

Tuberculosis Chemotherapy: the Influence of Bacillary Stress and Damage Response Pathways on Drug Efficacy

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INTRODUCTION

Tuberculosis (TB) was declared a global health emergency by the World Health Organization in 1993 and currently claims approximately 1.7 million lives per annum, more than can be attributed to any other bacterial infection (252). It is estimated that one-third of the world's population is infected with the causative agent, the obligate human pathogen *Mycobacterium tuberculosis* (76), with around 9 to 10 million new cases of TB being reported each year (252). Approximately 90 to 95% of initial infections are controlled by the cell-mediated immune response. However, TB immunity is static (171), and a residual population of viable bacteria may be maintained in a poorly understood state of clinical latency for extended periods (141). Approximately 5 to 10% of cases overall are thought to result from spontaneous reactivation of latent TB infection (219). The massive reservoir of viable bacteria in the estimated 2 billion asymptotically infected individuals worldwide is, therefore, of supreme importance for the epidemiology and control of TB. However, the potential for bacterial populations to occupy discrete lesions in a single host complicates targeting of the intractable pathogen (22). Furthermore, recent years have witnessed the lethal synergy between *M. tuberculosis* and the human immunodeficiency virus (HIV) (55) as well as an increasing emergence of multidrug-resistant (MDR) strains (251).

Infection with an *M. tuberculosis* strain that is resistant to the

two most commonly used frontline anti-TB drugs, isoniazid (INH) and rifampin, is defined as MDR TB and often culminates in incurable disease (109). Treatment of MDR TB is resource intensive, and the therapeutic strategies recommended for high-prevalence areas (81, 103) comprise combinations of second-line drugs that are more expensive, more toxic, and less effective than the drugs used in standard therapy (114). MDR strains constitute 1 to 3% of global TB isolates, and although worldwide distribution is characterized by localized "hot zones" (82, 183), MDR TB has been identified as a significant problem in every region under World Health Organization surveillance (251). Approximately 300,000 new cases of MDR TB emerge worldwide each year, with the most common pathway to multidrug resistance initiating with monoresistance to either INH or streptomycin (251). Furthermore, the prevalence of strains resistant to these single drugs correlates with those areas exhibiting the highest levels of MDR. Significantly, "super strains" resistant to at least three of the four frontline TB drugs make up 79% of all MDR TB cases.

CURRENT ANTI-TB CHEMOTHERAPY

The current standard chemotherapeutic regimen for active TB—directly observed therapy, short course (DOTS)—requires the supervised administration of a multidrug combination for a minimum period of 6 months. The frontline drugs are biased towards interference in cell wall integrity, with the actively dividing *M. tuberculosis* population in the lung cavity, which is key to transmission and emergence of drug resistance (101, 122), being the principal target. *M. tuberculosis* replication in pulmonary cavities most closely resembles optimal aerobic growth in vitro, and the effectiveness of the frontline drugs

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in treating acute TB is manifest in rapid bacillary clearance within the first 2 months of chemotherapy. Differences in drug susceptibilities between replicating and nondividing bacterial cells can be significant (112, 254), however, and the dependence of the frontline anti-TB drugs on actively replicating cells for activity is probably the greatest limitation of current therapy (119). This weakness is further reflected in the profound disparity between *in vitro* and *in vivo* efficacies of the majority of the frontline drugs (155), an additional factor dictating the complexity and duration of the DOTS regimen.

Poor antibiotic penetration, heterogeneity of host environments, and altered bacterial physiology and metabolic activity within those environments have all been blamed for impaired drug efficacies *in vivo*. However, the influence of the *in vivo* environment on drug efficacy should not be viewed as inevitably negative: pyrazinamide (PZA), for example, is inactive *in vitro* under standard culture conditions but displays strong sterilizing activity *in vivo* (257, 259). Moreover, the activity of PZA *in vivo* correlates with enhanced *in vitro* activity under low pH (257) and limiting oxygen (236), suggesting the relevance of these factors to the *in vivo* environment (discussed below). The absolute dependence on environmental factors for PZA activity has profound implications for the discovery of new anti-TB drugs, since it would almost certainly render this drug unidentifiable according to standard drug screening criteria, thereby eliminating a mainstay of current TB chemotherapy. At the very least, the differential effect of the *in vivo* environment on PZA versus the other frontline drugs emphasizes the need to understand the *in vivo* lifestyle of *M. tuberculosis* so that the factors influencing drug efficacy can be determined and new drug targets relevant to both latent and active disease can be identified.

BACILLARY SURVIVAL TACTICS

In the presence of a functional adaptive immune system, TB bacilli are sequestered in granulomas comprising differentiated macrophages and other immune cells maintained in complex structures by extracellular matrix components (58). Granulomas are thought to be characterized by hypoxia, low pH, and nutrient deprivation and are likely awash in inhibitory organic acids (145, 176, 177). Furthermore, the apparently static balance established between the host immune system and the pathogen's resistance (166, 196) suggests that bacilli might endure continual exposure to immune effectors, including reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (38, 131, 147, 169, 174, 239). Unfortunately, there is currently a critical lack of a tractable and inexpensive animal model which replicates all aspects of human TB (22, 125). However, despite the limitations, wild-type and mutant *M. tuberculosis* strains have been widely employed as bioprobes in the various animal models and *in vitro* to refine our understanding of the host environment as well as the mechanisms enabling long-term survival of *M. tuberculosis*.

Broad concepts which have emerged include the notion that persisting bacilli are metabolically active (26, 113, 209) and might adapt sufficiently to long-term nonreplication to reinstate cellular division (106). The stimuli for entry into a state of nonreplicating persistence have been explored by applying stresses such as nutrient starvation (14, 60), oxygen limitation

(21, 244), exposure to low-dose NO (234), and adaptation to stationary phase (106, 235). Analysis of gene expression profiles in response to these stresses, as well as phenotypic and physiological characterization, has largely validated the relevance of *in vitro* stresses to *in vivo* conditions. In addition, selective *in situ* analysis of RNA expression levels in murine and human lung tissue has corroborated key findings of the *in vitro* models (83, 213, 223, 227). Critically, the differential expression of selected *M. tuberculosis* genes in lung tissue of human TB patients has shown that *M. tuberculosis* possesses the capacity to respond to specific and variable microenvironments *in vivo*, and it might adopt a variety of metabolic states depending on host immune status and stage of infection (83, 122, 227). Some of the possible metabolic adaptations are considered briefly below, with emphasis on the types of DNA damage likely to be incurred by the bacilli during their occupation of granulomas. Mechanisms that have evolved to minimize or repair that damage, as well as the potential physiological and mutagenic consequences, are considered in subsequent sections, with particular reference to the emergence of drug-resistant clinical isolates.

THE *IN VIVO* ENVIRONMENT ENCOUNTERED BY *M. TUBERCULOSIS*

Persistence of *M. tuberculosis* hinges on its ability to survive (and replicate) for extended periods in pathogen-friendly phagosomes. After phagocytic uptake, *M. tuberculosis* interrupts the normal maturation pathway (48), preventing phagosome-lysosome fusion (168) and ensuring limited phagosomal acidification and a phagosomal compartment deficient in mature lysosomal hydrolases (201). Arrest of phagosomal maturation is a complex process which depends on the continual modulation of host cell trafficking events (48). Even where maturation is stalled, however, bacilli are exposed to a slight decrease in phagosomal pH (69). Notably, the transcriptional response of *M. tuberculosis* to acid stress *in vitro* (84) includes genes identified as being necessary for survival *in vivo* (205). In addition, several genes potentially involved in fatty acid metabolism are common to both the intraphagosomal and acid response transcriptomes of *M. tuberculosis* (74, 84, 207). Evidence of the dependence of PZA on acidic conditions for activity *in vitro* further reinforces the idea that bacilli are confronted by low pH during active disease (257, 259).

The notion that *M. tuberculosis* encounters environments of limiting oxygen availability during the course of human infection (62) has motivated efforts to characterize the physiological and metabolic changes associated with adaptation to hypoxia (242, 244). In particular, recent studies have identified a prominent role for the regulon controlled by the DosR-DosS/DosT two-component regulator system (179, 207, 211, 234). The DosR regulon includes genes associated with anaerobic metabolism and stabilization of cellular components and is induced in response to hypoxia and nontoxic NO concentrations (126, 179, 185, 199, 211, 234). Unfortunately, the induction of DosR in response to both hypoxia and NO exposure has complicated any interpretation of the specific contribution of each to the host environment. The identification of key features differentiating the respective transcriptomes (25) does, however, suggest that disruptions in respiratory pathways might

induce specific regulons (22). Evidence of the induction of DosR regulon genes in vitro in response to starvation (106) and other stresses (126), for example, is consistent with the presence of independent signaling pathways and raises the intriguing possibility that dormancy genes might be induced by other stimuli in vivo. Whatever the stimulus, the upregulation of DosR in various models of TB infection (123, 179, 185, 199, 207, 211, 213, 234) is, nevertheless, suggestive of its relevance to the adaptation of bacilli to persistence in humans.

M. tuberculosis is also thought to be deprived of nutrients and essential elements during occupation of the phagosome and in granulomas. Recent in vitro models of starvation have identified broad metabolic adjustments that are likely to apply during persistence in vivo (14, 60, 106) and which reinforce the suggestion that glucose deficiency and an abundance of fatty acids characterize the phagosome of activated macrophages (208). Genes involved in β -oxidation, the glyoxylate shunt, gluconeogenesis, amino acid/amine degradation, RNA synthesis and modification, and transcription are induced in response to nutrient stress, while carbon-degradative pathways, de novo ATP generation, and purine/pyrimidine synthesis are down-regulated. In addition, evidence suggests that sulfur (106) and iron (72, 97, 207, 227) are likely to become limiting, both of which might be required for the maintenance of redox balance. Finally, several genes associated with sodium dodecyl sulfate treatment and cell wall maintenance are upregulated in resting macrophages (207), suggesting that *M. tuberculosis* might also sustain damage to surface structures.

DNA Damage In Vivo

M. tuberculosis is expected to sustain a variety of potentially DNA-damaging assaults in vivo (24, 162), primarily from host-generated antimicrobial ROI and RNI (1, 3, 146, 171, 198). DNA is a biological target for RNI and ROI (33, 262), and interaction with toxic radicals is mutagenic (262). Furthermore, damage to cellular components required for the protection or propagation of DNA can indirectly affect chromosomal integrity, while detoxification reactions might themselves yield endogenous damaging adducts (171). Additional endogenous reactive intermediates are also likely to be generated by the switch between aerobic and anaerobic metabolism and from the partial reduction of terminal electron acceptors during respiration (22).

Exposure of *M. tuberculosis* to potent oxidative agents in vitro results in minimal differential gene expression (23, 72, 89, 133, 212), however, and has been attributed to the impaired ability of the organism to mount a coherent oxidative stress response as a result of a natural deficiency in *soxRS* and *oxyR* regulons (70, 73, 89, 212). It is possible, though, that loss of oxidative stress response regulation might confer a selective advantage by allowing constitutive expression of detoxification genes (104)—for example, the peroxiredoxin AhpC (30, 43, 207, 217), superoxide dismutase (107, 245), thioredoxin (194, 247), and the KatG catalase-peroxidase (150, 153, 173) are expressed in *M. tuberculosis* in the absence of the central regulators. An extension of this hypothesis holds that the selective inactivation of the canonical oxidative stress response in *M. tuberculosis* might facilitate immune avoidance by preventing the potentially detrimental expression of immunogens during

certain stages of the infection process (256). There is also the likelihood that, as in other organisms, some of the mechanisms implicated in antioxidant and antinitrosative defense function primarily to enable oxidative metabolism or in homeostatic signaling networks but fulfill a vital role in tolerance of host immune effectors (170, 256).

That the in vivo environment is DNA damaging is supported by several lines of evidence (23, 205, 207). Notably, damage repair pathways are essential for the virulence and survival of other intracellular pathogens (31). However, inactivation of alkylation repair and reversal (Rv1317c-*ogt*) (75) or recombinational repair (*recA*) (203, 204) pathways does not attenuate in vivo survival in mice, perhaps indicating that nitrosative or oxidative stresses do not induce cytotoxic DNA damage in the murine model. Alternatively, other repair pathways may function preferentially in mycobacteria; for example, a Ku-ligase system for double-strand break repair by nonhomologous end joining was recently characterized in *M. tuberculosis* (99) and might provide a possible alternative to homologous recombination for repair of such lesions (163). In addition, *M. tuberculosis* contains homologues of a putative DNA repair system that is highly conserved in thermophilic archaea and predicted to function in translesion synthesis (149). Several base excision repair enzymes have also been identified that are required for growth in vivo (205) but not in vitro under optimal conditions (206), implying a role in virulence. Furthermore, mycobacteria possess several conserved Fpg/Nei family DNA glycosylases (162), although the in vivo role of these homologues in base excision repair remains uncertain. Resistance to nitrosative stress in *Mycobacterium smegmatis* is dependent on a functional uracil DNA glycosylase (231). In addition, mutations in the *uvrB*-encoded excinuclease subunit result in elevated RNI susceptibility in vitro and reduced capacity to resist ROI and RNI in vivo (63). Together, these observations are suggestive of the increased importance of excision (base and nucleotide) repair pathways in mycobacteria (163). Moreover, the upregulation of *uvrB* and several other DNA repair genes in response to only certain damaging agents indicates that the type of lesion might determine the repair mechanism invoked (23).

Other mechanisms implicated in resistance against oxidative and nitrosative stress include the DosR-regulated α -crystallin (30, 77, 90, 182, 200, 211), while some of the physical properties characteristic of mycobacteria have also been implicated in the subversion of toxic ROI (13, 39, 40, 41, 87). *M. tuberculosis* produces high levels of mycothiol (172), the principal mycobacterial thiol that has been associated with resistance to various toxic species, including oxidants and frontline anti-TB drugs (32). The observation that mycobacteria in a state of low-oxygen-induced nonreplicating persistence are highly resistant to mitomycin C (188) also suggests that some protection of chromosomal DNA is provided by the NO- or hypoxia-mediated induction of *M. tuberculosis* *uspA*-like genes as part of the DosR regulon (179, 185, 211, 234). ROI and RNI inactivate proteins by targeting key residues, and a role for the *M. tuberculosis* peptide methionine sulfoxide reductase in the reversal of oxidative damage methionine residues has been demonstrated (218). Finally, it is possible that small biological molecules such as cysteine, methionine, and tyrosine, which interact with various reactive oxygen and nitrogen species, might function as biological traps for reactive intermediates;

however, their relevance to mycobacterial oxidative and nitrosative defense is unknown (171).

GENETIC DRUG RESISTANCE

All Resistance Determinants in *M. tuberculosis* Are Chromosomally Encoded

The preferential occupation of specific environments within the host restricts the opportunities for the acquisition of novel, transmissible genetic elements by *M. tuberculosis*. Furthermore, recent evidence indicates that *M. tuberculosis* infections are clonal (105, 240), and genome analyses have revealed only minor roles for horizontal gene transfer in the macroevolution of clinical and laboratory mycobacterial strains (53, 54, 85, 111, 124, 229). *M. tuberculosis* does not possess epigenetic information in the form of plasmids, and no evidence exists for its natural competence (68). Conjugal DNA transfer has been demonstrated in *M. smegmatis* (186) and is mediated by the specialized, RD1-encoded secretory apparatus (86). However, the relevance of conjugal DNA transfer in *M. tuberculosis* and its implications for genetic variability and drug resistance have yet to be established. Consistent with the genetic isolation of *M. tuberculosis*, all drug resistance determinants are chromosomally encoded (167, 195), arising exclusively through the acquisition and maintenance of spontaneous chromosomal mutations in target or complementary genes or, rarely, from the inactivation of a target gene by a mobile genetic element (61, 134).

Emergence and Costs of Drug Resistance

For other pathogens, extended antibiotic chemotherapy has been implicated in the evolution of multiple-antibiotic resistance (143), an association likely to be replicated during *M. tuberculosis* infections. The long duration of the DOTS regimen, together with the toxic side effects of the frontline drugs and the temptation to cease therapy as symptoms subside, often leads to patient noncompliance (161). Furthermore, even where treatment schedules are adhered to, limited efficacy of one or more drugs can compromise combination therapy (142); recent epidemiological evidence suggests that monotherapy, effective or actual, is common (251).

M. tuberculosis has a long generation time and can adapt to prevailing growth conditions through a regulated shift to an alternative metabolic state (60, 233, 241). These factors are considered key to the ability of the organism to establish latent asymptomatic infection, but they might also result in the selective activity of specific antibiotics against discrete subpopulations (Global Alliance for TB Drug Development [http://www.tballiance.org]). In addition, there is compelling evidence that infecting populations occupy diverse microenvironments within the host (34, 35, 83, 120, 122), some of which might be recalcitrant to antibiotic penetration or refractory to activity (78, 115). Significantly, the locally effective antibiotic concentration has been implicated in the evolution of low-level resistant variants during infection with other pathogens (11) and has been identified as an important determinant of mutation rate. Furthermore, different mutation rates and genotypes are thought to arise at intervals along a spectrum of applied con-

centrations (128, 260), a possibility with profound implications for the generation of diverse microbial populations in a single host (10). The parallel evolution of a single founder population into heterogeneous, antibiotic-resistant subpopulations within isolated loci has been demonstrated in patients undergoing active treatment for TB (122), for example.

The attachment of a fitness cost to resistance mutations (6) has led to the assumption that removal of antibiotic selective pressure will favor reversion as a result of a competitive replicative disadvantage. However, there is evidence that evolution in the absence of the selective antibiotic preferentially results in the acquisition of compensatory mutations that ameliorate the cost of resistance (5, 135, 136), rather than reversion. That is, the fitness cost more likely determines the stability and potential reversibility of the associated resistance mutation, with the ability to compensate genetically dictating the frequency of resistant mutants within a population. While the most fit mutants will be selected in a large population, lower-fitness, compensated mutants might become fixed during bottlenecks if they are formed at a higher rate than fitter, susceptible revertants (136, 148), particularly where genetic linkage exists between selected and nonselected resistance markers (80).

Information on the relative fitness of MDR TB isolates is limited (65); however, there is evidence that compensatory mutations can restore reproductive potential in monoresistant *M. tuberculosis* strains (210, 215). In addition, while drug-resistant *M. tuberculosis* strains more frequently possess low-rather than high-cost mutations (202), studies investigating the effects of resistance on virulence (16, 159, 181, 192, 202) have failed to establish a direct correlation (50). Instead, relative fitness *in vitro* appears to depend not only on the particular resistance mutation but also on the specific assay (151). Of course, there is the possibility that a resistance mutation might affect the ability of the pathogen to interact with the host environment and so might remain undetected *in vitro* (5). Mutations impairing virulence, for example, such as deletion of *katG* (140), will not survive selection in areas of high transmission (51).

The effects of resistance mutations on the fitness of *M. tuberculosis* are crucial to epidemiological predictions of the spread of MDR isolates (50). This concept has been further refined by recent evidence from mathematical models which suggests that, provided the relative fitness of an MDR strain remains above a defined threshold, a subpopulation of the low-fitness MDR strain will outcompete both the drug-sensitive strains and other, less fit MDR strains when confronted by a functioning TB control program (19, 49). As a result, the distribution of fitness (49) among circulating *M. tuberculosis* strains might be considered a more accurate predictive measure of resistance emergence. This, in turn, has led to the proposal that DOTS regimens be supplemented with anti-MDR strategies to limit resistance amplification, as well as further transmission of MDR strains (19, 49).

Coincident MDR TB prevalences and HIV infection rates (252) add a further degree of complexity and are suggestive of a positive correlation between MDR and HIV seropositivity. Although the identification of HIV as an independent risk factor for MDR TB is contentious (251), characteristic features of HIV/TB-associated clinical disease might favor the

emergence of resistance. It has been suggested, for example, that disease outcomes in the treated TB patient are determined by a combination of host defense mechanisms and antimicrobial activity (98). That is, a functional immune system might be required to potentiate drug activity, thus preventing the evolution of resistance. Although direct evidence is scant, the enhanced sterilizing activity of PZA *in vivo*, in contrast with its poor activity *in vitro* (257), is suggestive of a synergistic interplay between drug and host (236).

The large bacterial populations associated with *M. tuberculosis*-infected immunocompromised individuals might provide an expanded subset for selection and transmission of rare mutation events. Furthermore, it has been suggested that the absence of a functioning immune response in those individuals might exacerbate the conditions implicated in the exposure of bacteria to monotherapy in immunocompetent patients (93); for example, uncontrolled replication and dissemination could produce drug-inaccessible compartments, while drug absorption might be compromised by other HIV-associated chronic infections. It has also been proposed that drug-resistant strains of reduced fitness might undergo compensatory adaptation during passage through a population of immunocompromised individuals, ultimately restoring their capacity to infect immunocompetent hosts (93). In general, it seems likely that increased TB incidence rates associated with high HIV prevalence will facilitate the spread of both susceptible and MDR strains (55); while slower to emerge in immunocompetent individuals, MDR strains could result in huge burdens of disease in the future (93). However, there is evidence to suggest that the impact of HIV on TB transmission (and therefore prevalence) is more complex and might depend on factors such as the duration of infectious period and the presence of a functioning TB control program (56, 57).

Mutation Rates and the Role of Mutators

Based on *in vitro* measures of rates of mutation to single drug resistance in *M. tuberculosis* (64), the emergence of MDR TB appears to require a larger bacillary population than is usually present during infection. However, the assumption that the risk of acquiring multiple resistance equals the product of individual mutation rates is likely an oversimplification, considering the complex interplay of factors that might operate to increase mutation rates *in vivo*. Small subpopulations of mutators characterize commensal and pathogenic bacterial populations *in vivo* (18, 67, 154, 180, 221, 232), consistent with the idea that elevated mutation rates may promote adaptation to the fluctuating host environment. However, the deleterious consequences of a constitutive mutator phenotype (20) ensure that maintenance of the mutator allele depends on genetic linkage to the resultant beneficial mutation (20, 232). In general, the selective advantage of a high mutation rate is transient, and regaining the wild-type genotype is essential to the long-term survival of the population (94, 95).

Stable, acquisitive evolution is thought to depend on minimal disturbances to established bacterial pathways and host-pathogen interactions (253), a concept consistent with the suggested adaptation of separate *M. tuberculosis* lineages to particular host populations (111). Instead, the long-term selection or counterselection of small-effect mutators is thought

likely to exert greater influence on bacterial evolution (221), perhaps explaining the failure to observe a mutator phenotype in *M. tuberculosis*, despite its natural deficiency in several pathways associated with hypermutability in other organisms (162). A possible exception is provided by the W-Beijing genotype, which is most frequently associated with emergence of MDR TB (96). A high proportion of W-Beijing isolates contain mutations in genes required for elimination of damaged nucleotides (*mutT*) and reversal of alkylation damage to DNA (*ogt*) (193). *In vitro* assays of mutation rates have so far failed to demonstrate increased mutagenesis in W-Beijing isolates (246), although it is possible that the strains tested did not carry the characteristic "mutator" mutations. Furthermore, the prevalence of the W-Beijing lineage among MDR strains makes it tempting to speculate that the mutator phenotype might be manifest only under (stressful) *in vivo* conditions.

Inducible (environment-dependent) mutators, in contrast, increase global mutation rates specifically in response to applied stress (158). Those cells that survive produce progeny cells with normal mutation rates, thereby reducing the risk of unchecked mutagenesis. Whereas the acquisition of a mutator phenotype is a random event, it has been proposed that inducible mutagenesis is an adaptive response that has evolved by second-order selection to modulate mutation rates while limiting the costs associated with a constitutive mutator phenotype (158, 225, 226). That inducible mutator mechanisms are subject to selection has been inferred from the negative correlation between stress-induced mutagenesis and constitutive mutators (17). This idea is further reinforced by the observation that where stresses are frequent or of long duration, inducible mutators are selected as efficiently as mutator alleles (17). The variation in the strength, frequency, and nature of inducible mutagenesis mechanisms is thought to be reflective of the dynamic response of different pathogens to specific local environments (225).

The role of stationary-phase or stress-induced mutagenesis in bacterial adaptation has been subject to considerable recent attention (225). In particular, an association between inducible mutation pathways and the emergence of drug-resistant isolates of pathogenic bacteria has been described (4, 18, 189, 197), which might be especially relevant to the generation of antibiotic and stress resistance mutations in *M. tuberculosis*, whose microevolution within the host environment (as stated above) is driven by genetic rearrangement and point mutations (105, 195). In most bacterial systems studied to date, adaptation to environmental stress is predicated on the activity of SOS-inducible, error-prone repair polymerases of the Y polymerase superfamily (157, 220, 224, 255). Members of the Y family of DNA polymerases likely evolved to promote mutation avoidance and damage tolerance through a specialized ability to replicate across a variety of DNA lesions; however, the flip side of this ability is that the very properties enabling translesion synthesis are implicated in mutagenesis (88). The *M. tuberculosis* genome encodes two putative Y family polymerases of the DinB subclass (178), but, unusually, neither is upregulated in response to DNA damage (23, 66). Instead, their predicted physiological roles are fulfilled in *M. tuberculosis* by a novel, damage-inducible C family polymerase, DnaE2, which is solely responsible for damage-induced base substitution mutagenesis (23). Significantly, deletion of *dnaE2*

results in damage hypersensitivity and eliminates damage-induced base mutagenesis in vitro and is associated with late-stage attenuation as well as reduced emergence of drug resistance mutations in a murine infection model (23).

Coupled with the induction of *dnaE2* during stationary-phase infection in mice (23), these observations suggest that genetically encoded antibiotic resistance mutations may arise as the result of DnaE2-mediated repair synthesis during persistent infection. According to this hypothesis, a range of host immune effectors and other environmental damaging agents, as well as endogenous oxidative and nitrosative metabolic stresses or antibiotics, might induce damage lesions. Stalled replication at a lesion induces *dnaE2* expression either as part of the mycobacterial SOS response or by unknown regulatory mechanisms analogous to novobiocin-mediated *dnaE2* induction (25). Error-prone repair synthesis by DnaE2 might fix mutations in chromosomal DNA at the site of the damage, in some cases conferring antibiotic resistance which is then selected.

INTRINSIC DRUG RESISTANCE

M. tuberculosis is intrinsically resistant to many of the antibiotics and chemotherapeutics in current medical use (117). This intrinsic resistance is, at least in part, because of the relative impermeability of the mycolic acid-rich mycobacterial cell envelope (27). The contribution of each of the various classes of mycolic acids to cell wall composition has been shown to vary depending on growth phase and oxygen tension and differs for in vitro versus intraphagosomal survival (13). In addition, adaptation to the stationary phase is characterized by a thickening of the mycobacterial cell wall (59), which might be especially relevant to antimicrobial tolerance in vivo. However, limited permeability accounts to only a certain degree for the inherent antibiotic resistance of mycobacteria (117), and mycobacteria possess several general mechanisms which might limit the intracellular accumulation, or interfere with the activity, of those antimycobacterial compounds that are able to penetrate the cell wall. For example, a recently identified mycobacterial protein, MfpA, has been implicated in low-level fluoroquinolone resistance (108). Although the precise physiological role of MfpA is not known, its structure resembles that of double-stranded DNA, thus enabling interference in fluoroquinolone drug action by binding to the cellular target, DNA gyrase.

The concentration achieved by antibiotics inside bacterial cells is a function of the number of efflux systems capable of extruding toxic compounds (175). Although hydrophilic diffusion in *M. tuberculosis* is constrained by the limited number and restrictive structure of mycobacterial porins (79, 228), mycobacteria contain a large number of putative drug transporters (138). Significantly, recent evidence suggests a role for active efflux in diminished intracellular drug concentrations (190). Expression of the *efpA*-encoded drug transport homologue is induced in INH-treated *M. tuberculosis* (248), for example, while exposure of *M. tuberculosis* to either INH or ethambutol results in upregulation of *iniA*, encoding a transmembrane protein implicated in tolerance to both frontline anti-TB drugs through an MDR pump-like mechanism (52). Overexpression of certain transport genes has similarly been shown to confer

low-level resistance to specific antimicrobials (2, 44, 71, 144, 214, 222). Conversely, targeted disruption of specific efflux pump genes is associated with increased antibiotic susceptibility (139). Finally, the demonstration that disruptions in efflux gene regulation markedly increase resistance to a variety of drugs is suggestive of a potential evolutionary mechanism for the development of multidrug resistance in pathogenic mycobacteria (139). Indirect support for this contention might be provided by the recent identification of the WhiB7-regulated multidrug resistance system in *M. tuberculosis* (164).

PHENOTYPIC DRUG TOLERANCE

Specialized Persister Cells

The ability of bacterial populations to resist killing by bactericidal factors has been the subject of renewed interest recently, particularly because of the potential implications for drug tolerance (9, 127, 160). Most in vitro antibiotic kill curves are biphasic, comprising early exponential decay followed by bacteriostasis and reduced or delayed bactericidal activity (36, 102, 250). The surviving subpopulation comprises phenotypically tolerant bacteria, or persisters, that can be enriched by prolonged antibiotic exposure (250). In contrast to the case for heritable resistance, however, persisters retain genetic susceptibility to the drug.

Although primarily associated with biofilm or stationary-phase populations (28, 137, 216), persisters were originally observed in populations of rapidly growing planktonic bacteria (15). In fact, separate persister classes have been classified (9) according to the requirement for stationary-phase passage, with type I persisters emerging slowly during stationary-phase exit and type II persisters arising spontaneously in growing populations as a result of a reversible metabolic switch. Of course, because it is not associated with a long-term fitness cost, reversible antibiotic tolerance confers a selective advantage; that is, phenotypic heterogeneity offers a genetically homogeneous population an "insurance policy" (130) against elimination by antibiotics or other stresses.

Mathematical modeling suggests that the optimal switching rate between normal and type II persister cells is a function of the frequency of environmental changes, implying that bacterial persistence mechanisms constitute an adaptation to the distribution of environmental change (130). There is some evidence, however, that the switch to a persistent state might be preferentially induced by certain classes of antibiotics (152, 160), in some cases involving the SOS response (160). Furthermore, the switch to a persistent phenotype might be favored where bactericidal antibiotics are ineffective against slowly dividing or nondividing cells (9, 160), a characteristic of current frontline antituberculosis drugs.

The Stringent Response, Toxin-Antitoxin Loci, and Applicability to Phenotypic Drug Tolerance in *M. tuberculosis*

The mechanisms governing the switch to a persistent phenotype remain to be identified; however, accumulating evidence implicates the activity of the prokaryotic stringent response regulator, RelA (100, 230). RelA catalyzes the

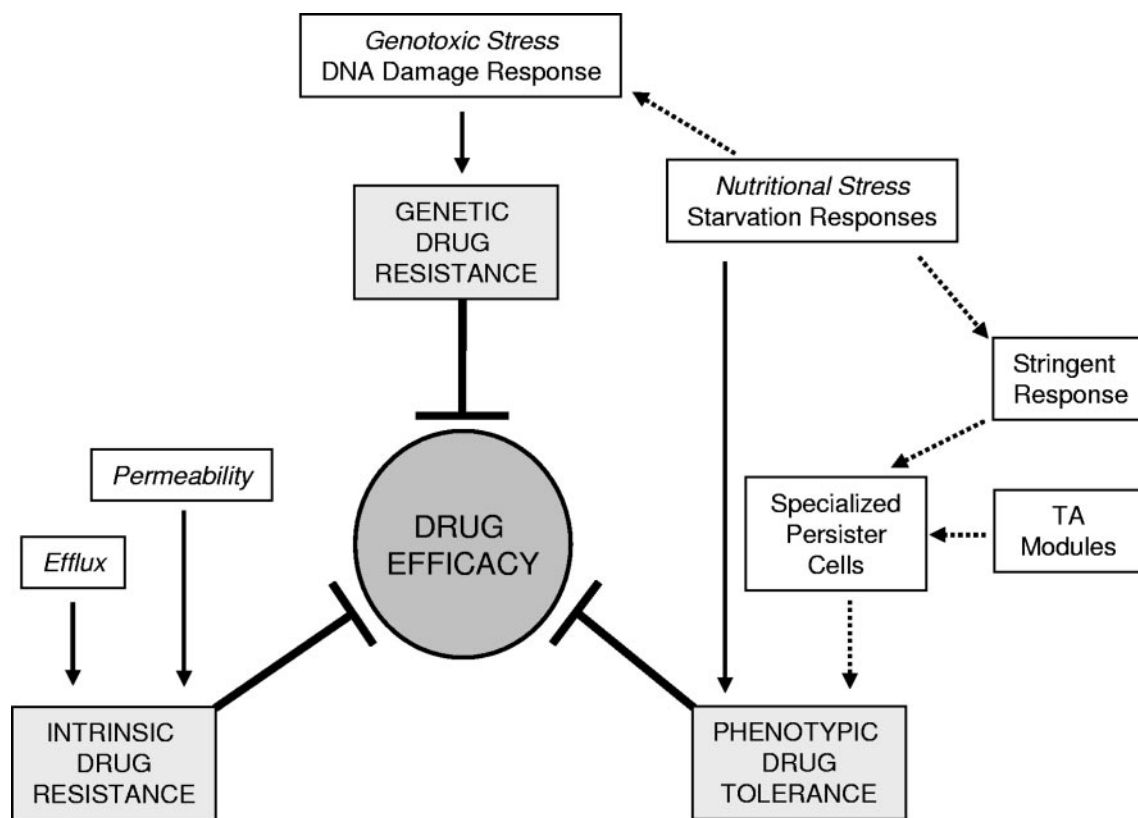


FIG. 1. Mechanisms and pathways limiting drug efficacy in *M. tuberculosis*. Solid lines represent known interactions; broken lines represent those assumed or postulated.

hyperphosphorylation of GTP to (p)ppGpp during amino acid and carbon source starvation (37). Binding of the (p)ppGpp alarmone to the RNA polymerase β subunit inhibits transcription of translation machinery components, stimulates amino acid biosynthesis and transport operons, and decreases transcription rates (8, 12, 37, 42, 237). In addition, (p)ppGpp affects the global transcriptional response to changing environmental conditions by mediating association of alternative σ factors with RNA polymerase (121, 132). Given the similarities between persistent and stringent physiology, the requirement for RelA is not surprising. However, the identification of a high proportion of toxin components of toxin-antitoxin (TA) modules among those genes induced in a persister population (127) suggests that RelA, through the activity of (p)ppGpp, might not constitute the sole mediator of persistence.

TA modules were originally characterized as factors ensuring episomal stability by postsegregational killing of cured segregants (91, 116). However, subsequent analysis has revealed the widespread distribution of chromosomally encoded TA modules in prokaryotic genomes (184). The stable toxins inhibit transcription and translation by various mechanisms, including inhibition of DNA gyrase activity (118) and cleavage of mRNA (45–47, 187, 258) and require neutralization by labile antitoxins. Compelling evidence of the potential role of TA-like modules in persistence is provided by the recent characterization of the *Escherichia coli* *hipA7* allele (129), originally identified in a screen for high-persistence mutants (165). Significantly, abrogation of (p)ppGpp synthetase activity in the

hipA7 mutant strain eliminates the high-persistence phenotype (129), implying the requirement for a functional stringent response in the persister state. These observations have been incorporated in a general model of stress physiology (92) in which the (p)ppGpp-mediated stringent response, in combination with TA activity, effects a regulated switch to a persistent phenotype.

The failure of frontline drugs to sterilize *M. tuberculosis* infections in vivo (156) is reminiscent of incomplete killing in vitro (110) and suggests that a switch to persistent physiology might hinder anti-TB drug efficacy. Significantly, resistance to frontline antimicrobial compounds emerges during long-term in vitro adaptation of *M. tuberculosis* (110, 238) and mimics resistance in bacilli recovered from tuberculous lesions in vivo (241, 243). Similarly, starvation of *M. tuberculosis* in vitro has recently been shown to increase tolerance of a wide range of antimycobacterial compounds (254). The stringent response in *M. tuberculosis* mediated by the *M. tuberculosis* Rel protein has been the subject of detailed investigation and has been shown to be required for survival of *M. tuberculosis* during long-term starvation in vitro (191) and for persistence in mice (60, 123). *M. tuberculosis* also contains an unusually large number of putative TA loci in its genome (7, 184). Although most of these TA loci have yet to be investigated, sequence-specific mRNA interferase activity has recently been demonstrated in two *M. tuberculosis* MazF homologues (261). That study further showed that four of the putative *M. tuberculosis* MazF proteins cause cell growth arrest when overexpressed in *E. coli* (261).

Coupled with their abundance in *M. tuberculosis*, as well as the functionality and physiological importance of the stringent response regulator, these observations are strongly suggestive of possible involvement of the TA genes in phenotypic drug tolerance in *M. tuberculosis*.

CONCLUDING REMARKS

The efficacy of TB chemotherapy is a function of drug activity against potentially heterogeneous populations of *M. tuberculosis* in disparate anatomical loci within the host, as well as the ability of the organism to subvert that activity. The current frontline drugs are a modern intervention, however, and *M. tuberculosis* has evolved mechanisms over thousands of years (29) to enable survival in the variable and hostile in vivo environment. In this review, it has been proposed that the same mechanisms might affect drug efficacy (Fig. 1). Specifically, inherent characteristics such as cell wall physiology and active efflux of toxic metabolites, persistence mechanisms such as (p)ppGpp- and TA-mediated translational inhibition, and adaptive mechanisms such as induced mutagenesis are potentially implicated in impaired antibiotic activity or the emergence of chromosomally encoded resistance mutations. Furthermore, although this review has categorized separate tolerance or resistance mechanisms, it is likely that a complex interaction governs survival in vivo; for example, phenotypic tolerance likely enables the emergence of antibiotic-resistant mutants by ensuring that a subpopulation survives the extended course of chemotherapy. Finally, key assumptions require further investigation. For example, there is limited information on the ability of the different anti-TB drugs to penetrate lesions in vivo, while the contribution of *M. tuberculosis* Rel and the TA modules to drug tolerance has emerged as an important area for future study.

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