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Review

Modeling of *Mycobacterium tuberculosis* dormancy in bacterial cultures

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ABSTRACT

The currently available methods are unable to directly detect dormant forms of *Mycobacterium tuberculosis* (Mtb) in vivo. The persistence of Mtb in the host body is detectable only in an indirect manner via the immunological response to Mtb-specific antigens. It is commonly recognized that the pathogen prevalently exists in the human body in a latent stage. Additional research efforts focusing on the Mtb dormancy are needed for development of sterilizing drugs, which are necessary to control LTBI and stop TB epidemic. To this end, the in vitro models of Mtb dormancy may be useful. This review briefly describes the phenomenon of Mtb dormancy and its role in the context of tuberculosis as a persistent bacterial infection; then the article characterizes in details the in vitro methods used for modeling the Mtb dormancy in bacterial cultures.

1. Pathogen dormancy as a factor of persistence of tuberculosis infections

Currently, tuberculosis (TB) is regarded as a bacterial infection with a long-term pathogen persistence in the host body in a latent or an active form [1,2]. The mechanisms inducing and maintaining the persistence of a pathogen in latent tuberculosis infection (LTBI) are still unclear. Nevertheless, the relevant research data suggest that a key factor responsible for the persistence of *Mycobacterium tuberculosis* (Mtb) in an infected organism is its ability to exist in a dormant form [2,3]. The dormancy is the state when bacteria cease to divide yet do not die, thereby preventing eradication of the pathogen from the infected body.

As is believed, the host immune response induces Mtb to enter a dormant state. In particular, an important role of hypoxia, acidification when trapped in the phagosome of alveolar macrophages, and a nutrient deficiency in caseous necrosis foci formed during immune-mediated granuloma formation [4] has been shown. A result of the immune response to Mtb infection is formation of a specific granuloma where the pathogen resides in the cell and/or in its central caseous zone. As is assumed, these sites of infection, with the conditions adverse for Mtb, induce and maintain dormancy of the bacilli for years and even decades [5–7].

Dormancy is a potentially reversible state so that the mycobacterial cell remains viable and can restore its ability to divide. In an infected organism, the Mtb recovery from dormancy may result in LTBI reactivation and subsequent development of active TB. The estimated risk of LTBI reactivation is 5–10% across the lifespan of an individual [8,9]. The risk of LTBI reactivation is manifold increased in the immunocompromised individuals (HIV-infection and immunosuppression induced by the treatment with TNF-α inhibitors and corticosteroids or caused by comorbidities, such as diabetes, silicosis, and chronic renal failure, or the conditions, such as smoking, drug abuse, and homelessness) [10–12].

The LTBI prevalence in the human population is higher than the TB prevalence. According to epidemiological data, as much as 1.6–1.9 billion people globally have LTBI [13]. In some regions, the LTBI prevalence reaches 50% of the total population [14] and in certain population groups is extremely high too [15–19]. Thus, LTBI is a rather significant source of the active TB, which feeds into the epidemic process.

The role of Mtb dormant phenotype in an infected body is not limited to maintenance of LTBI alone. Another clinically relevant issue is the problems in eradicating the pathogen from lesions using a standard anti-TB therapy. The dormant Mtb forms can reside at the sites of post-TB morphological alterations even in the case of completely successful treatment or spontaneous cure [20–22]. Presumably, the Mtb persistence upon curing of active TB is related to the drug tolerance of Mtb dormant forms. In essence, the overwhelming majority of anti-Mtb drugs are efficient against the metabolically active and replicating bacterial cells. The reduction of active metabolic processes and so the deficiency of drug targets in dormant mycobacteria impede eradication of the pathogen and rather enhances its further persistence.

Of importance, the impact of certain antibacterials can trigger a persistent (dormant) state of Mtb [23,24]. From a clinical standpoint, persistence of the pathogen in post-tubercular lesions is a risk factor for

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TB relapse. The studies involving Mtb genomic identification show that endogenous reactivation account for 81% (49–100%) of the TB relapses [25–27]. The risk of relapse widely varies and is associated with cavitary pulmonary TB, comorbidities (HIV infection and diabetes), Mtb drug resistance, Beijing genotype, and other factors [25–28]. The TB relapse rate upon the completion of anti-TB therapy is 0.4–47% in different regions [25,29], which considerably worsens the epidemic situation [30].

Thus, the Mtb dormancy should be considered as both one of the key factors of pathogen persistence in the body and a factor feeding the TB epidemic process. It is unlikely that the goal of global TB eradication is achievable without a deep insight into the mechanisms of Mtb dormancy and the host response to persistent Mtb forms as well as without the development of the efficient antibacterials against dormant Mtb [31].

In the LTBI natural course in humans, any microscopic, cultural or molecular methods are unable to detect the pathogen or its direct markers. Moreover, the persistent/dormant Mtb forms are similarly undetectable in the active or cured TB subjects. Therefore, in vitro and/or in vivo modeling of an Mtb dormant state is of a paramount importance for the insight into the mechanisms underlying the Mtb dormancy and persistence as well as the LTBI development.

This review systemizes the information about the Mtb dormancy models and describes the experimental approaches to in vitro modeling of dormancy state.

2. The phenomenon of bacterial dormancy and its main features

In general, dormancy is defined as a reversible functional state of bacterial cell(s) characterized by reduced metabolic activity and cessation of cell division [32]. Since many researchers have observed this phenomenon in a variety of microorganisms both in their natural habitats and in vitro systems, there are tens of descriptions and comments on the nature of dormancy [3,33,34]. Current views on the mycobacterial dormancy are based on the results of numerous studies utilizing microbiological, biochemical, physicochemical, and molecular genetic approaches. It is currently accepted that the dormant phenotype of mycobacteria is characterized by a set of the following features.

2.1. Inability to replicate

Cessation of cell proliferation is demonstrated by the absence of or a decrease in the ability to grow in culture media under experimental conditions. Although the inability to replicate is a key, “fundamental”, feature of the dormant state of bacteria, it is insufficient to regard a bacterial cell or a population as dormant. The failure in cultivating bacteria may well be explained by other reasons. For example, the long-lasting efforts to cultivate *M. leprae* failed although the replication of this pathogen in the infected IL-10–suppressed macrophages and/or experimental animals (nine-banded armadillos) has been confirmed [35]. Moreover, live bacterial cells and their vital functions are identifiable by noncultural methods, for example, with the help of functional fluorescent dyes or PCR strategies [36–38] even in the absence of bacterial growth in culture medium. This state of bacteria is defined as “viable but nonculturable” (VBNC) [39,40] or “nongrowing but metabolically active” (NGMA) [41]; these definitions more accurately denote the inability of bacteria to proliferate when cultivated in growth media yet remaining alive.

2.2. Expression of dormancy genes

The transition of the bacterial cell to a dormant state is associated with certain changes in the transcriptome. Specific gene expression is induced by the exposure to various stimuli, typically external and adverse for the pathogen. This is a key intracellular mechanism initially involved in formation of a dormant phenotype. The Mtb (strain H37Rv) genome comprises 4173 genes at least several hundred of which are involved in dormancy [42–44].

The attempts have been made to arrange the genes involved in dormancy into gene networks and identify specific regulons [45]. The involvement of the DosR (dormancy survival regulator) regulon in the development and maintenance of dormant state has been studied more comprehensively as compared with other genes [46–49]. The significance of the genes beyond this regulon has been also shown [42–53]. However, the full and precise knowledge about expression of the genes associated with Mtb dormancy is yet to be obtained.

2.3. Metabolic shift

Expression of certain genes changes the metabolism of bacterial cells. A number of studies have demonstrated that the transformation into dormant (nonreplicating) forms involves major cellular metabolic pathways. In general, characteristic of the metabolic shift is slowdown of all principal cellular processes, which results in the arrest of cell growth and division [54–56]. Changes in the protein synthesis as a direct consequence of the changes in the transcriptome determine the further cell functioning. The whole proteome profiling studies have shown a downregulated expression of over 1500 proteins during Mtb dormancy. In addition, a dormant Mtb synthesizes the proteins (including enzymes) that are not typically expressed during active replication [57–59].

Activation of the glyoxylate shunt, which allows Mtb to use fat-derived carbon as a source for synthesis of complex carbohydrates has been repeatedly reported [60–63]. As is known, the energy metabolism is changed towards a decline in the intracellular ATP level and an increase in the NADH/NAD ratio [64,65]. The changes in the lipid metabolism of the Mtb cell wall has been described [55,66,67]. The nitrate metabolism has been also shown to contribute to Mtb dormancy [68–70]. However, the available information is insufficient to envisage a complete picture of the dormant *Mtb* metabolism both in vitro and in vivo.

2.4. Changes in cell morphology

These alterations often appear simultaneously with the functional reorganization of nonreplicating bacteria and affect the size, shape, and ultrastructure of microorganisms [71]. The most striking example is sporulation of some gram-positive bacteria, which the authors consider as a true dormant state [72]. The inability of Mtb to sporulate is well known. However, altered forms of dormant Mtb, including ovoid cells [73], as well as the forms containing intracellular inclusions (lipid bodies) [74,75] or the forms with altered staining properties [76], have been described.

2.5. Phenotypic drug tolerance

The drug resistance not associated with mutations is usually explained by “inactivity” of the target for antibacterials [77]. A phenotypic tolerance of dormant Mtb to isoniazid has been repeatedly observed [78–80]. Isoniazid is a pro-drug, which requires a catalase–peroxidase-mediated activation in the bacterial cell [81]. In a nonreplicating dormant Mtb, the catalase–peroxidase synthesis is impaired [82]. The data on the effects of other antibacterials on a dormant Mtb are rather limited and contradictory [83–88]. The molecular and cellular mechanisms underlying the Mtb phenotypic drug tolerance to the majority of anti-TB drugs are yet vague. However, a detailed understanding of this phenomenon is necessary for the development of efficient preventive anti-TB treatment and prevention of post-treatment pathogen persistence.

2.6. Reversibility

From a biological point of view, the phenomenon of dormancy is regarded as the ability of certain species to maintain their viability under adverse conditions. In other words, the purpose of transition of a microorganism to dormancy is to survive, cease this condition some time later, and restore proliferation. The term resuscitation is used most frequently to define this process [89,90]. The mechanisms underlying the Mtb resuscitation are much less studied as compared with the mechanisms of transition to dormancy. Under experimental conditions, elimination of the factor(s) that caused dormancy makes it possible to obtain the data on genetic, metabolic, and phenotypic characteristics of resuscitating Mtb [57,58,91]. It is still unclear how the biological processes are activated and replication of microorganisms is recovered in an infected organism [92]. However, prevention of LTBI reactivation or TB relapse demands the knowledge about what triggers the Mtb resuscitation in vivo, what share of mycobacterial population is capable for recovering, how long the pathogen is able to stay in dormancy, whether the dormant Mtb has been killed and eliminated from the body, or whether the immunological (TST or/and IGRA) tests have converted along with the recovery.

Currently, most of the information available on the Mtb dormancy is obtained with the help of the models described below.

3. In vitro modeling of Mtb dormancy in bacterial culture: methods and findings

Since Mtb often persists within macrophages and/or granulomas, the in vitro experimental models of Mtb dormancy involve not only pure mycobacterial cultures, but also infected cultures of macrophages or other immunocompetent cells as well as 3D granuloma-like structures simulating real granulomas in vivo.

The Mtb dormancy models in pure bacterial cultures are the easiest to perform and the least expensive. In general, these models represent a mere cultivation which enable a mycobacterial population to enter dormancy under the impact of external stimuli and a characterization of this state. The object of such modeling is a pure fresh mycobacterial broth culture; usually, the Mtb reference strain H37Rv is used. For this purpose researchers select the factors that influence the pathogen in vivo (proven or hypothetical) as dormancy inducers. Historically, the studies of Mtb dormancy began as in vitro experiments. As early as in 1933, Loebel et al. [93] showed a reduction in oxygen consumption by Mtb during a long-term cultivation in the medium without nutrients or in the presence of high hydrogen ion concentration. The loss of the ability of mycobacterial culture to grow after 3- to 15-day exposure to anaerobic conditions in both culture medium and saline [93] was also demonstrated. Thus, the factors detailed below are able to trigger the Mtb transition to dormancy under in vitro conditions.

3.1. Progredient hypoxia

A low oxygen concentration influences Mtb at its residence in a macroorganism, namely, in macrophages and the caseous sites inside granulomas, nondestructive infiltrative foci, or tuberculomas [94]. Mtb bacilli are obligate aerobic microorganisms; oxygen concentration gradually decreases over the cultivation time in a liquid medium. This is widely used by researchers for modeling the Mtb dormancy. The simplest model is an extended Mtb incubation in a broth culture. Obvious limitations of this model are its inconsistency with the actual conditions inside an infected body and a low rate of hypoxia generation. As a result, a stationary growth phase mycobacterial population is generated (during which both actively dividing and already dead cells coexist) rather than a population with a dormant phenotype. For example, a sevenfold decrease in the metabolic activity of a 100-day old Mtb culture in the stationary growth phase as compared to a log phase culture according to [^{3}H] uridine incorporation into mycobacterial RNA was observed [95]. However, the replication capacity of the stationary culture remained unchanged [95]. A significant difference in the Mtb gene expression between a stationary phase culture and that cultivated under hypoxic conditions was shown [96]. This team observed an evident induction of all DosR regulon genes under the anaerobic cultivation conditions, whereas only part of DosR regulon genes was expressed in a stationary phase aerobic culture. In addition, certain differences were observed in the expression of PE/PPE family genes, involved in the regulation of antioxidant defense, as well as the *ahpC* and *desA3* genes, involved in the synthesis of cell wall mycolic acids. The authors assume that the transcriptional changes observed in an anaerobic culture are the in vivo signatures of Mtb dormancy. Nevertheless, they note that the dormant state was not confirmed by cessation of replication and metabolic changes [96].

The culture models of Mtb dormancy utilizing active generation of hypoxia are more advanced as compared with the stationary phase models.

The original model by Wayne demonstrated a two-step Mtb transition to a nonreplicating persistence state during its extended incubation in broth in a sealed rotatable vial induced by self-generated decrease in oxygen concentration. The first stage (a microaerophilic stage by the definition of authors) took place when oxygen saturation in the medium reached 1% and was characterized by a delay in Mtb replication as well as a slowdown of the nucleic acid replication and an increase in ATP content in bacterial cells. An anaerobic stage commenced when oxygen saturation was below 0.06%. Under these conditions, the Mtb replication ceased and a sharp decrease in glycine dehydrogenase concentration was observed [97]. In addition, the Mtb nitrate reductase activity in the microaerophilic stage significantly increased [68]. The Mtb cell division resumed in 32 h after part of nonreplicating culture was transferred to a fresh medium with a high oxygen concentration. The authors assume that a slow decrease in the oxygen concentration at the microaerophilic stage contributes to the Mtb adaptation to the developing anaerobic conditions; similar factors influence the bacilli inside the specific granuloma; and adaptation to microaerophilic conditions stimulates maintenance of the pathogen's viability.

An increased reduction of nitrates is most likely associated with accumulation of this compound, generated as a result of nitric oxide degradation and contributing to the maintenance of mycobacterial respiratory function in the foci of specific inflammation/necrosis. Over 50% of the Mtb cells incubated under hypoxic conditions survived exposure to isoniazid and ciprofloxacin at concentrations affecting aerobic cultures. The bactericidal effect of rifampin decreased less significantly. Metronidazole showed a bactericidal activity only in the anaerobic cultures [97].

The role of hypoxia in the induction of Mtb dormancy in vitro was also demonstrated in some modifications of the model described above. Leistikow et al. [65] developed a rapid anaerobic dormancy model. The authors used the rotation of *Mtb* culture at higher speed than in the model by Wayne. One of the used experimental strains was an Mtb mutant unable to express the DosR regulon genes. The ability of the mutant strain to recover its growth was decreased by 58 and 88% by days 10 and 20 of anaerobic incubation on agar media as compared with the wild-type strain. However, staining of the mutant strain cells with fluorescein diacetate demonstrated preserved metabolic activity. This suggests that the DosR regulon is involved in certain processes associated with oxygen consumption, maintenance of ATP level, and redox balance during the hypoxia-induced Mtb transition to dormancy. In addition, the strain with a limited function of the DosR regulon showed decreased growth recovery when transferred to aerobic culture conditions as compared with the wild-type strain. The expression levels of the DosR regulon genes were not determined in this study [65].

Since 1996, the Wayne model has been used in hundreds of studies, which greatly increased our knowledge about the Mtb dormancy. For example, Rustad et al. [98] described expression of the 230 Mtb genes referred to as EHR (Enduring Hypoxic Response) genes: a rise of gene

expression followed the induced DosR-mediated hypoxic response on days 4–7 of hypoxia [98]. The transcription kinetics in mycobacterial cells was observed at different stages of hypoxia [51] and the Wayne model was used for proteomic analysis of dormant Mtb [57]. Several technical modifications of the hypoxia-induced Mtb dormancy model have been proposed, in particular, the Mtb cultivation with permanently controlled 1% oxygen concentration in a chemostat [99]; keeping Mtb culture in the vacutainers with colorimetric detection of oxygen consumption by resazurin reduction assay [100]; and adaptation of the Wayne model to a microplate format with mycobacterium viability detection according to nitrate reductase activity [101].

3.2. Nitric oxide (NO)

As has been shown, low NO concentrations generated by adding DETA (diethylenetriamine, an NO donor) to culture medium caused a dose-dependent bacteriostatic effect [102]. Delayed Mtb growth was observed for no less than 100 h but it retained its viability later. A two- to eight-fold increase in the expression of 48 DevR/DosR regulon genes was also observed. Since the in vitro models of hypoxia-induced Mtb dormancy gave similar results, the authors assumed that low NO doses inhibited the Mtb respiratory function, thereby limiting their replication [102]. Similar results were obtained using NOR-3 and SPER as NO donors [103]. A 4-h exposure of mycobacterial culture to each reagent had no affect the Mtb ability to grow in culture media although it induced a 6- to 112-fold increase in the expression of 15 genes (14 of which were related to the DosR regulon). The authors consider that the observed transcriptional changes represent an integral adaptive response of mycobacterial cells to the combined effect of hypoxia and reactive nitrogen species within granulomas [103]. In addition, a similar upregulation of DosR regulon genes in the Mtb in IFN-γ–activated macrophages was observed in in vivo LTBI/TB models [104]. These data suggest that NO and/or its derivatives are involved in the induction of Mtb dormant state during host immune response.

3.3. Carbon monoxide (CO)

In vitro and in vivo studies [105,106] have shown that an increase in the hemoxygenase (HO-1) expression level and activity in Mtb-infected macrophages resulted in CO generation. CO is another likely trigger for the Mtb intracellular survival in a dormant state. To confirm this hypothesis, an Mtb culture was exposed to CO for 48 h by filling the headspace with CO using a CO generator. Only high CO concentrations induced a slight decrease in the Mtb growth rate although the observation time was only 150 h. A transcriptome analysis of experimental cultures showed a dose-dependent expression of the majority of DosR regulon genes. It was also shown that the CO-mediated pathway of DosR gene activation in macrophages was independent of IFN-γ–regulated NO production and lasted for over 24 h versus 2 h observed in the NO response. This suggests that NO is a triggering factor for the Mtb transition to a dormant state in the early LTBI phase, while CO and hypoxia are involved in the maintenance of this state [105].

3.4. Nutrient starvation

While inside the macrophages and placed into a relatively isolated space of phagosomes, Mtb experiences a deficiency in the supply of nutrients required for its life activities and replication. Bacterial cultures are the most convenient object for modeling the conditions of nutrient deficiency for the pathogen. Accordingly, a number of microbiological models simulate Mtb starvation. As early as 1974, it was reported that the Mtb incubation in distilled water for 30 days caused the loss of acid resistance in Ziehl-Neelsen staining, while making Mtb stainable with aniline dyes [107]. In this state defined as a chromophobic by the author, Mtb cells retained their viability for 2 years and restored their staining affinities and ability to replicate during the first subcultivation in culture medium.

The 6-week incubation of Mtb suspension in phosphate buffered saline decreased the Mtb oxygen consumption and delayed its growth without reducing the viability [108]. In addition to a decrease in the respiratory activity in experimental culture, the contrasting changes in the expression levels of seven proteins were observed, including an increase in the synthesis of 16 kDa α-crystallin homolog (*acr, HspX*), a protein the expression of which in Mtb is enhanced in hypoxia, in vitro NO exposure, and in Mtb residing in macrophages [109–111]. An RNA/DNA analysis has shown a low level of activation of the genes associated with DNA replication, repair, transcription, and translation; synthesis of amino acids, lipids, cofactors, and prosthetic groups of proteins; energy metabolism; and virulence. Conversely, the expression of genes responsible for the degradation of small molecules, production of antibiotics, and resistance as well as the genes with the functions unknown at the time of study, was increased [108]. As was demonstrated by M. Gengenbacher et al. [112], a 5-day Mtb nutrient starvation induced a fivefold decrease in the concentration of intracellular ATP. A stable low ATP level was maintained for 42 days of observation and this was necessary and sufficient to preserve the Mtb viability.

A decline in the ATP synthesis was a general condition for the Mtb persistence in a nonreplicating state in both the hypoxia-induced and nutrient deprivation models [112]. The model by Hampshire et al. [113] utilizes deficiency of nutrients in the stationary phase of Mtb cultivation; the oxygen content, temperature, and pH are maintained constant (under chemostat conditions). As is demonstrated, glycerol vanished by day 15 of incubation and the glucose concentrations dropped 25.5-fold by day 100. Concurrently, the replicative capacity gradually decreased by four orders of magnitude by day 80 of persistence in the stationary phase. The authors assume that the number of viable mycobacteria in culture after 100-day cultivation increased fourfold owing to the Mtb adaptation to nutrient deficiency. In addition, the Mtb growth arrest was accompanied by expression of the genes associated with β-oxidation of fatty acids, glyoxylate shunt, anaerobic respiration, ATP synthesis, peptide acid and carbon-carbon cleavage, RNA synthesis, and transcription. Despite the permanent aeration of experimental cultures, an increased expression of two (*Rv2629* and *Rv2630*) DosR regulon genes was observed [113].

A new starvation model was developed by one of the authors of this review, Batyrshina (application for patent of invention #2018140171 to the Federal Institute of Industrial Property). In this model, the Mtb dormant phenotype is formed as a result of deprivation of three essential components of a conventional nutrient medium for cultivation of mycobacteria. ADC supplement (albumin, dextrose, sodium chloride, and catalase) is excluded from the Middlebrook 7H9 complete medium. This gave the Mtb phenotype with dormancy characteristics. Particularly, the delay of growth in nutrient-deprived medium was observed from day 2 of Mtb incubation, as assayed by optical density (OD_{600}) values. The ability to grow on solid media (agar Middlebrook 7H10) decreased by 1–1.5 log by days 2–3 of incubation. After 4 days of deprivation, Mtb completely lost its ability to replicate. In addition, the recovery rate of the deprived Mtb following its transfer to fresh Middlebrook 7H9 medium decreased (16–29 days versus 1.5–2.5 days for the control culture). However, a significant decrease in oxygen consumption was recorded in a BACTEC system. The morphological alterations of dormant Mtb, such as a 2.0–2.5-fold decrease in the mycobacterial cell size and the development of coarse intracellular inclusions, were observed. The end of starvation by adding the above mentioned components to growth medium recovered the Mtb replicative capacity in both solid and liquid media after 5–11 and 5–17 days, respectively. In addition to nutrient deficiency, dormant phenotype in this model is apparently induced by the energy imbalance in the mycobacterial cell due to the absence of carbohydrates as well as the reduced osmolarity of the medium.

3.5. Deficiency or excess of metal ions

3.5.1. Iron ion deprivation

Iron (Fe) is an indispensable cofactor for the Mtb enzymes catalyzing the redox reactions. Mtb is able to synthesize siderophores, the molecules directly binding to the Fe of the host heme [114]. High concentrations of iron-sequestrating proteins, such as transferrin, haptoglobin, hemopexin, lactoferrin, and lipocalin, were identified in the necrotic cores of TB granulomas [115,116]. A high level of these proteins suggests a limited of Fe^{3+} availability in the caseous compartments. To confirm the hypothesis that iron deprivation can cause metabolic and replicative changes in Mtb, Kurthkoti et al. [117] cultivated the Mtb strain H37Rv in the media (1) with a minimal (~1 μM) Fe content and (2) supplemented with a heterologous siderophore, deferoxamine. In the iron-deprived medium without deferoxamine, the Mtb growth was observed for 2 weeks of incubation and only slightly lagged behind the growth of the control culture, whereas the cultivation in the presence of a siderophore completely arrested the growth. Nevertheless, Mtb remained viable on solid culture media over 9 weeks of observation. Adding $FeCl_3$ or heme to an iron-deprived medium rapidly recovered the growth. Fluorescein diacetate or propidium iodide staining of 14-day-old iron-starved cultures showed that approximately 70–80% of the Mtb population retained the metabolic activity and the intact cell wall. Iron-starving cultures were drug-tolerant to high concentrations of isoniazid, ciprofloxacin, kanamycin, ethionamide, and cycloserine but remained sensitive to rifampin. RNA sequencing revealed different expression patterns of several hundreds of genes, 240 of which were expressed only under iron deprivation and 124 were also expressed in nutrient deprivation, hypoxia, and stationary phase models [108,118,119]. Overexpression of the genes involved in the iron binding lasted for 14 days of deprivation. Expression of the energy metabolism and respiration genes was considerably downregulated, while expression of the genes involved in carbohydrate and amino acid metabolism was changed in different manners. In particular, the genes coding for enzymes involved in the tricarboxylic acid cycle, oxidative phosphorylation, glycolysis, and methionine and ornithine synthesis were repressed. Expression of the genes of arginine and lysine synthesis pathways (the latter is involved in the production of siderophores) was upregulated. The genes involved in the synthesis of mycolic acids and ethers of the Mtb cell wall were repressed; however, the genes encoding the cholesterol synthesis associated with the in vivo Mtb persistence [120] were overexpressed [117].

3.5.2. Magnesium ion deprivation

Magnesium ions (Mg^{2+}) are necessary for bacterial growth, reproduction, and maintenance of the cell wall stability being cofactors in many ATP-dependent reactions in addition to their involvement in the formation of tRNA and ribosome tertiary structure [121,122]. Early studies have shown that the intraphagosomal Mg^{2+} deficiency in IFN-γ–activated macrophages is an additional factor potentially influencing the Mtb replication [123]. A wild-type Mtb strain and its *perM* mutant with disrupted PerM membrane protein were used to assess the effect of magnesium ions on viability [124]; note that this protein is associated with persistence of the pathogen in the chronic experimental infection in mice [125]. The strains were cultivated in the Soton medium supplemented with Mg^{2+} at concentrations ranging from 0 to 2000 μM. The wild-type Mtb failed to survive the absence of Mg^{2+} but retained the ability to replicate in the medium containing 25 μM or higher Mg^{2+} concentrations; on the contrary, the *perM* mutant slowed down its replication in the presence of 250–500 μM Mg^{2+}. In the *perM* mutant cultivated in the medium with a low Mg^{2+} concentration (250 μM), 40 genes were overexpressed as compared with the wild-type Mtb; 16 of these genes are associated with cell division and/or cell wall synthesis. Scanning electron microscopy showed an increase in the mean cell length and formation of cell wall protrusions by the mutant strain following its 5-day incubation in the Mg-reduced medium. The *perM* mutant had no phenotypic tolerance to isoniazid and ethambutol but displayed an increased sensitivity to β-lactam antibiotics, especially cephalexin and piperacillin, specifically inhibiting FtsI transpeptidase, which is involved in the synthesis of peptidoglycan of the cell wall during bacterial cell division. Actually, the study by Goodsmith et al. [124] did not demonstrate any clear signs of a dormant Mtb phenotype but found that the Mtb adaptation to Mg^{2+} deficiency in macrophages involved a cell division restriction as well.

3.5.3. Copper excess

Copper (Cu) is a cofactor of at least two Mtb enzymes, namely, cytochrome *c* oxidase and Cu/Zn superoxide dismutase (SodC). The former catalyzes the final stage of the electron transfer to oxygen in oxidative phosphorylation and the latter dismutates superoxide radical (O^{2-}) to either molecular oxygen (O_2) or hydrogen peroxide (H_2O_2), thus protecting Mtb from oxidative stress [126]. Copper is required for bacterial growth but high copper ion concentrations are toxic [126,127]. In the Mtb-infected macrophages, copper contributes to oxidative stress inside phagolysosomes and/or has a direct toxic effect when penetrating into the mycobacterial cell. The physiological levels of copper in the phagolysosomes of macrophages vary in the range of 25–500 μM [128]. Ward et al. [129] studied the effect of copper on the growth and transcriptional response of Mtb H37Rv cultivated in the Middlebrook 7H9 medium with 0–500 μM Cu. Only the maximum physiological level (500 μM) inhibited the Mtb growth in culture and had a partial bactericidal effect reducing 100-fold the number of viable Mtb cells. In response to an increased copper content in the medium, the expression levels of *csoR* (Cu metal regulator gene) and *ctpV* (putative copper exporting p-type ATPase V gene) increased ~180- and ~55-fold, respectively. In addition to overexpression of the specific genes involved in copper metabolism, the cultures incubated with the maximum copper concentration and in a copper-free medium significantly differed in the expression of 30 Mtb genes. Among these genes, 16 were unique to the copper-induced Mtb response, while the expression of the other 14 was also observed in the models of oxidative stress [129]. Neither this study nor the previous one showed any formation of a dormant phenotype induced by high copper concentrations. Nevertheless, the results suggest the role of copper in the Mtb adaptation to the survival within phagosomes.

3.6. Deficiency of potassium ions

A decrease in the ability of Mtb to form colonies on solid culture media after a 40-day incubation in a medium free of potassium ions has been demonstrated [130]. Analysis of the transcriptome of Mtb unculturable phenotype has shown upregulation of 830 and downregulation of 864 genes as compared with a log phase mycobacterial culture. These genes are mainly associated with the transition of bacterial cells to anaerobic respiration and with the shifts in lipid and amino acid metabolism. In addition, the authors described morphological transformations appearing as shortening and spherical shape of mycobacterial cells as well as ultrastructural changes in their cytoplasmic membranes and found a decrease in the sensitivity of experimental culture to isoniazid. The transfer of washed unculturable Mtb cells to a standard culture medium containing potassium ions was accompanied by an increase in the metabolic activity (according to uracil incorporation) within 10–24 h after the transfer and subsequent rapid recovery of unculturability on dense media. The authors assumed that the restoration of cultural properties of the experimental culture was associated with a stimulating effect of certain factors contained in the supernatant.

3.7. Low pH environment

Many researchers regard an increase in the acidity of environment as one of the main potential factors inducing the in vivo Mtb transition

to a dormant state [4,131]. As for in vitro models, it has been used both alone and in combination with hypoxia, starvation, and other inducers of a Mtb dormant state [73,132,133]. In particular, the pH of post-stationary phase Mtb cultures was maintained in the range of 6.2–5.0 [134]. Mtb was shown to lose the ability to restore its growth on solid culture media 180 days after the incubation at low pH. In addition, ovoid forms of mycobacterial cells were formed, which displayed a 200-fold decrease in RNA synthesis (according to the rate of 5.6-3H-uracil incorporation in the cells) as compared with vegetative forms of bacteria or a ~40-fold decrease as compared with an Mtb stationary phase culture. The intracellular ATP concentration decreased 58- and 35-fold, respectively. Administration of the obtained ovoid cells to laboratory mice induced a typical active Mtb form in the animal organs, which developed 1.5 years later [134].

3.8. Multiple stress

An in vitro model concurrently utilizing four factors able to transform Mtb to a dormant state—relatively low oxygen saturation (5%), high CO concentration (10%), acidification of the medium to pH = 5, and decrease in the nutrients—was presented in 2009 [132]. The authors observed cessation of Mtb replication and a gradual loss of acid resistance with a proportional increase in the accumulation of lipids (triacylglycerols and waxes). In total, as much as 331 genes with more than twofold changed expression were revealed by transcriptome analysis of the experimental culture. A significant increase in the expression of the genes coding for the glyoxylate cycle enzymes, anaerobic cell respiration, and the response to stress (for example, *hspX/acr; Rv2031c*) has been recorded. Expression of the genes associated with energy metabolism, in particular, the genes coding for ATP synthase and NADH dehydrogenase, was suppressed. The authors believe that this model reflects a dormant state of the Mtb in vivo more adequately as compared with the models utilizing only one of the potential dormancy triggers [132].

3.9. Ascorbic acid

The role of vitamin C in the induction of Mtb dormancy in vitro was demonstrated in two studies of Indian researchers. Vitamin C (10 mM) added to the culture medium as in other models led to pronounced changes in transcription—upregulation of 280 and the repression of 283 genes, which, in total, make up about a quarter of the entire Mtb genome. The most significant changes were observed in the lipid metabolism genes (34 upregulated and 19 downregulated genes); virulence, detoxification, and adaptation genes (34 upregulated and 21 downregulated genes); and in intermediate metabolism and respiration genes (81 upregulated and 56 downregulated genes). Expression of the genes involved in the regulation of intracellular processes and the processes associated with the Mtb cell wall function was either suppressed (for 55 genes) or enhanced (for 30 genes). In a comparative analysis of the DosR regulon, the authors observed similar changes in the expression of over 20 genes of dormant Mtb in the models of hypoxia, CO and NO stresses, and low pH values [133,135]. Obviously, these changes were associated with the ability of ascorbic acid to bind oxygen and, simultaneously, to acidify the culture medium. Under these conditions, the response of the DevR/DosR regulon genes is most likely a universal response of Mtb to various stress factors and their combinations.

3.10. Phosphate deficiency

The data on a critical involvement of inorganic phosphate in the cell functioning and on a limited availability of phosphates inside macrophage phagosomes in vivo [136] suggested the choice of this factor as an inducer of the Mtb dormancy. Mtb was cultivated in a liquid medium supplemented with different concentrations of inorganic phosphate while maintaining constant neutral pH; a dose-dependent decrease in (but not the loss of) the Mtb ability to grow on dense media starting from day 7 of incubation in a phosphate-limited medium was observed. A 24-h incubation resulted in expression up- and downregulation of 340 and 275 genes, respectively; a 72-h exposure up- and downregulated expression of 369 and 448 genes, respectively; and expression of 149 genes was changed at both time points. An increase in the expression of the PE/PPE family genes was the most pronounced. The *SenX3-RegX3*–dependent induction of the phosphate-specific transport operon, *pstS3-pstC2-pstA1,* was observed. In addition, a phosphate-poor medium induced morphological changes in Mtb, including a loss of acid resistance by day 14 and doubling of the bacterial cell length by day 28. The minimum bactericidal concentration of isoniazid was 20 μg/ml for a 28-day phosphate-deprived culture versus 0.06 μg/ml for the same strain in the logarithmic growth phase. The phenotypic tolerance to rifampin did not develop (MBC = 0.25 μg/ml). The authors inferred that the *RegX3*-induced Mtb response to a low phosphate content is important for its persistence in specific lesions.

3.11. Lipids as the sole carbon source

Previous studies have shown that a potential Mtb reservoir within TB granulomas is foamy macrophages, which contain numerous lipid droplets in the cytoplasm [137]. A study of the ability of in vitro cultivated Mtb to adapt to high concentrations of fatty acids [138] utilized a mixture of oleic, palmitic and stearic acids (0.001% each) instead of dextrose as the traditional source of carbohydrates in the medium. The Mtb growth curves insignificantly differed from the control. However, a total RNA sequencing revealed a change in the expression of about 10% of the coding genes. The overexpression of 14 genes, five of which belonged to the DosR regulon and the remaining ones were involved in glyoxylate cycle, lipid metabolism, and the response to metabolic reductive stress, was specifically associated with the Mtb cultivation conditions at a high fatty acid concentration. In addition, a significant overexpression of the tRNAs involved in modification of mycobacterial cell wall lipids was found for the first time in this model. Electron microscopy demonstrated formation of lipid bodies in the cytoplasm of mycobacterial cells. A 95% bactericidal effect of rifampin, moxifloxacin, amikacin, and metronidazole was observed in the Mtb of lipid-enriched cultures 7–14 days later than in the control cultures. Thus, a decrease in the Mtb replication did not occur in this model but a particular Mtb phenotype displaying some other signs of dormancy was formed.

In another modification of this model, 0.01% cholesterol was added to the medium in addition to fatty acids and the Mtb cells were cultivated under hypoxic conditions [44]. This time, a global transcriptome analysis revealed either up- and downregulation of expression of 185 and 183 genes, respectively; a number of these genes were functionally related to cellular intermediate metabolism and respiration, lipid metabolism, and cell wall modification, while the functions of one-third of these genes were unknown. The most significant changes in the expression of six genes (*Rv3161c, Rv3160c, Rv0678, Rv1217c, PPE53.* and *che1*) were observed at all stages of cultivation (exponential, stationary, and microaerophilic). The authors referred to this set of genes as “the main lipid response genes”; they are functionally involved in the Mtb cell wall transport systems, detoxification from antibacterials, iron uptake, and sulfur metabolism. In a combined exposure to hypoxia and a high lipid concentration, the *Rv3160c, Rv3161c*, and *PPE53* expression was increased 327-, 372-, and 19-fold, respectively [44]. Other studies have shown comparable overexpression of these genes when exposed to the Mtb inhibitors of cell wall synthesis and cell respiration [139,140]; this has suggested the authors that the genes studied are involved in the mechanisms of Mtb drug tolerance associated with impaired antibacterial drug penetration through the cell wall and efflux effect [44].

3.12. Antibacterials

Dozens of studies on the effects of antibacterial drugs on the dormant forms of mycobacteria utilize the above models; the Wayne and starvation models are used most often. Keren et al. [141] were the first to show formation of the Mtb subpopulation with signs of dormancy induced in the culture by anti-TB drugs. About 1% of the cells from originally drug-sensitive Mtb H37Rv population evolved into drug-tolerant ones following 7–14-day exposure to streptomycin, ciprofloxacin, isoniazid, or rifampin at 5 × , 10 × , 20 × , or 50 × MIC. After 1 week of Mtb cultivation in the presence of streptomycin, ciprofloxacin, or D-cycloserine, low numbers of drug-tolerant Mtb cells (referred to as persisters) were present in the lag and early exponential phases and their abundance sharply increased in the late exponential and stationary phases. The Mtb exposure to D-cycloserine at a dose of 100 μg/ml for 7 days caused over twofold upregulation of 282 and downregulation of 1408 genes. The genes associated with ribosomal protein synthesis, respiration, and ATP production were mainly downregulated. Expression of the genes involved in glycolysis, glyoxylate shunt, pyruvate dehydrogenase synthesis, and electron transport was more variable. Eight genes of the Dos regulon were overexpressed, while other five genes (*acr2, pdhA, lat, Rv1152,* and *Rv3290c*) were expressed in this model similar to the hypoxia and starvation models. As for the expression of drug target genes, it changed as follows: one isoniazid target, *katG,* was upregulated but another target, *inhA,* downregulated; *gyrA* and *gyrB* for ciprofloxacin and ribosomal protein genes for streptomycin were downregulated as well, while *rpoB* for rifampin did not significantly change. It is assumed that the drug tolerant Mtb is in dormancy expressing a small set of genes of the core dormancy response [141]. Similar experiments would give a deeper insight into the mechanisms underlying the Mtb persistence and suggest the approaches to overcome the persisters in the patient's body.

State-of-the-art research methods, such as flow cytometry, time-lapse fluorescence microscopy, microfluidic systems, and environmental scanning electron microscopy, have given a variety of data on the biology of microorganisms at the level of individual cells and their ultrastructure, subpopulations, and whole populations. In particular, in the experiment by Wakamoto et al. [142] with *M. smegmatis,* microaliquots of mycobacterial suspension were passed through the capillaries of a microfluidic device at a rate of 35 μl/min in the presence of 50 μg/ml isoniazid (10 × isoniazid MIC for *M. smegmatis*). At certain time intervals, the viable mycobacteria were counted according to the CFUs on agar. Additionally, the *M. smegmatis* division dynamics at the level of individual cells was recorded by time-lapse microscopy. During the first 20 h of isoniazid exposure, the replicable mycobacterium counts sharply decreased by 1 log; then, the decline significantly slowed down and more than a half of the original population remained viable after 60 h. The authors identified three phases in the drug exposure dynamics—delay, destruction, and persistence of mycobacteria— and showed that the persistence at a population level results from a decrease in cell death rate rather than an increase in the division rate. Microscopy has shown a gradual lysis of mycobacterial cells over the 6–144 h of isoniazid exposure but individual cells remained intact. After removal of isoniazid, the remaining intact cells quickly resumed replication; when their offspring was exposed to isoniazid, the kinetics of the bactericidal effect was repeated. The researchers concluded that the *M. smegmatis* persistence was associated with a reversible phenotypic tolerance of part of the bacterial population. In addition, it was shown using the overexpression of the recombinant *M. smegmatis* strain with *katG* that a twofold increase in catalase–peroxidase expression gave a 1000-fold increase in the bactericidal effect of isoniazid in the persistence phase [142].

3.12.1. Hollow fiber system model

The hollow fiber system was developed by Blaser et al. as early as 1985 [143] to simulate in vitro pharmacokinetics/pharmacodynamics of antibacterials and their combinations in relation to Gram positive and Gram negative bacteria. Later, this model was adapted to Mtb when studying the effect of ciprofloxacin in the Mtb logarithmic growth phase [144]. An undoubted advantage of the hollow fiber system model is the possibility to study the dynamic effects of antibiotics on Mtb in various physiological and metabolic states and a quantification of both sensitive and resistant subpopulations. The Mtb cultivated in Middlebrook 7H9 medium for 28 days at pH 5.0–6.8 displayed no growth at the pH below 5.8 and slowed down its growth at pH 5.8 as compared with that at pH 6.8 [145]. An Mtb population obtained at pH 5.8 was classified as semidormant, that is, containing replicated, dormant, and dead subpopulations in relatively stable proportions [146]. It was shown using pyrazinamide in the hollow fiber system model that its sterilizing effect appeared at the concentrations corresponding to a dose of over 60 mg/kg in contrast to the recommended dose of 15–30 mg/kg [145]. A hypoxia-induced dormant Mtb was generated using the Wayne method to study the dynamic effect of moxifloxacin on these mycobacteria. Moxifloxacin showed a high bactericidal activity against nonreplicating Mtb at the “anaerobic” stage [147].

3.13. Genetic mutations

Currently, genetically modified Mtb strains are often used for in vitro and in vivo studies aiming to determine the role of a particular gene/operon in the induction and maintenance of its dormant state. As a rule, researchers use a removal or overexpression of a target gene, for example, *DosR* [65,105,106,133]. The culture models using Mtb strains with a dormant phenotype obtained by genome editing (caused by a certain external factor) would be a useful tool for studying the effects of antibacterials on dormant Mtb forms. Genetic engineering approaches to the development of such models for Mtb have not yet been tested. A model using Mtb strain 18b was proposed [148]. This streptomycin-dependent strain was isolated in 1955 [149]; 40 years later, this isolate was found to be incapable of growing in a streptomycin-free medium but its replication capacity restored when transferred to a streptomycin-containing medium. Coincidently, a mutation (an insertion of cytosine in the 530 rRNA loop) determining streptomycin dependence was detected [150]. Sala et al. [148] observed a slowed Mtb 18b growth during a 10-day incubation in a streptomycin-free medium and restoration of the ability to replicate within 3–5 days after adding the drug; in parallel, the number of colonies changed. The streptomycin-deprived nonreplicating Mtb 18b displayed a decreased sensitivity to isoniazid, increased sensitivity to rifampin and pretomanid, and equivalent sensitivities to moxifloxacin, bedaquiline, and meropenem as compared to Mtb 18b cultivated in streptomycin-containing medium [148].

Later, a genome-wide analysis of the streptomycin-deprived Mtb 18b was performed involving RNA sequencing. The Mtb 18b transcriptional profile of was similar to the Mtb transcriptome profile for the microaerophilic phase of hypoxia models [96,151] and macrophage models [102]. In these models, the DosR regulon genes showed the greatest degree of concordance [152].

Various bacterial pathogens are capable of forming a subpopulation phenotypically resistant to antibacterials; the cells of such subpopulations are referred to as persisters. Unlike the bacteria with genetically determined resistance, persisters do not grow in culture media in the presence of antibacterials but are able to restore the population to original level after the transfer to the culture medium without the drugs [153]. The studies of transcriptional and translational profiles of *E. coli* persisters have demonstrated that they are in a dormant state [154]. To identify the genes associated with the formation of Mtb persisters, a full genomic sequencing and transcriptome analysis of mutant Mtb mc2 6020 strain were performed [155]; this strain was produced using an auxotrophic strain with a high level of phenotypic resistance to streptomycin and rifampin. The mutants produced 100–1000-fold more persisters than the wild-type strains including the bacteria with a drug

resistance to the antibiotics not used when producing the persisters. The sequencing identified several tens of genes associated with the persistent state, including the genes involved in lipid biosynthesis, carbohydrate metabolism, toxin–antitoxin system, and transcription regulation. This suggested that Mtb persisters are formed via multiple metabolic shifts, including those associated with the state of bacterial dormancy. The survival of a subpopulation of persisters in vivo during anti-TB therapy may contribute to the recurrence of the disease. However, Torrey et al. [155] did not study any other characteristics of a dormant phenotype.

4. Concluding remarks

The data presented above demonstrate that the development of Mtb dormancy is a rather complex process, which radically remodels the genetic, metabolic, and functional systems of the mycobacterial cell. Obviously, the results obtained with the in vitro dormancy models cannot be directly extrapolated to the processes in an Mtb-infected organism, since such models are unable to reproduce the multifactorial cell and tissue microenvironments and the impact of the immune system on the pathogen. In fact, these models have their intrinsic limitations similar to any other culture system. Nevertheless, the culture models are valuable for obtaining the data on the mechanisms of dormancy at the level of a bacterial cell, including the molecular ones. In addition, the culture models are the first and necessary step in the study of the effects of antibacterials on the dormant forms of pathogens.

Another limitation of the culture models is associated with assessment of the effects of a dormancy inducer on the Mtb replicative capacity with evaluation of the bacterial growth in culture media. Only a retrospective estimation is possible in this case, while a precise evaluation of the studied population for heterogeneity in dormancy is unfeasible. More promising are the noncultural assays allowing for quantification of viable microbial subpopulation, for example, fluorescence microscopy or flow cytometry with functional dyes. It is also necessary to assess the relationship between the metabolic and functional shifts of mycobacterial cells and their replicative capacity.

Note that the cultural methods used for the modeling of Mtb dormancy are constantly evolving from the simplest techniques based on the deficiency of nutrients to multifactorial multiple stress models utilizing special technical devices and targeted chemical reactions [102,103,105,132,142]. The use of state-of-the-art DNA and RNA sequencing methods and proteomic profiling enables obtaining a great amount of data on the Mtb biology. In this context, culture models remain one of the principal sources of information about the nature of Mtb dormancy.

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Appendix A. Supplementary data

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