairs the ability of *M. tuberculosis* to induce the accumulation of lipids in the macrophage[107]. *M. tuberculosis* growth was notably diminished in mouse lungs as a result of PhoPR downregulation. Treatment with the compound AC2P36 induced thiol stress at acidic pH, which enhanced *M. tuberculosis* killing and potentiated the activity of isoniazid, clofazimine and diamide[115].

Oxidative stress.

As a part of the innate immune response, macrophages generate reactive oxygen species (ROS) to slow *M. tuberculosis* growth or kill it. Macrophages produce ROS through the action of Nox enzymes, the mitochondrial matrix and acidification of the phagosome[116-118]. Oxidative stress is highly toxic to *M. tuberculosis* as it damages DNA, protein and lipids[119], and impedes thiol redox homeostasis[120,121]. ROS-mediated damage can cause cell death by the oxidation of the guanine nucleotide pool and its incorporation into DNA and RNA[122]. Also, thiol-specific oxidizing stress can be caused by the impaired thiol redox homeostasis, which results in growth disruption and cell death[120,121]. To combat oxidative stress, *M. tuberculosis* has developed a distinctly thick cell wall consisting primarily of mycolic acid, which acts as a robust physical barrier to protect from ROS[35,123]. Additionally, some mycobacterial genes have dedicated roles to counteract DNA damage, particularly those involved in Fe–S cluster biogenesis[36,124]. Many genes related to the oxidative stress response can protect against the harmful effects of ROS upon detection, but others are responsible for repair after damage[125-129]. In this Review, we do not discuss the genetic mechanisms involved in the responses, as a recent review thoroughly explains how *M. tuberculosis* responds to oxidative stress[130]. Some of the virulence factors secreted by *M. tuberculosis* play a part in protecting *M. tuberculosis* from ROS. A phosphate transporter, PstS, is one of the glycolipoproteins that induces phagocytosis and reduces ROS production by binding to the macrophage mannose receptor[131,132]. *M. tuberculosis* nucleoside diphosphate kinase (Ndk) also contributes to decreased ROS production in the host macrophage by attenuating NADPH oxidase-mediated host innate immunity[133]. Thus, *M. tuberculosis* responds to host stressors by both adapting their own state and manipulating the host response.

The importance of the oxidative stress response for *M. tuberculosis* pathogenesis is well studied, and thus, drug treatments that curtail these protective mechanisms have been designed. For example, a compound associated with thiol stress, AC2P36 (see above), enhances the accumulation of ROS, selectively killing *M. tuberculosis* at low pH[115]. As AC2P36 depletes free thiol pools of *M. tuberculosis*, it synergizes with isoniazid, clofazimine and diamide by causing a ROS burst and thwarts defense of *M. tuberculosis* against oxidative damage[115]. Along with new drug development, the use of nutritional supplements for the host, such as vitamin C, in combination with antibiotics is also being investigated as a way to potentiate the bactericidal ability of treatments[134,135]. It was found that *M. tuberculosis* is more sensitive to vitamin C than other bacterial pathogens because it promotes high ferrous ion levels and ROS production through the Fenton

reaction[134]. Moreover, when vitamin C is used in combination with pyrazinamide, bacterial heterogeneity is reduced by the killing of subpopulations that are tolerant to other drugs such as isoniazid and rifampicin[135].

Hypoxia.

Drug tolerance is enhanced in *M. tuberculosis* in hypoxic granulomas[68]. Hypoxic conditions have been detected in lesions where cellular necrosis occurred, notably within the necrotic caseum[136,137] (FIG. 2). Even though *M. tuberculosis* is an aerobe, it can survive hypoxia by transcriptionally transitioning to an anaerobic dormant state[138-140]. Because *M. tuberculosis* requires oxygen for its growth, hypoxia rapidly establishes a non-replicating, drug-tolerant state[12,138,141,142] (Table 2). Hypoxia induces the deacetylation of DosR, a dormancy survival regulator, promoting anaerobic dormancy by inhibiting aerobic respiration and preventing replication[138,140]. During this anaerobic dormancy, *M. tuberculosis* alters its morphology and metabolism, which results in changes in cell wall structure, lipid metabolism and gene expression, and increasing tolerance to rifampicin and clofazimine[31,67,142,143]. For example, the outer layer of the cell wall of *M. tuberculosis* is uneven and thicker in low-oxygen growth conditions compared to normoxia[67]. This morphological change restricts the entry of rifampicin, conferring tolerance. Moreover, this population of *M. tuberculosis* had increased survival rates against clofazimine treatment in the lungs of C3HeB/FeJ mice which form hypoxic, necrotic granulomas compared to the lungs of BALB/c mice, which only present cellular granulomas[143]. These data suggest that hypoxic environments promote the accumulation of drug-tolerant *M. tuberculosis* subpopulations as a consequence of their survival mechanisms to this host-mediated stress. The susceptibility of *M. tuberculosis* in hypoxia to treatment is dependent on the drug mechanism of action, as hypoxia induces susceptibility of non-replicating *M. tuberculosis* to metronidazole in both *in vitro* and non-human primate models[136,144-146]. Also, it was found that the combination of rifampicin and nitazoxanide showed enhanced *M. tuberculosis* killing in hypoxia compared to the first-line treatment regimen of rifampicin, isoniazid, pyrazinamide and ethambutol[146].

Consequences of heterogeneity.

Population structure.

The different sources and types of variation arising from innate bacterial heterogeneity and adaptations to the local environment generate a range of physiological states in mycobacteria. Subpopulations of *M. tuberculosis* differ from each other in many measurable factors (such as growth, metabolism, damage and gene expression) so that they exhibit different phenotypes. One example is persisters; that is, members of a drug-tolerant subpopulation survive in the presence of an antibiotic without genetic resistance and can regrow after removing antibiotics[147,148]. As understanding of the mechanisms underlying heterogeneity improves, mycobacteriologists are beginning to transition away from investigating gross changes in population response towards interrogating subpopulation and single-cell behaviors. This approach is well established in other fields including immunology, for example, in their use of flow cytometry to measure a population of cells as the aggregate of many multivariate single-cell measures. Flow cytometry plots, which

plot individual cells over physiological measurements, can then be used to determine the distributions of subpopulations. In this section, we propose interrogating physiological states of *M. tuberculosis* through a conceptual framework similar to that of a flow cytometry plot. We refer to this as a 'population structure', which is a map of individual cells across multiple measurable states in a phase diagram format (FIG. 3). We illustrate how population structure can be visualized to enable parsing of subpopulations of *M. tuberculosis* with similar physiological states and phenotypes as they vary with host microenvironments. For example, one well-characterized subpopulation of replicating *M. tuberculosis* is formed in response to the oxidative stress of the phagosome[117]. This group of cells can be mapped onto a diagram of population structure to help understand the parameters of drug tolerance in the phagosome (FIG. 3). In this example, the growth state (property A) is high because the *M. tuberculosis* are replicating (x-axis), their metabolic state (property B) is chemically reduced (y-axis), and these bacilli are tolerant to isoniazid and rifampicin treatment[117].

In principle, exhaustive measurement of every feature of a population of *M. tuberculosis* is the only way to comprehensively describe the subpopulations that form in these environments; this is clearly not feasible, even with different -omic profiling techniques. Thus, we can only probe subsets of properties, and we must identify those most pertinent for reporting on the physiology relevant to disease and treatment response. Composites of measurements, derived from dimensionality reduction algorithms such as uniform-manifold approximation (UMAP) or principal component analysis (PCA) may be used to probe cellular properties that distinguish subpopulations from each other. As an abstract example, we map population structure using two measurements: property A and property B which can be replaced with any cellular states such as cell size, gene expression or virulence (FIG. 3). Some environments may shift the average cellular states (condition 1) and may either amplify (condition 2) or attenuate (condition 3) cell-to-cell variation. Through population structures, we aim to understand how bacterial and environmental heterogeneity intersects to generate distinct *M. tuberculosis* subpopulations and single-cell behaviors.

Consequences of bacterial heterogeneity.

Mycobacteria produce bacterial heterogeneity by asymmetric cell growth and division, genetic mutations, and through variation at the transcriptional and post-translational levels. To date, studies of cell-to-cell heterogeneity in *M. tuberculosis* and mycobacteria indicate that there are distinct subpopulations of *M. tuberculosis* that can be characterized by different cellular states, such as growth rate, asymmetry in growth and division and metabolism. Although these features correlate with drug susceptibility among subpopulations, we have yet to comprehensively describe the specific states and growth properties that propagate this complex landscape of responses.

Asymmetric growth, division and cellular organization give rise to sister cells that inherit different cell wall ages, lipid bodies, growth properties and protein aggregates from their mother. Together, these differences stratify cellular states within a single population, which we attempt to correlate with phenotypes such as drug tolerance so that we can understand how *M. tuberculosis* adapt at population- and single-cell levels to tolerate stressors. For example, asymmetric distribution of cellular components such as irreversibly

oxidized proteins (IOPs) causes different drug susceptibility owing to different levels of oxidative stress between sister cells[149] (FIG. 1d). IOPs are extensively oxidized proteins that irreversibly accumulate carbonyls on amino acid side chains and gain toxicity through illicit binding[150,151]. Researchers have discovered that the sister cells with a high amount of IOPs are more sensitive to drugs such as kanamycin, streptomycin and isoniazid, which promote oxidative damage[149,155,156]. Thus, presenting the population structure of *M. tuberculosis* and the measurement of cellular IOP concentration in a diagram would reveal a subset of drug-susceptible bacteria characterized by high amounts of IOPs (FIG. 3).

Though the mechanisms are not understood, cell size correlates with drug susceptibility at a single-cell level[3,8,11,42]. It may be that cell size is an informative and measurable factor of individual cells that integrates several elements of growth behavior and is variable from cell-to-cell. Long cells (such as accelerator cells) are more sensitive to cell wall-acting drugs, such as cycloserine, meropenem and isoniazid, relative to smaller cells (alternator cells)[3]. However, cells born small are more susceptible to rifampicin, which targets transcription[8,42]. Using the framework of a population structure, if we plot cell size, we would expect the low end of the distribution to be susceptible to transcriptional inhibitors and the high end to be susceptible to cell wall-acting agents (FIG. 3). The breadth of this cell size distribution is also a determinant of drug tolerance as knockdown of *lamA,* which reduces cell size variation, increases rifampicin and vancomycin susceptibility in *M. smegmatis* and *M. tuberculosis*[8]. Depending on the degree of such asymmetry, the distribution of heterogeneity may also vary from population-to-population. In a population structure framework, decreasing asymmetry (and heterogeneity) may reduce the proportion of the population in the ‘survivor’ region (for example, shifting from the structure in condition 2 to 3 with drug A) (FIG. 3). However, we have yet to understand how drug-tolerant subpopulations may be characterized using patterns of growth state in different host-relevant growth conditions. Rational approaches to designing shortened treatment therapies for TB must address the origins and vulnerabilities of persisters, and how growth and division asymmetry in these conditions promote tolerance.

Consequences of environmental heterogeneity.

M. tuberculosis are versatile, with the flexibility to readily adapt to different host environments. We illustrate examples of how adaptation to different host environments changes the population structure. One example is a simple shift to cholesterol as a carbon source. In this example, one axis (property A) in FIG. 3 can be used to represent the expression level of *mce4*, which encodes the cholesterol import system[88]. We presume that the population would shift to the right on a cholesterol diet as *mce4* expression increases. Growth state can be used as property B in which population structure movement relative to the y-axis will depend on the cholesterol levels, as *M. tuberculosis* doubles faster in higher concentrations of cholesterol[28]. Changes to specific carbon sources may increase density in the population structure plot (FIG. 3), whereas others may cause scattering.

The parameters of the population structure of *M. tuberculosis* in the acidified phagosome have yet to be thoroughly defined. However, data suggests that we can map drug and stress tolerance to metabolism and growth state. For example, a strong link between non-growing

cells (for example, a growth state as property A) and increased use of the glyoxylate shunt (metabolism as property B) at low pH was identified, which confers rifampicin and isoniazid tolerance[157] (FIG. 3). The subpopulations formed in this environment are gradually being characterized and ongoing work focuses on understanding the changes in metabolism caused by *M. tuberculosis* altering its carbon sources[104].

The framework of a population structure may also explain the physiological state of *M. tuberculosis* in the hypoxic conditions of the granuloma. As one example, expression of genes related to lipid catabolism is upregulated in hypoxia, increasing energy stores in a non-replicating state. Together, these changes create phenotypic drug tolerance[142]. Applying this example to the framework, if one axis presents lipid catabolism-related gene expression, the population will move to a greater value on this axis in hypoxia. On another axis, which represents growth state, the population would move to a smaller value as they are non-replicating (Fig. 3). Further studies are necessary to define these dynamic population structures in complex lesion conditions and their interplay with drug tolerance.

Consequence of heterogeneity in the clinic.

In patients with TB, subpopulations resulting from both bacterial and environmental heterogeneity are observed. One example is differentially detectable (DD) Mtb/differentially culturable tubercule bacteria (DCTB) found in the patient's sputum. DD Mtb/DCTB are subpopulations of *M. tuberculosis* bacilli that are undetectable in standard *in vitro* culture medium but can grow and therefore be detected in medium supplemented with culture filtrate[10,158]. DD Mtb/DCTB has been identified in fresh clinical isolates and culture filtrate-added media conditions *in vitro*[10,159]. Because standard medical laboratory assays do not detect DD Mtb/DCTB, this hidden subpopulation may be responsible for disease relapse in patients after treatment[10,158,160]. We have yet to understand how DD Mtb/DCTB cells are generated and the relative proportions of DD Mtb/DCTB in *M. tuberculosis* populations from different types of lesions. It may also be that other clinically important subpopulations that have yet-to-be defined are undetectable in either medium. Once we understand the factors that foster growth of these subpopulations we can begin to characterize their growth and metabolic properties in the format of a population structure (FIG. 3).

Different microenvironments not only affect the formation of heterogeneous *M. tuberculosis* subpopulations, including DD Mtb/DCTB, but may also affect the pharmacokinetics–pharmacodynamics **[G]** (PK–PD). For example, complex granuloma structure and poor vascularization obstruct drug distribution in specific sites[78]. As we learn more about drug penetrance and the microenvironment of different lesion compartments, we may improve multi-drug regimen designs to contain effective antibiotics and access all *M. tuberculosis* niches[23,28,161-163].

The range of environmental conditions and stressors imposed in different granulomas generates a heterogeneous *M. tuberculosis* population with remarkable differences in growth and metabolic states and drug susceptibility patterns (FIG. 2). However, population adaptation to different microenvironments does not necessarily make one *M. tuberculosis* subpopulation tolerant to all types of antibiotics. Persistence is condition- and drug-dependent. We should take advantage of our current knowledge on the range of persisters

to rationally design multi-drug therapies that comprehensively target this ensemble. To do so, we must map *M. tuberculosis* state to patterns of drug efficacy and understand what subpopulations are formed in each lesion type at the single-cell level.

Conclusions and outlook:

Variation in mycobacterial virulence and drug response is achieved by subpopulations that can survive and thrive in the presence of host immune and drug stressors. The characteristics of the stress-tolerant bacilli are dependent on the stressor. Therefore, to have a flexible response to a range of stressors, mycobacteria use variation as a survival strategy. Mycobacterial heterogeneity is generated, in part, through stochastic processes as in other species, but it is amplified in mycobacteria through asymmetric growth and division patterns, and genetic and epigenetic modifications. The pathology of TB also organizes *M. tuberculosis* into physically separate subpopulations via granulomas, causing resident *M. tuberculosis* to encounter different and dynamic microenvironments. The adaptation of *M. tuberculosis* in these physically separate and compositionally distinct lesions forces heterogeneity in the population. In addition, this heterogeneity can be further compounded by innate bacterial variation.

The lengthy, multi-drug treatment regimen for TB is necessary to sterilize every niche of the lung, underscoring how important it is to focus on developing new therapies against the vulnerabilities of the resident drug-tolerant subpopulations. However, we still do not understand how this variation is developed and maintained from both bacterial and lesional sources. This task is difficult not only due to technical challenges in working with a slow-growing pathogen that clumps readily in culture, but also because of the complexity of *M. tuberculosis* heterogeneity. Cell state and variation are multivariate, dynamic, and condition-specific. For this reason, we propose that mycobacteriologists utilize an integrated approach to aggregate and interpret these data in future studies. These population structures (for example, phase diagrams) will enable us to define different features (axes) of importance and track how single-cell and population-level behaviors can be reconciled (FIG. 3). In the physical sciences (and increasingly in biology), phase diagrams are used to navigate multivariate spaces to understand critical points where qualitative behaviors change. We anticipate that *M. tuberculosis* will not only shift between different regions on the phase diagram as they adapt to changing host environments but that the degree of heterogeneity (that is; scatter) will also be regulated. In particular, the phase diagram may help specify the changes and effects of these factors that make TB treatment difficult. As TB scientists make more discoveries, visualizing the population structure in this way will help us characterize the relevant features of the *M. tuberculosis* subpopulations that lead to treatment failure and subsequently inform new treatment strategies to target them.

Technological improvements are poised to accelerate our understanding of heterogeneity in TB and how variation affects disease and treatment outcomes. For example, we expect more widespread use of new imaging methods, including deep immunophenotyping and higher throughput time-lapse imaging accompanied by deep-learning-assisted image segmentation and analysis approaches. Genetic tools such as CRISPR interference, collaborative cross mice, and sets of clinical *M. tuberculosis* isolates will enable us to understand variation in

both the host and pathogen. Together, the increase in depth and breadth in TB studies will help us design improved TB interventions by understanding how *M. tuberculosis* creates and exploits heterogeneity.

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Glossary:

Granulomas
Complex lesion structures that form during TB pathogenesis and consist of immune cells, epithelioid cells, and necrotic tissue

Macrophage activation
A change in physiology in response to signals from adaptive or innate immune mechanisms that enable macrophages to perform specialized effector functions

Necrotic caseum
Lipid-rich foci within granulomas primarily derived from necrotic cellular debris

Divisome
Protein complex responsible for performing membrane- and cell wall-division functions

Accelerator cell
Cells that inherit the pole from which its mother cell grew and usually elongate at a higher rate than alternator cells

Alternator cells
Cells that generate a new growth pole upon birth and elongate at a lower rate than accelerator cells

Glycopeptidolipid (GPL)
A type of lipid present on the outer leaflet mycobacterial envelopes that influences biofilm formation, growth, and pathogenicity

Isogenic sister cells
Cells born from the same mother and share identical genotypes

Minimum inhibitory concentration (MIC)
The lowest concentration of a drug that will inhibit growth

Collaborative crosses
Panels of mouse strains with significant genomic variation designed to mimic the heterogeneity of the human population and its spectrum of disease phenotypes

Foamy macrophages

Macrophages that are abundant in lipid droplets formed from pathogen-induced perturbation of lipid biosynthesis

Virulence factors
Components of a pathogen that enable it to invade and colonize a host

Secretion systems
Molecular nanomachines present on the outer surface of a pathogen that secrete substrates to promote pathogenicity

Pharmacokinetics–pharmacodynamics (PK–PD)
The relationship between drug distribution, absorption, metabolism, and concentration in the human body over time

Antimicrobial breakpoints
The lowest concentration of a drug that will inhibit the growth of almost all (95%) wild type strains of the organism but does not inhibit clinical strains that are resistant

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Text Box 1:

Mycobacterium tuberculosis heterogeneity and drug susceptibility testing.

Antimicrobial susceptibility testing (AST) is widely implemented in the treatment and management of tuberculosis (TB) cases that fail the frontline treatment of rifampicin and isoniazid[166,167]. Such testing is required for surveillance and designing an effective regimen of second-line drugs for individual patients to prevent poor treatment outcomes[168,169]. There are two central methods for diagnosing drug-resistant TB infection: genotypic testing and phenotypic culture-based AST. Though it may take more than 3 weeks before final interpretation, phenotypic testing is often the preferred method for determining drug susceptibility as it is quantitative and overcomes the failure of molecular assays to detect under-appreciated resistance mutations[170,171].

Current phenotypic testing methods are nevertheless affected by the extensive heterogeneity of *Mycobacterium tuberculosis*. Phenotypic AST relies on assessing the growth of a clinical isolate in traditional solid media in the presence of a critical concentration of a drug[170]. To determine critical concentration and set antimicrobial breakpoints **[G]** for the classification of isolates as resistant, international committees use data from minimum inhibitory concentration (MIC) distributions of wild-type *M. tuberculosis* in nutrient-rich growth medium[172-174]. However, growth in standard conditions does not accurately represent the drug response of *M. tuberculosis in vivo*[21,28,139,160,71-78].

Using results from current AST methods as guidance for clinical regimens has caveats because standard *in vitro* conditions do not foster the growth of the complete array of *M. tuberculosis* subpopulations present in the lung. One such example is differentially detectable (DD) Mtb/ differentially culturable tubercle bacilli (DCTB)[175-179]. Such bacilli require additional, unknown resuscitation-promoting factors not present in rich-growth medium and, thus, these bacilli are not tested for susceptibility using current methods[159].

Other subpopulations such as persister cells, or phenotypically drug-tolerant cells, may also confound efforts to utilize AST results to guide clinical regimens. Bulk population growth inhibition metrics like MIC used in AST fail to evaluate the killing of these persisters, the cells that eventually lead to regrowth of *M. tuberculosis*[148]. Thus, two *M. tuberculosis* isolates might have similar MICs but disparate proportions of persisters, which may lead to different treatment outcomes in patients[147]. A growing appreciation of all of these factors may prompt a re-evaluation of the methods used to determine antimicrobial breakpoints, critical concentrations, and susceptibility of clinical isolates.

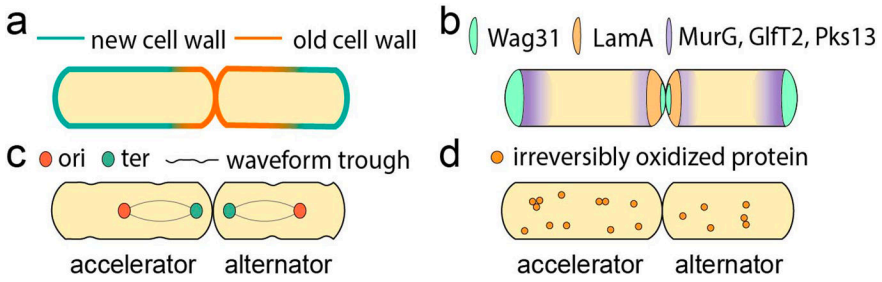

Figure 1. Mycobacterial asymmetry.

(a) In mycobacteria, growth is asymmetric: the old pole elongates more than the new pole. The sister that inherits the old pole from the mother called the accelerator cell) is born longer and elongates more than the sister that inherits the new pole (the alternator cell). **(b)** The cell elongation and division protein Wag31 and growth inhibitor LamA are regulators of asymmetric growth in mycobacteria. Inhibition of growth at the new pole by LamA is relieved after a time lag. MurG, GlfT2 and Pks13 are cell wall synthesis complexes in *Mycobacterium smegmatis* that are localized in the subpolar regions with their highest local concentration at the old pole. **(c)** In *M. smegmatis*, chromosome positioning is asymmetric with the terminus (ter) closer to the new pole than the origin of replication (ori) is to the old pole. The asymmetric positioning of the chromosome is proportional to cell size. The future sites of asymmetric division are determined in mother and grandmother cells and can be observed by surface wave troughs that are inherited from previous generations. **(d)** Asymmetric distribution of irreversibly oxidized proteins (IOPs) in *Mycobacterium tuberculosis* creates sister cells with different growth properties and sensitivities against antibiotic stresses depending on their IOP-burdens. Specifically, sister cells with a high amount of IOPs are more sensitive to drugs.

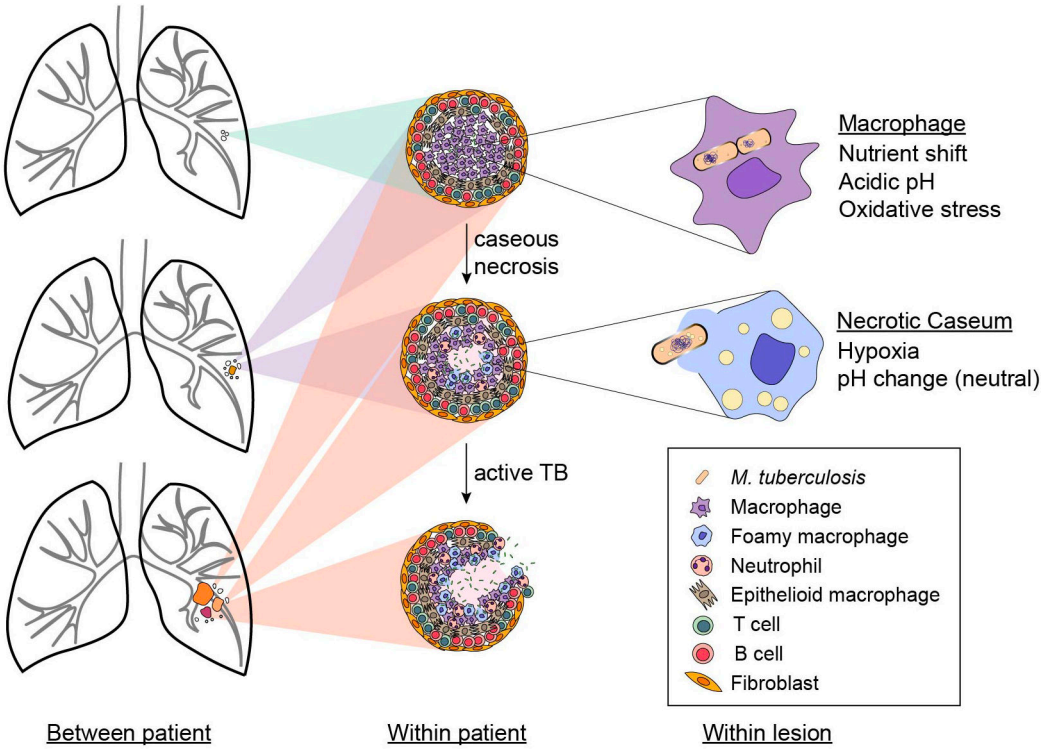

Figure 2. Environmental heterogeneity.

Microenvironments in tuberculosis lesions (granulomas) vary among patients, within one patient, and even within a lesion as shown with examples in the first, second, and third columns, respectively. Lesion composition is dependent on the stage of disease progression and the network of immune cells present. Many types of cells respond to infection with *Mycobacterium tuberculosis*, including macrophages, neutrophils, T and B cells, epithelioid cells, and fibroblasts, and populate cellular granulomas. *M. tuberculosis* in infected macrophages encounter an environment characterized by low pH, oxidative stress and abundant lipids. As *M. tuberculosis* utilizes these host lipids, it secretes lipid vesicles into the macrophage cytoplasm, inducing some of the infected macrophages to become foamy with lipid droplets. Cytoplasmic debris from necrotic macrophages form the granuloma's caseous center, which may be acidic or neutral and vary in oxygen content.

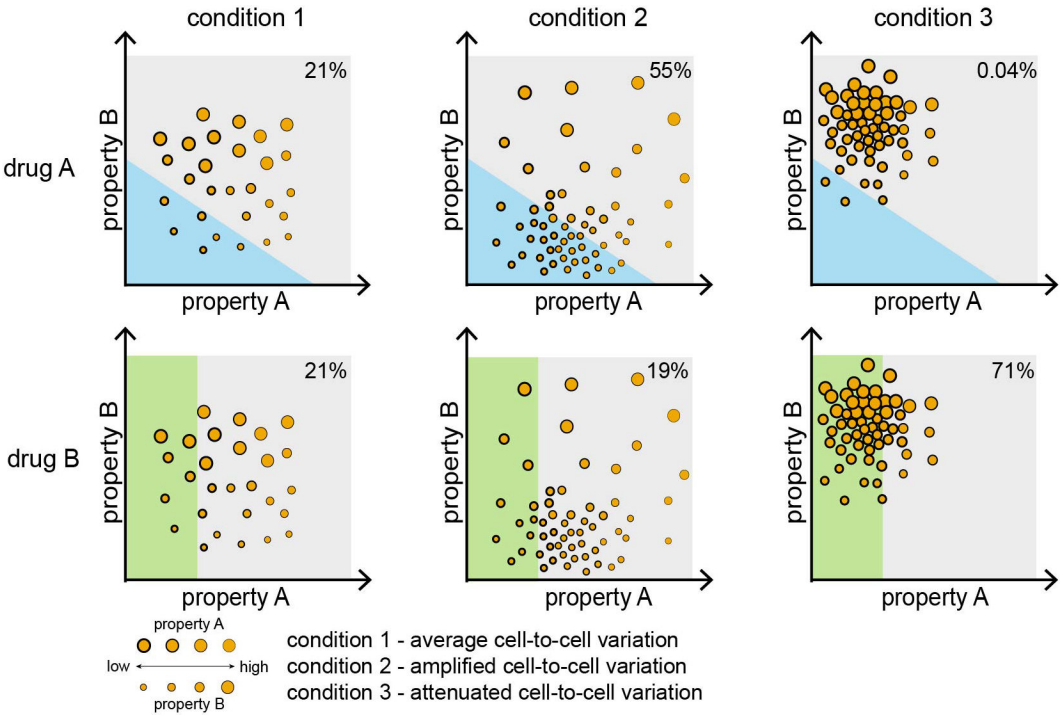

Figure 3. Population structure and complex *Mycobacterium tuberculosis* phenotypes.

A hypothetical population structure framework maps single-cell and population-level cellular states and phenotypes across multiple processes. The locations, dense regions and spread in this phase diagram change due to adaptation to different environmental conditions, including drug treatment. Each axis (property A and property B) can represent cellular states, such as cell size, gene expression or virulence. Abstracted axes, as being used for illustrative examples here, may be made quantitative using weighted sums of specific cellular features through dimensionality reduction techniques such as principal component analysis. Condition 1 is an example wherein cells are evenly distributed throughout the range of properties A and B. During adaptation to new environments and stressors, the population structure can be shifted and/or concentrated to other regions of the phase diagram. The degree of bacterial heterogeneity can become amplified (condition 2) or attenuated (condition 3). Following antibiotic treatment, the size of the tolerant subpopulation may vary depending on which condition *Mycobacterium tuberculosis* has adapted to (for example, 21% in condition 1, 55% in condition 2, 0.04% in condition 3 following treatment with drug A). The region that encompasses drug-tolerant cell states will be dependent on the activity and mechanism of action of the drug (compare drug A versus drug B), highlighting that there are different kinds of persister cells.

Table 1.

Genetic, transcriptional, translational and post-translational modifications involved in bacterial heterogeneity

Type of variation	Cause	Outcome	Reference
Genetic variation	INDEL and SNPs in *mps1-mps2-gap*, *mmpl4b*	Switch of colony morphology from smooth to rough form, leading to more severe, persistent infections	52
	SNPs in *prpR*	Multidrug tolerance to INH, RIF, OFX under propionate-rich condition	12
	Gene mutations in *Rv0565c*	Tolerance to ETH and PTH	13
	Frameshift mutations in *glpk*	Slower growth, reduced sensitivity to drug regimens containing PZA	53,54
Transcriptional level	Induced expression of *rpoB* promoter II in response to RIF	Tolerance to RIF	57
	Various expression levels of KatG	Better survival following treatment with INH	56
Translational level	Substitution of amino acids in RNA polymerase	Phenotypic resistance to RIF	58
	Substitution of amino acid (S315T) in KatG	Resistance to INH	59-62
Post-translational level	Lysine acetylation and methylation of HupB	Phenotypic resistance to INH	63-65
	Phosphorylation at Y102 in MtrA	Tolerance to INH and VAN	66
	Phosphorylation at T34 in PonA1	Increased MIC of RIF	39

ETH, ethambutol; INDEL, insertion or deletion;INH, isoniazid;MIC, minimum inhibitory concentration; OFX, ofloxacin; PTH, prothionamide; PZA, pyrazinamide; RIF, rifampicin; SNP, single nucleotide polymorphism; VAN, vancomycin.

Table 2.

Mycobacterium tuberculosis in different host microenvironments and effective drugs

Host environment	Specific lesion	Impact on *M. tuberculosis* and adaptation of *M. tuberculosis*	Effective drugs and supplements	Refs
Nutrient shift	Macrophages and caseum (lipid-rich environment)	Altered metabolism and growth rate	Vitamin D	28,29,88,32
		Changed growth control and morphology	NA	7,89,164
		Induces enzymes to utilize lipids		30,91,92
		Accumulation of lipid droplets		31,32,88
Acidic pH	Macrophage	Growth arrest	Ethoxzolamide	33,107
		Altered physiology by regulating *phoPR* two component system	AC2P36	33,34,105,115
		Modulation of virulence factors and lipid anabolism	NA	106-111,114
Oxidative stress	Macrophage	Thickening of the cell wall as a physical barrier	AC2P36	35, 115,123
		Altered gene regulation to protect after ROS recognition	Vitamin C	36,124,134,135
		Damage repair	NA	125-129
		Regulation of virulence factors	NA	131-133
Hypoxia	Caseous center of necrotic lesions and cavities	Growth arrest and dormancy	Metronidazole	136, 139, 144,145, 165
		Phenotypic drug tolerance	Rifampicin-nitazoxanide	140-142,146
		Altered morphology and metabolism; changes in cell wall structure, lipid metabolism and gene expression	NA	31,67,142,143

NA, not applicable; ROS, reactive oxygen species.