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REVIEWS

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Molecular Mechanisms of Drug Tolerance in *Mycobacterium tuberculosis*

A. V. Antonova[a], D. A. Gryadunov[a], and D. V. Zimenkov[a, *]

[a] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, 119991 Russia*

**e-mail: z@biochip.ru*

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Abstract—A dramatic increase in drug-resistant forms of tuberculosis (TB) stimulates a search for novel anti-TB drugs and studies of the drug resistance acquisition. One of the possible causes is a phenotypic resistance or drug tolerance which is not associated with genomic changes. The majority of anti-TB drugs eliminate 99% of MTB cells in 3–5 days, but the remaining subpopulation becomes unsusceptible to treatment and capable for long-term persistence with ability to resuscitate once the external adverse factor is removed. This evasion of the stress factor facilitates selection of resistant forms, thus warranting long-term treatment with at least four antibacterial drugs in TB. The review considers the main mechanisms of bacterial tolerance that are due to alterations in the cell wall, activation of efflux pumps, induction of transcriptional regulons, changes in metabolic flows, and modification of molecular machineries.

Keywords: Mycobacterium tuberculosis, regulation of gene expression, phenotypic resistance, drug tolerance, efflux pumps

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INTRODUCTION

Tuberculosis (TB) was a fatal disease and caused 1.5 million deaths annually until antibiotics became available. The discovery of streptomycin made it possible to start chemotherapy for TB. Combined treatment with potent anti-TB drugs (streptomycin, isoniazid, and rifampicin) came to be used soon afterwards, greatly reducing the TB incidence. Notwithstanding, the disease came back because multiple drug resistance had developed in certain *Mycobacterium tuberculosis* (MBT) strains. Standard therapy for drug-sensitive TB includes taking at least four drugs for 6 months. The treatment duration was empirically estimated at 18–24 months for resistant TB [1].

First resistant MTB strains were resistant to only one drug, streptomycin. However, a step-wise accumulation of resistance-associated mutations has yielded strains that express multiple drug resistance (MDR) or extensive drug resistance (EDR), and totally drug resistant strains have recently emerged and are virtually incurable because of limited number of new antibiotics.

EXPERIMENTAL OBSERVATIONS OF TOLERANCE

A definition of drug tolerance is based on several experimental observations, and the main of them is a biphasic nature of mortality curve of a microbial population exposed to a bactericidal agent. A major part of the bacterial population is rapidly eliminated in the first phase, which is followed by a long-term persistence phase with the surviving cell number remaining almost unchanged [2]. Surviving cells start growing exponentially as soon as the antibacterial agent has ceased to act and rapidly restore the population, which still remains sensitive to the drug. The effect was initially described in streptococci and later observed in various microorganisms exposed to antibacterial drugs of various classes; in particular, it was observed in MBT both in vitro and in vivo [3]. It is of interest to note that a similarly biphasic survival curve was described for the effect of cytotoxic drugs on cancer cells [4].

Similar curves were obtained for the microbial survival as a function of the drug concentration; i.e., the surviving cell amount stops decreasing once a certain threshold has been exceeded and reaches a plateau or even starts increasing [5]. This paradoxical phenomenon of tolerance to high concentrations, which is known as the Eagle effect after the name of its discoverer [6], was initially described for the effect of penicillin on various bacteria. The universal character of the effect was confirmed more recently, in particular, in mycobacteria [7].

A low (approximately 10^{-6}) frequency of tolerant cells in a growing culture made it difficult to study the phenomenon. However, high persistence (*hip*) mutants

were identified in *Escherichia coli* in the early 1980; their titer remained high, 10^{-2}–10^{-3}, in the second phase in survival curves obtained with various antibiotics [8], while the minimal inhibitory concentration (MIC) remained the same. More recent studies associated the *hipA7* mutation with the toxin–antitoxin (TA) module, which is responsible for the formation of a slowly proliferating, resistant cell fraction [9].

CONCEPTS OF TOLERANCE AND PERSISTENCE

When discussing bacterial tolerance of antibacterial drugs, tolerance due to changes in the transcription profile of drug resistance genes is essential to distinguish from the existence of the so-called dormant cell subpopulation, which is characterized by low metabolic activity and a potential for long-term persistence. Dormant cells are capable of starting intense growth once the external adverse factor is removed. A minor part of the cell population occurs originally in the dormant form as a result of stochastic differences in expression of regulatory genes and asymmetric divisions, while a fraction of metabolically active cells evade stress exposure and enter dormancy [10]. It should be noted that the term dormancy yields to the term persistence because tolerant persistent cells are not absolutely dormant.

Persistent forms are most likely responsible for chronic infections and disease relapses. Resistant forms of pathogens are selected during therapy owing to persistence, which leads to antibiotic tolerance [11]. MBT tolerance is a cause of why TB needs long-term treatment with several drugs. In addition, the facts that TB is difficult to cure and that resistance develops are partly related to a genetic heterogeneity of the pathogen population in a host; the heterogeneity is due to occasional mutations, which confer resistance to a particular drug on a minor cell fraction before therapy starts, provided that the population is large enough.

Genetically determined tolerance is a particular phenotypic resistance type and is characterized by an unchanged MIC and an elevated cell survival at higher concentrations of an antibacterial agent. An example is provided by the cells that display changes in growth parameters and are selected via cyclic passages of an *E. coli* culture in antibiotic-containing and antibiotic-free media (a model of daily medication). Phenotypically, the cells display an extended lag time and an unchanged MIC [12]. Tolerance precedes and substantially facilitates the development of resistant forms [13]. An extended lag time is not the only mechanism that increases tolerance, and a low proliferation rate of MBT cells is possible to consider as a tolerance-increasing factor [14]. The *hip E. coli* mutants can also be classed as selected forms with genetically increased tolerance due to a higher fraction of ampicillin-resistant persisters. However, attempts of one-step selection of mutants with a lower persister fraction were unsuccessful, and only consecutive deletion of at least five TA modules appreciably decreased the persister titer, suggesting multiplicity of persisting subpopulations [15].

A set of mutations was identified to affect the development of MBT persisters in vitro and in a mouse model [16]. The results showed the roles of phthiocerol dimycocerosate (PDIM) and phospholipid biosyntheses, glycerol metabolism, glyoxylate shunt, and microaerobic cytochrome *bd*; mutations of TA modules were detected. An important observation was made; i.e., mutations responsible for adaptation to continuous exposure to isoniazid can adversely affect the fitness in vitro in carrier strains so that the strains are less efficiently detected via laboratory testing [16].

EXPERIMENTAL STUDIES OF TOLERANCE

Resistance is quantitatively characterized using the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) [17]. Antibacterial agents are classified into bactericidal and bacteriostatic according to the MBC/MIC ratio, but the classification is questionable because the MBC depends on the exposure duration, drug concentration, cell count, strain growth rate, and microbial species [18]. MIC assays are less laborious and less subjective and are broadly used in research and medicine. A survival curve analysis is thought to provide a gold standard in evaluating tolerance and persistence and consists in culturing bacteria for a long period of time and plating the culture in a serial manner to obtain colony-forming unit counts [19]. A method to estimate the minimum duration for killing (MDK) was proposed recently [20].

Various experimental stress models are used to evaluate persistence and drug tolerance and to search for new antibacterial agents that affect dormant forms. In particular, cells are cultured in buffered saline in the absence of nutrients (Loebel model); deprived of oxygen (Wayne model), iron, magnesium, amino acids, or vitamins; or exposed to copper, reactive oxygen species, nitrogen monoxide (NO), carbon monoxide (CO), or sublethal concentrations of antibacterial agents [21]. All these models facilitate the cell transition to a low-active dormant form capable of long-term persistence.

Nutrient deficiency, which is modeled by incubating cells in a phosphate buffer, induces tolerance of the majority of anti-TB drugs, including rifampicin, isoniazid, streptomycin, moxifloxacin, ethambutol, bedaquiline, PA-824, clofazimine, thioridazine, and even a combination of four agents [22]. Elevated MICs of anti-TB drugs in ex vivo model hypoxic conditions and a better survival in macrophages were observed for approximately one-third of clinical isolates, but a possible association with the genotype was not analyzed [23].

Transcription studies of cells subjected to various stress factors identified the main regulatory pathways of adaptation, such as expression of the σ^H subunit [24], DosR regulon [25], and enduring hypoxic response regulon [26]. Several limitations of the available methods should be noted; e.g., a correlation between microarray and transcriptome sequencing data was only 0.4–0.5 in spite of the coincidence of upregulated gene sets [24]. Moreover, a transcriptional response to stress does not necessarily cause proportional changes in proteome. In the NO stress model, protein contents did not change within the first 24 h, while transcriptional changes were detectable as early as 20 min of exposure, and certain proteins, especially those with Fe–S clusters, showed a negative correlation between transcription and translation data as a result of targeted protein degradation [27].

Physiology of M. tuberculosis in vivo

Mycobacterium tuberculosis is a facultative intracellular pathogen that causes various, including both pulmonary and extrapulmonary clinical forms of TB. Several stages of granuloma formation are often observed together in the lungs in active TB, and heterogeneity of the MTB surrounding additionally depends on the intracellular persistence stage: primary infection of macrophages, phagosome steps, phagolysosome formation and maturation, exit into the cytoplasm, and apoptosis and autophagy processes [28]. Sputum samples from patients who release bacteria are a main biological material tested in TB, and it is therefore an open question as to whether the results of microbiological, transcriptional, and proteomic MTB testing *ex vivo* correspond to the actual characteristics of cells occurring within the host organism.

Intensely proliferating cells were earlier believed to prevail in the sputum. However, dormant forms were shown to predominate in the sputum in 2008, and lipid inclusion bodies were observed in 3–86% of sputum cells [29]. Transcription profiling of sputum MBT cells revealed complex adaptive changes both prior to and during therapy. Cells showed a decrease in metabolism, translation, DNA replication, and production of cell-wall proteins and induction of genes for oxidative stress proteins, PE/PPE family proteins, alternative σ factors, and several toxin proteins of TA pairs [30]. Upregulation of genes of the DosR regulon, which is associated with the hypoxic response, was observed before therapy and decreased 14 days after the start of therapy. A more intricate expression profile was established for *relA*, which regulates the stringent response to starvation. Its induction became detectable only on the second day and was followed by slow repression [31]. Metabolic adaptations included a switch from aerobic to anaerobic respiration, block of the tricarboxylic acid cycle, and activation of the glyoxylate shunt and methylcitrate cycle [30].

The factors that affect MBT during the pathological process include changed nutrient availability, oxidative stress, and a low pH in phagolysosomes [32]; oxygen starvation was observed in animal granulomas in vivo [33]. Iron deficiency is characteristic of necrotic granulomas and is overcome using siderophores, which are iron-capturing molecules [34]. MBT cells are presumably exposed to NO, hypoxia, and phosphate deficiency when persisting within the host cell [35].

Phenotypic Heterogeneity

The clinical picture of TB is difficult to understand not only because the pathological process is complex, but also because MBT shows phenotypic heterogeneity, which provides an important nonspecific mechanism of adaptation to various conditions and stress. Heterogeneity is observed at the levels of individual molecules and cells even when environmental conditions are homogeneous, as is the case with in vitro cultures [10]. A variety of persister populations was detected in both exponential cultures and sputum samples from patients in experiments with fluorescent phages and showed dynamic changes upon isoniazid treatment [10]. Heterogeneity formed a certain period after infection in a macrophagal model [36].

The presence of MBT subpopulations in clinical samples was confirmed in cytological studies, reflecting heterogeneity of MBT growth in vivo [29]. A large percentage of viable but nonculturable MBT cells was observed in a mouse model of chronic infection and sputum samples from most patients [37]. Such cells are capable of growing in a liquid medium, but do not produce colonies on agar media, leading to false negative results in TB diagnosis.

The supernatant collected from a MBT strain H37Rv culture in the early stationary phase contains an acid-labile and heat-stable resuscitation factor and was found to increase the cell viability in aged cultures and to allow small inocula to initiate growth in liquid cultures [38]. Resuscitation-promoting factors (Rpf proteins) were the first proteins associated with reactivation of chronic infection. Five Rpf-coding genes (*rpfA–rpfE*) are contained in the MBT genome. The Rpf proteins stimulate growth restoration in nonreplicating cells in vitro and facilitate the bacterial survival in mouse models in vivo [39]. However, cell reactivation depends on other factors as well, suggesting multiplicity of persister cell fractions, the profile of which is shaped by both the host immune system and the genotype of the pathogen [40].

Phenotypic heterogeneity of a monogenic population is partly due to stochastic fluctuations in transcription of the genes for key regulatory factors. There is only a minor number of regulatory proteins and corresponding operator sites in an individual cell, and gene expression is consequently affected by stochastic

processes, which were earlier believed to average out and thus make no contribution to the phenotype [41]. Expression of a transcription factor subject to positive autoregulation provides the simplest system where stochastic noise is amplified. Two metastable states of transcriptional repression and derepression are possible in this case, and the system can switch at random between them [42], leading to the formation of two phenotypically different subpopulations in an isogenic bacterial population. Living cells were observed to possess more complex regulatory networks with autoregulation, mutual regulation, and hierarchic regulation of various transcription factors at posttranscriptional, translational, and posttranslational levels.

In particular, a bimodal character of transcription of the *hipA* toxin gene is determined by transcription noise and leads to differences in growth rage in an *E. coli* cell population and ampicillin tolerance of slow-growing cells, as was demonstrated in the *hipA7 E. coli* mutants [9]. However, the replication rate alone did not affect the MBT tolerance at least with isoniazid, pyrazinamide, and ethambutol [43].

The role of stochastic processes in the development of MBT tolerance was confirmed in studies of isoniazid tolerance; i.e., the MBT survival on exposure to isoniazid was associated with temporal stochastic increases in the concentration of KatG catalase-peroxidase, which converts isoniazid to an active form [44]. Periods of a low KatG content in the cell occur as a result of KatG instability, allowing metabolically active proliferating cells to appear in the presence of isoniazid.

Phenotypic heterogeneity is seen at the macromolecular level as well. A characteristic example is provided by modification of part of the ribosomes in response to the MazF toxin, as considered in detail below. A contribution to heterogeneity is additionally made by mistranslation, or ambiguous decoding, which is a mechanism of genetic code flexibility and results in that newly synthesized polypeptide chains have amino acid substitutions due to mistakes made by the ribosome or aminoacyl-tRNA synthetases [45]. The mistake rate is inconstant and may increase to several percent when cells grow at a low pH or are in the stationary phase [46]. Experiments with *M. smegmatis* showed that mistranslation plays a role in rifampicin tolerance [46], which arises because amino acid substitutions in a fraction of newly synthesized RNA polymerases make the enzymes resistant to rifampicin action.

General phenotypic flexibility due to continuously changing heterogeneity allows cells to withstand a great variety of stress factors without possessing specifically induced adaptive mechanisms, which require continuous energy-spending expression of a receptor and receptor-inducible operon responsible for the stress response [44].

It is thought that approximately two milliard people are currently infected with MBT in the global population, but infection is latent and lacks clinical manifestation in the majority of infected patients. The TB pathogen is capable of asymptomatic persistence in the host body in a dormant form, which is controlled by the immune system. The main diagnostic tests for latent TB are an immunological test for specific MBT antigens (tuberculin test) and a more recent test for interferon γ production. It became clear with new diagnostic tests that latent MBT infection has a range of different forms, which further contribute to heterogeneity of external conditions at which MBT persists in vivo [47].

MECHANISMS OF DRUG TOLERANCE

Metabolic Adaptation

The MBT division time increases to 100 h or even greater values in response to a stress *in vivo* or in animal models of chronic infection [48]. Growth arrest is an adaptive strategy that helps bacteria to survive in stress, as was demonstrated experimentally by identifying the mutant strains that replicate in hypoxia [49]. Bacteria with mutations of the *dosR* transcriptional regulator gene and *tgs1* triglyceride synthase gene showed higher titers in a 6-week hypoxic culture [49]. DosR-dependent transcriptional activation of *tgs1* is responsible for a competitive accumulation of triglyceride stores and a redistribution of metabolic fluxes to reduce the flux through the tricarboxylic acid cycle. Mutations were additionally detected in other genes, including those involved in syntheses of triglycerides, pyruvate dehydrogenase, succinate dehydrogenase, phosphor hexokinase, and transcriptional regulators. An analysis of the *tgs1* mutant and a strain overexpressing citrate synthase, which is involved in the tricarboxylic acid cycle, made it possible to assume that a redistribution of acetyl-CoA to triglyceride synthesis affects the growth rate and tolerance to various antibiotics [49]. To provide independent support, cells were observed to store carbon and nitrogen in the form of triglycerides when their growth was arrested [50].

Several experiments showed that fatty acids and cholesterol play an important role in MBT cells during the pathological process [51]. In fact, MBT is capable of utilizing fatty acids as a carbon and energy source. Acetyl-CoA resulting from fatty acid β-oxidation is primarily involved in glyoxylate shunt reactions, which trigger subsequent gluconeogenesis, while NADH equivalents provide for ATP generation via oxidative phosphorylation in the electron transport chain. The MBT genome harbors more than 100 duplicated genes for enzymes involved in the five steps of the fatty acid β-oxidation cycle, thus being capable of catabolizing a broad range of fatty acids; the fact indicates that lipids are an important source of carbon and energy for MBT [52].

Actinobacterial lipid bodies, which consist of triglycerides and waxes, are analogs of eukaryotic lipid bodies [53]. In hypoxia, macrophages accumulate triglycerides, which serve as a carbon and energy source for intracellular MBT; in addition, cholesterol and fatty acids are abundant in the intercelular space of a granuloma [54]. Inclusion bodies are found in a substantial fraction of MBT cells in sputum samples from patients [29]. Cells with inclusion bodies have higher tolerance to rifampicin, isoniazid, ethambutol, and ciprofloxacin [55], and their presence in the sputum is associated with a poor treatment prognosis. Cultures of MBT cells containing lipid inclusions were found to form in vitro in phosphate depletion and multistress models [56].

A *mce4* (mammalian cell entry) gene cluster was detected in MBT and shown to play an important role in cell survival upon chronic infection in mice. The *mce4* gene codes for a cholesterol import system, which allows MBT to extract both carbon and energy from cholesterol contained in host cell membranes [57]. Cholesterol utilization is coordinated with fatty acid utilization via the Rv3723/LucA regulatory protein [58].

The glyoxylate shunt of the tricarboxylic acid cycle is essential for maintaining the slow-growing cell state. Apart from fatty acid catabolism, maintaining the redox balance in the cell is also a role of the glyoxylate cycle [59, 60]. MBT treatment with isoniazid, rifampicin, and streptomycin induces *icl1* and *icl2,* which code for isocitrate lyase, which catalyzes the first step of the cycle; and *icl* mutants have a higher sensitivity to the above drugs [61]. Both of the isoforms (Icl1 and Icl2) additionally possess 2-methylcitrate lyase activity, thus coupling the clyoxylate and methylcitrate cycles, and this coupling is essential for balanced fatty acid utilization without accumulating toxic propionyl-CoA [62]. Cells with mutations of malate synthase (GlcB), which is the second enzyme of the glyoxylate cycle, are more sensitive to oxidative stress, nitrogen stress, and rifampicin and have a lower potential to form biofilms, to pass into a persisting state, and to survive in macrophages [63].

In an oxygen-deficient environment, MBT cells are capable of switching to respiration using nitrate as a terminal electron acceptor [64]. Secretion of succinate synthesized in the course of fermentation is used by MBT to maintain the membrane potential in the absence of respiration [65]. Remodeling of central metabolism towards succinate synthesis in hypoxia is of importance for maintaining the ATP level and catalyzing anaplerotic reactions and is due to isocitrate lyase and succinate dehydrogenase activities [66]. However, syntheses of citrate and fumarate, which are other compounds of the tricarboxylic acid cycle, increase in MBT cells in iron deficiency, indicating that other mechanisms are used to maintain the membrane potential [67].

Microscopic images of MBT cell sections show not only lipid bodies, but also other organelle-like bodies, including polyphosphate-containing structures [68], which presumably serve to store energy. Polyphosphates and guanosine tetra(penta)phosphates (p)ppGpp act as signal molecules of the stringent control system. The polyphosphate level increases upon a cell transition to the stationary phase and then returns to its norm [69]. As experiments with *E. coli* demonstrated, (p)ppGpp stochastically induces the TA modules, leading to the formation of persister subpopulations [70]. The signaling pathway includes the interaction of intracellular polyphosphates with Lon protease and targeted degradation of antitoxins. Putative positive feedback, which ensures a bistable phenotype, might be due to toxin HipA-dependent phosphorylation of glutamyl-tRNA synthase, which is the event that prevents aminoacylation and enhances the stringent response [71], or due to activation of the MprAB two-component system, which induces *sigE* and *relA* [72]. Studies of strains carrying mutations of polyphosphate kinases and exopolyphosphatases, which regulate the polyphosphate level, showed that polyphosphate accumulation increased antibiotic tolerance and vice versa [72]. In addition, a polyphosphate-accumulating strain showed changes in metabolism and cell wall and had impaired capability of biofilm formation [72].

Biofilms

Most microbes are capable of growing in biofilms, which is a special form of bacterial existence wherein bacteria are embedded in an extracellular polymeric matrix and undergo differentiation to produce pores and channels. In contrast to the planktonic form, biofilms are far more resistant to various stress factors, including antibiotics. Biofilm formation is thought to provide an important strategy of adaptation in chronic infections [73].

Biofilm-like structures were observed for MBT upon its culturing in vitro. In detergent-free cultures, MBT forms special structures at the air–medium interface. The structures are known as pellicles and contain an extracellular matrix, which holds cells together [74]. Bacteria have a lower phenotypic sensitivity to isoniazid and rifampicin in pellicles; i.e., approximately 10% of cells survive exposure to the drugs used at concentrations several hundreds of times higher than the respective MIC [75].

A role in producing the extracellular matrix was confirmed for free mycolic acids, keto-mycolic acids, and lipids synthesized by polyketide synthase Pks1 [76]. Studies with a reductive stress model implicated exopolysaccharides, proteins, and DNA, which are components of biofilms formed by other bacteria [77]. MBT cell treatment with dithiothreitol was shown to cause biofilm formation within a short period of time, 29 h [78]. Like in pellicles, cells living in these biofilms are tolerant to various anti-TB agents.

Several genetic determinants of MBT biofilms were identified; their regulation and association with quorum sensing were described [79]. However, the question of whether MBT forms true biofilms in the human body is still open [78].

Role of the Cell Wall

The impermeable MBT cell wall functions to provide an efficient barrier to antibiotic penetration. Although MBT is a Gram-positive bacterium, its cell wall consists of many layers, is thick, and varies in hydrophobicity. The between-layer space is similar to the cell-wall periplasm of Gram-negative bacteria. A peptidoglycan layer is linked with an arabidogalactan one in the cell wall, and the two layers together form a hydrophilic barrier, which prevents penetration of hydrophobic molecules. The two layers are covalently linked with an outer mycolic acid layer; long fatty acid chains of the layer form a wax barrier, which prevents penetration of both hydrophobic and hydrophilic molecules. For instance, β-lactam diffusion through the cell wall in MBT is several hundreds of times slower than in *E. coli* [80].

Crosslinks between residues 4 and 3 mostly arise in peptidoglycan molecules during the exponential growth, while 3 → 3 crosslinks come to predominate upon a transition to the stationary growth phase [81]. Inactivation of L,D-transpeptidase Rv2518c, which is responsible for 3 → 3 crosslinking, increases the cell sensitivity to amoxicillin and attenuates persistence in animal models [82]. A thickening of the cell wall in hypoxia was observed as early as the first models were obtained [83], and lower permeability of the cell wall is at least partly responsible for tolerance to antibiotics of various classes, as demonstrated with a starvation model [84].

Efflux Pumps

Bacterial efflux pumps are found in all bacteria, and their function of actively transporting various molecules across the cell-wall membranes is related to pathogenesis, homeostasis maintenance, intercellular signaling, biofilm formation, detoxification, and drug resistance, including multiple drug resistance. A total of 267 transporters were predicted to exist in MBT, of which 129 belong to the ATB-binding cassette (ABC) family; 30, to the major facilitator superfamily (MFS); and 14, to the resistance-nodulation-cell division (RND) family [85]. Approximately 40 transporters are involved in excreting various antibacterial agents, thus providing a baseline resistance to wild-type strains. Expression of several transporters in a MBT strain with MDR was found to be higher than in a sensitive isolate [86]. However, the regulatory signaling pathways and the transport specificity range are still poorly understood [87]. More than 20 efflux pumps are upregulated in MBT cells persisting in macrophages, and at least 12 of them are absolutely essential for MBT survival within a macrophage [88].

Tap (Rv1258c) is one of the efflux proteins that is upregulated within macrophages and has been studied most comprehensively. Tap is responsible for a lower MBT sensitivity to tetracyclines, rifampicin, clofazimine, etc., and its WhiB7-dependent regulation plays a role in the development of rifamycin tolerance upon intracellular parasitic persistence [89]. A natural substrate of Rv1258c is still unidentified, as is the case with the majority of efflux pumps. It is possible to assume that antimicrobial peptides (AMPs) are responsible for upregulation of efflux pumps. For instance, macrophage-induced tolerance of *M. marinum* is not suppressed by dexamethasone, which inhibits the majority of macrophagal defense mechanisms except AMP expression. In *Streptococcus pneumoniae*, the AMP LL-37 induces transcription of *mefE*, which codes for a component of a Rv1258c-related efflux pump [90]. Free-living mycobacteria, such as *M. smegmatis*, may utilize the pumps to protect themselves from small molecules, such as lantibiotics and antibiotics produced by their ecological competitors, and to enhance their growth within amebae.

Induced Modification of Drugs and Drug Targets

Pathogenic bacteria are capable of evading the effect of antibiotics by structurally modifying their targets. A typical example of such resistance is provided by MBT resistance to macrolides and lincosamides. MBT and other mycobacteria are naturally resistant to macrolides and lincosamides. These antibiotics inhibit protein synthesis to stop bacterial cell growth. The antibiotics bind reversibly to a certain rRNA site in the 50S subunit of the bacterial ribosome and thus inhibit the peptidyl-tRNA translocation. A BCG strain was found to be sensitive to many macrolides and lincosamides, while its parental *M. bovis* strain and other vaccine strains are antibiotic resistant. Comparative genomic studies associated the macrolide and lincosamine sensitivity of the BCG strain with a deletion of *erm37*, which codes for rRNA methyltransferase [91].

The *erm(37)* gene is in a large chromosome locus known as Region of Difference 2 (RD2), which is absent from the *M. bovis* BCG genome. The enzyme encoded by *erm(37)* changes the ribosome structure by methylating the 23S rRNA in MBT. Experiments with macrolide binding in vitro confirmed that Erm reduces macrolide affinity for ribosomes and thereby suppresses the inhibitory effect of macrolides on protein synthesis. Homologous proteins that facilitate the resistance to macrolides and lincosamides were found in *M. smegmatis* and *M. fortuitum*. Expression of *erm* is upregulated in mycobacteria exposed to macrolides and lincosamides, the effect being possibly mediated by the WhiB7 transcriptional regulator [92].

Direct chemical modification is another mechanism that mycobacteria utilize to inactivate antibiotics. Recent studies identified acetylation as an important factor in acquired resistance to aminoglycosides, which are broad-range antibiotics and exert a bactericidal or bacteriostatic effect depending on their concentration [93]. Intrinsic aminoglycoside resistance of mycobacteria is attributed to various acetyltransferases. The enhanced intracellular survival (Eis) protein was initially identified as a factor of mycobacterial survival within host macrophages. In vitro studies showed that Eis uses acetyl-CoA as an acetyl group donor and acetylates several amino groups in aminoglycosides, thus inactivating antibiotics [94].

REGULATORY MECHANISMS OF TOLERANCE

RNA Polymerase Factors

There are many (13) σ-subunit genes in the MBT genome [95], suggesting a flexible adaptive response to various stress factors. Eleven out of the 13 subunits belong to the extracytoplasmic family (ECF) and are capable of acting as receptors of external influences [96]. Yet the total set of σ subunits is expressed in the log phase, which is thought to be free of stress, and the cell is consequently capable of rapid adaptation via posttranslational regulatory mechanisms based on interactions with anti-σ factors [97]. Induction of alternative σ subunits is observed in various kinds of stress, but only one of them, σ^F, responds to known antibacterial drugs, such as ethambutol, rifampicin, streptomycin, and cycloserine [98]. The σ^F regulon includes genes involved in central metabolism and synthesis of the cell wall, as well as genes for transport proteins and several transcriptional regulators [99].

On transition to a metabolically low-active form, adaptation to new conditions involves all levels of cell organization, including transcription and translation macromolecular machineries. RNA polymerase activity is not totally inhibited in cells exposed to rifampicin in vitro, and the MIC of polymerases isolated from stationary-phase cells is twice as high as that of exponentially growing cell'. A study of the stationary-phase transcription complex identified several RNA polymerase-associated proteins: chaperone GroEL1, DNA polymerase 1, and several transcription factors [100]. Interactions with these proteins, as well as with the replication initiation factor DnaA, protects RNA polymerase from the effect of rifampicin [101].

One of the above factors, RbpA, was initially identified in *Streptomyces coelicolor* as a factor responsible for basal rifampicin resistance and rifampicin tolerance of RNA polymerase [102]. A mycobacterial homolog of RbpA showed the same properties, and introduction of an additional RbpA gene copy and induction of its transcription increased the MIC of rifampicin in *M. smegmatis* cells [103]. RbpA interacts with the β subunit and protects polymerases from rifampicin-dependent inhibition. In addition, RbpA binds with the major σ subunit σ^A and stress-inducible σ^B, thus stabilizing the transcription initiation complex and stimulating DNA melting and a transition of the initiation complex into an open form [104]. Transcription of *rbpA* is controlled by σ^E and is activated in oxidative stress [105]. The interaction between σ^A and RbpA is essential for the oxidative stress response. While the exact mechanism and the range of activated genes remain obscure, RbpA is known to activate transcription of the *furA–katG* operon in response to hydrogen peroxide [105]. In stress, the autoregulated, hydrogen peroxide-sensitive, iron uptake-controlling transcription factor FurA additionally activates transcription of the downstream gene for catalase-peroxidase, which is responsible for peroxide inactivation and isoniazid conversion into an active form.

Toxin–Antitoxin System

The TA system was the first mechanism implicated in bacterial drug tolerance. A TA module includes a pair of cotranscribed genes, which code for a toxic protein, whose activity or expression level depends on the protein or RNA that act as an antitoxin. The antitoxin is unstable and responds to various stress factors to allow the toxin to exert its activity. In MBT, AT modules are induced in response to temperature shock, hypoxia, DNA damage, starvation, intramacrophage conditions, and antibiotics [106].

Several hypotheses have been advanced to explain the role that TA modules play in the physiology of MBT. The TA modules were assumed to play a role in programed death of part of the population, protection from foreign DNA, and maintenance of genomic DNA stability. A major hypothesis is based on several findings and suggests that the TA elements convert part of the population into a dormant form to avoid the effect of stress [107].

The majority of protein toxins possess endoribonuclease activity [108] and recognize specific sequences in mRNAs, rRNAs, or tRNAs [109]. The MBT genome harbors more than 80 putative TA modules, far more than the genome of any other intracellular pathogen. This multiplicity of TA modules allows MBT to adapt to various stress factors during infection, facilitating long-term persistence until the conditions favor the growth of MBT cells [110].

Most of the MBT TA modules belong to the VapBC family, which includes 47 elements; the MazEF family is the second largest and includes nine loci [109]. The molecular mechanisms of action of toxins and antitoxins were established for only a minor portion of TA modules. This fact, along with the multiplicity of TA modules and their possible interference, renders it unfeasible to construct an exhaustive model of stress signal reception and adaptive responses.

However, the main TA modules that are activated on exposure to the majority of stress factors were identified in a recent systematic study [111].

Among all free-living bacteria, MBT possesses the greatest number of TA modules of the virulence-associated protein (VapBC) family. The VapC toxins contain a PIN nuclease domain, suggesting ribonuclease activity as their main function [112]. The VapB antitoxin is a protein and interacts directly with the toxin; i.e., VapBC is a type II TA system.

In total, 14 out of the 47 VapBC-family toxins were characterized in MBT. VapC20 and VapC26 specifically cleave the sarcin-ricin loop of the 23S rRNA [113]. VapC45 displays low-specific ribonuclease activity towards the 16S and 23S rRNAs and mRNAs [114]. VapC4 and the other toxins characterized in the family specifically cleave various tRNAs at the anticodon loop [109]. VapC affects fMet-tRNA in other microorganisms [109]. Although several MBT VapC proteins were found to interact with fMet-tRNA, tRNA cleavage was not detected in vitro or in vivo [115]. The roles of the other VapC toxins were not identified because the toxins did not exert their toxic effect upon heterologous expression in *M. smegmatis* [115].

Stress conditions that downregulate MazEF expression, such as exposure to the antibiotics that affect translation or transcription or an amino acid starvation-induced increase in intracellular ppGpp, lead to ClpAP-dependent degradation of MazE, and the MazF toxin is consequently released. MazF inhibits translation of most mRNAs by exerting its ribonuclease activity, which specifically produces leaderless mRNAs, and modifying the ribosome complex by cleaving the 16S rRNA to remove its 43 terminal nucleotides, which are responsible for recognition of the Shine–Dalgarno sequence and canonical translation initiation [116]. Thus, MazF generates a subpopulation of modified ribosome complexes that perform specialized translation of stress-related leaderless mRNAs in the cell. A deletion of three out of the nine MazEF modules identified in MBT was shown to substantially reduce the persister fraction in vitro and to increase the sensitivity to rifampicin, levofloxacin, and gentamycin, but not isoniazid [117].

As mentioned above, leaderless transcripts are produced by the majority of TA modules [118]. It is of interest to note that leaderless mRNAs are far more efficiently translated by MazF-modified ribosomes [116] and 70S ribosome complexes, which prevail in the stationary phase [118]. RNA ligase RtcB utilizes its noncanonical activity to revert the ribotoxin effect in the cell, thus saving substantial resources that would be necessary for synthesizing ribosomes or tRNAs de novo. Experiments with *E. coli* showed that RtcB restores the modified ribosomes by ligating the MazE-cleaved 3' fragment to the 16S rRNA and is capable of ligating the tRNAs cleaved at the anticodon loop [119]. In MBT, the RtcB homolog Rv2631 belongs to the DosR regulon.

DosR–DosT Two-Component System

The DosR–DosS (dormancy survival regulator), or DevR–DevS, two-component system is thought to provide a key regulatory mechanism that underlies mycobacterial dormancy. Heme-containing histidine kinases DosS (DevS) and DosT of MBT phosphorylate and activate the regulator DosR in response to changes in redox potential, NO, or CO [120]. In addition, the DosR-dependent regulon is induced in other growth-limiting conditions [56], including exposure to nitric oxide, carbon oxide, and ascorbic acid and infection of macrophages. Similar induction is observed during the acute and chronic infection phases in mice in response to cell treatment with rifampicin, bedaquiline [121], and isoniazid [61]. DosR is presumably one of the key regulators that facilitate cell survival in granulomas, and a greater number of genes in the DosR regulon and their higher upregulation are thought to be responsible for the evolutionary success of Beihing-family strains [121].

Discrepant results are available for the role that DosR plays in the response to hypoxia. Transcription of the DosR regulon was not induced until 20 days of hypoxia in a clinical isolate [122], while expression of the regulon genes was upregulated only during primary adaptation and returned to its baseline level 24 h after in a wild-type strain [26]. DosR repression was additionally observed in clinical samples [10]. In long-term hypoxic cultures, DosR activation occurred on days 30 and 60. This mechanism was assumed to ensure 95% survival over one year of culturing in hypoxic conditions [123].

The DosR regulon comprises 48 dormancy-related genes [40], including the heat shock protein gene *acr* (chaperone hspX), *narX*, *narK2*, *fdxA* (nitrate accumulation and alternative electron transport), *nrdZ* (deoxynucleoside triphosphate synthesis in microaerobic conditions), *tgs1* (triglyceride accumulation), and six mycobacterial orthologs of universal stress proteins protecting DNA from damage. Several genes involved in adaptation of translation machinery to dormancy also belong to the DosR regulon. Two of them code for ribosome-associated S30AE-family proteins Rv0079 and Rv1738 [124]. The Rv0079 homolog RafH stabilizes the 70S ribosome and prevents its dissociation in *M. smegmatis,* which is related to MBT [124]. A noncanonical mechanism of translation initiation on leaderless mRNAs in 70S ribosomes was discovered in the early 2000s. More recent studies identified this mechanism as important for MBT survival in stress [118]. In addition, 70S ribosomes are capable of reinitiating translation of downstream open reading frames in polycistron mRNAs via fMet-tRNA-dependent scanning, which does not require energy [125]. Transcription of 26% of the MBT genes is initated at an anno-

tated start codon, and the relative content of the resulting mRNAs substantially increases in starvation or growth arrest [118]. The mean life of leaderless mRNAs is higher than that of the mRNAs that have the Shine–Dalgarno sequence at the 5' end [126]. A high portion of leaderless mRNAs is found in genes of the TA modules, the σ^E regulon, and enzymes of the methylcitrate cycle [118]. Rv1738, the other protein of the S30AE family, binds to the 70S ribosome and inhibits translation; *rv1738* shows one of the highest upregulation levels in the DosR regulon [127].

Two proteins of the cAMP receptor/fumarate and nitrate reduction regulator (CRP/FNR) family were identified in MBT: Rv3676 (Crp) and Rv1675c (Cmr). In contrast to major representatives of the family, Cmr does not bind cAMP, and its activity is regulated by reactive oxygen and nitrogen species, which affect two cysteine residues to convert Cmr into a form incapable of DNA binding [128]. Apart from regulating transcription of its own gene and divergently oriented *rv1676*, Cmr suppresses the *rv1434c–dosR–dosT* operon and thus partly ensures the response of the DosR regulon genes to O_2 and NO [128].

Transcriptional Regulators of the WhiB Family

Proteins of the WhiB family are found in many actinomycetes and belong to the family of Fe–S cluster-containing transcriptional regulators. WhiB was first identified as a sporulation regulator in *S. coelicolor* [129]. As is known today, the WhiB proteins are associated with biosynthesis of antibiotics and regulation of the redox balance. There are seven WhiB-family transcription factors in MBT.

The Fe–S cluster of WhiB1 is sensitive to NO, and transcription of its gene is regulated by CRP-cAMP. WhiB2 expression is upregulated in starvation and on exposure to cell wall-affecting drugs, such as isoniazid, ethambutol, and cycloserine. WhiB2 was implicated in cell division in *M. smegmatis*. The protein is most likely associated with the cell cycle in MBT as well because its expression decreases in the late stationary phase. In contrast, WhiB3 is activated in the stationary phase, its activation being possibly related to the sensitivity to lower pH values because the medium is acidified in the stationary phase in MBT cultures. WhiB3 interacts with σ^A to regulate the pathogenicity genes, and *whiB3* deletion mutants are incapable of infecting guinea pigs. Expression of *whiB4* is possibly associated with stress-inducible σ^F. WhiB5 regulates expression of 58 genes, which are involved in MBT virulence and reactivation mechanisms. WhiB6 responds to temperature shock, oxidative stress, and exposure to ethanol or sodium dodecyl sulfate [97].

The *whiB7* gene is the best characterized among the *whiB*-family genes. Substitutions in its extended 5' region are associated with kanamycin resistance [130]. The WhiB7 regulon is induced within macrophages, the effect being possibly due to the influence of fatty acids [92], in heat shock, and at low iron concentrations and is repressed in the presence of ethanol or cycloserine. WhiB7 is induced by subinhibitory concentrations of translation-affecting antibiotics, and their MICs are significantly lower in a *whiB7* deletion mutant [92]. The WhiB7 regulon includes not only *eis, erm,* and *rv1258*, but also the gene for the efflux protein Rv1473, which is responsible for macrolide elimination from MBT cells; *cut2,* which codes for cutinase presumably capable of releasing fatty acids from extracellular lipids [92]; and *hflX*, which codes for a ribosome-splitting factor that rescues stalled ribosomes [131].

CONCLUSIONS

Antibiotic-resistant MBT forms and design of new antimicrobial drugs are the focus of recent research. However, effective drugs do not always result in successful therapy. Forms that possess phenotypic tolerance to antibiotics and cause latent asymptomatic TB are still a great problem. Tolerant MBT cells were earlier believed to be inactive both metabolically and transcriptionally. Yet recent studies showed that tolerant MBT cells are not absolutely dormant and are capable of spreading to produce new lesions in the host body. This phenomenon still lacks an exhaustive explanation in spite of the advances in studying the drug tolerance mechanisms. It remains unclear how the host immune response affects MBT tolerance. An important problem is to understand the pathways whereby external signals are transmitted to persistence and antibiotic tolerance mechanisms. Identification of the key pathways is essential for developing new strategies to eliminate tolerant MBT cells. Further studies of the role of efflux pumps in acquired drug tolerance of bacterial subpopulations and the possibility of adding efflux pump inhibitors to standard anti-TB medications will improve the efficacy of therapy in TB.

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