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REVIEW PAPER

Dormant Forms of Mycobacteria

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Abstract—Dormant states of bacteria with drastically decreased metabolic activity, enhanced resistance to harmful factors, and absence of cell division is a form for surviving unfavorable environmental conditions. This state does not necessarily imply formation of highly differentiated spores and cysts; it has been demonstrated for non-spore-forming bacteria, including pathogenic ones. The latency of a number of infectious diseases is generally believed to be related to the capacity of bacteria (including *Mycobacterium tuberculosis*, an infective agent of tuberculosis) to produce dormant forms. Indeed, some results of histological investigation and modeling of latent infections in animals, as well as results obtained with in vitro models, support the hypothesis of production of dormant forms by tuberculosis bacteria. In the present review, existing experimental models of dormant form production in mycobacteria are considered, as well as modern data concerning the mechanisms of their formation and their relation to the "nonculturable" state. The mechanisms of reversion to culturability and the role of extracellular factors in reactivation of dormant forms are discussed in detail.

Key words: mycobacteria, nonculturable cells, dormant forms, resuscitation.

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Under unfavorable growth conditions, bacteria are known to pass into a dormant state with drastically decreased metabolic activity, enhanced resistance to harmful factors, and absence of cell division. Dormant states of bacteria have been traditionally associated with formation of highly differentiated spores or cysts. However, the possibility of transition into a dormant state was recently experimentally confirmed for non-spore-forming bacteria; it results in formation of less differentiated, cystlike forms which are different from spores [1].

Production of dormant forms by non-spore-forming mycobacteria, including *Mycobacterium tuberculosis*, an infective agent of tuberculosis, is of special interest, since the latent form of tuberculosis infection is believed to be related to transition of bacteria to a dormant state inside the host organism [2]. The nature of the latent state of tuberculosis bacteria and the mechanisms of its regulation are still poorly understood. According to a widespread notion, slowly growing pathogenic mycobacteria *M. tuberculosis* and *Mycobacterium leprae* may survive in vivo in a dormant state for a long time after infection [2, 3]. The dormant forms of *M. tuberculosis* probably persist for years in a host organism with subsequent transition into the active state and, therefore, activation of the disease.

The dormant state is understood here as a *reversible* state of a bacterial cell, when the level of metabolic activity is decreased and the cell is able to survive for a long time without division [4]. Such dormant cells usually exhibit peculiar morphology, a thickened cell wall, and enhanced resistance to harmful external factors [1]. Standard plating techniques are often inadequate for accurate enumeration of microbial dormant forms, because some of them may be in a "nonculturable" state. However, the "unaccounted for" bacteria may resume growth under favorable conditions.

In the course of evolution, microorganisms developed various strategies to counteract unfavorable environmental factors [4]. These factors include nutrient and oxygen limitation and extreme temperatures, as well as the effect of nonoptimal pH values, oxidants, and specific antibacterial metabolites produced by other bacteria or by specialized cells of the host [5, 6]. Bacteria possess a specific system for autoregulation of growth and development; it involves extracellular metabolites, anabiosis autoinducers (d_1 factors or alkylhydroxybenzene derivatives), which participate in control of cell differentiation at the level of regulation of the functional activity of subcellular components and of the cell as a whole [1, 7–9].

Decreased metabolic activity is usually the first response to a stress factor. If this slowdown is not prolonged, bacteria may restore their normal activity after the conditions have reverted to optimum. In the case of persistent stress, transition to a dormant state is possible, either as highly differentiated forms (e.g., spores; in this case metabolism is not detected) or as weakly differentiated resting cells of non-spore-forming microorganisms that exhibit low or undetectable metabolic activity. To obtain such dormant cells, limi-

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tation by some nutrient source is usually applied, which switches on the mechanisms of division arrest and resistance. The starving cell population may persist either due to survival of a small number of bacteria at the expense of the death of most of the cells (cryptic growth) [10] or due to the dormant (anabiotic) state of some of the cells in the population [4].

In some cases the resting cells become "nonculturable" (NC) while retaining their viability; i.e., they lose capacity for germination and growth under conditions which are optimal for the vegetative cell of the species (including standard agar media). For NC cells to revert to growth, a reactivation process is required; similarly to transition to the NC state, the latter is still poorly understood.

In this context, the term "nonculturable" has an operational meaning ("nonculturable" under given conditions and for a given physiological state) [11]. This is not a necessary characteristics of a dormant state. Generally, the "nonculturable" condition is related to a specific strategy of a bacterial cell, preventing its exposure while preserving its capacity for reversion of the growth processes under specific, favorable conditions.

MYCOBACTERIA AND LATENCY OF INFECTION

Numerous pieces of direct and indirect evidence suggest that nongrowing tuberculosis bacteria are present in the human organism. Understanding of the processes occurring between infection of a human by *M. tuberculosis* and activation of the disease is based on the results of a tuberculin test, which indicates undoubted presence of the infectious agent. Comstock et al. [13] analyzed the data obtained for about 0.5 million patients and concluded that the incubation period for tuberculosis may be from several weeks to an entire life [12]. Age plays an important role in the postinfection development of the disease: the first peak occurs in early childhood; the second, in late adolescence; and the third, in the population 65 years old or more. This late peak is due mostly to the presence of dormant forms in the organism, which persisted after the initial infection (50 years earlier) and activated as the result of decreasing immune protection of the aging host organism [13].

In 1952, Medlar et al. [14] reported that, while microscopically detectable mycobacteria were present in most of damaged organs (lungs and spleen), they did not form colonies on agar media. Since therapeutic and surgical actions depend on whether the cells are dead or are capable of reactivation causing a relapse of the disease [16], these observations, confirmed by a number of other authors [15], are of special importance. Khomenko et al. have been investigating the different cell forms of *M. tuberculosis* in human tissues. He concluded that small (filterable) forms of *M. tuberculosis* exist, which are probably responsible for persistence of the tuberculosis agent. The forms isolated by Khomenko did not grow on standard media. However, when introduced into an animal organism, the filterable forms caused development of the tuberculosis process in many cases and the organism was culturable on standard media [17].

Biketov et al. demonstrated that *M. tuberculosis* cells isolated from cultured macrophages were unable to form colonies on solid media but could grow in liquid media after incubation with the Rpf reactivating protein [18]. Dhillon et al demonstrated that only 1–5% of the mycobacteria isolated from the tissues of animals with chronic tuberculosis formed colonies on solid media, while resuscitation of these cells in liquid medium resulted in a 20- to 100-times higher number of culturable cells [19]. In general, the results of these works suggest the role of "nonculturable" bacteria in development of "latent" tuberculosis.

Certain features of "nonculturable" cells of *M. tuberculosis*, including their decreased metabolic activity, characterize them as dormant forms.

Dormant Forms of M. tuberculosis in Experiments on Animals

The following experimental models are regularly used to obtain dormant forms of *M. tuberculosis* in an animal organism in imitation of latent tuberculosis: the model of chronic tuberculosis, the Cornell model, and the model of artificial granuloma.

Models of chronic tuberculosis. Sever and Youmans [20] infected mice intravenously with different doses of *M. tuberculosis* and observed the development of the disease. The division rate of tuberculosis mycobacteria in the lungs remained stable for about 2 weeks. The number of bacteria then stabilized and remained constant for at least 80 days (4×10^4–4×10^6 CFU depending on the initial infective dose). The authors and other researchers [20, 21] have demonstrated that nutrient deficiency in the tissues was not the only factor responsible for repression of bacterial metabolic activity, so that transition to a metabolically inactive state most probably resulted from the immune response of a host organism to infection [21].

Importantly, in the model of chronic tuberculosis mycobacterial cells are potentially capable of proliferation. For example, Orme et al. [22–24] infected 3-month-old mice with less than ten bacteria and observed the course of infection over the rest of their life. After a usual period of initial growth in lungs and subsequent transition into a nongrowing state, the bacteria remained inactive until the mice reached an age of 18 months, when they commenced rapid growth resulting in the death of the animals. Resumed growth of the mycobacterial population in 18-month-old mice was found to coincide with the weakened immune response in aging animals [22, 24].

Importantly, in the models of chronic tuberculosis, the bacterial cells were culturable.

The Cornell model. McCune and Tompsett demonstrated that in the mice injected with a combination of isoniazide and pyrazinamide at the day of infection, mycobacteria were not revealed on solid media inoculated with lung and spleen samples [25]. However, culturable tuberculosis bacteria were found in mice tissues after the termination of antibiotic treatment; the author explained this phenomenon as reversion of the dormant "nonculturable" forms to growth [26]. In a similar model, application of greater volumes of infective material and lower doses of antibiotics resulted in a 10^7-fold decrease of colony formation on solid media for the mycobacteria from the tissues of infected mice [27]. At the same time, the titer of genomic copies (PCR) revealed an only 30-fold decrease in the number of bacterial cells. Rifampicin, an inhibitor of RNA polymerase, was found to effectively suppress relapses of a latent infection [28]. Thus, RNA synthesis occurs in "nonculturable" bacteria and is required for survival of *M. tuberculosis* in the latent phase of the infection.

The absence of data on resuscitation of dormant bacteria in the Cornell model in vitro creates certain difficulties of interpretation. While PCR enables detection of numerous "nonculturable" cells, which may subsequently be reactivated in vivo, this method may also detect numerous dead cells. An increase in the number of colony-forming units (CFU) may result from growth of a very low number of the cells retaining their viability [29].

Model of artificial granuloma. The in vivo model of artificial granuloma in mice involving the implantation of polyvinylidene fluoride fibers with *M. tuberculosis* cells was developed in 2004 [30]. After some time, the granuloma was formed around these fibers. Mycobacteria within the granuloma were found to exhibit very low metabolic activity, although they remained culturable. Since the duration of the experiments on this model was limited to 28 days, this term was probably insufficient for transition of *M. tuberculosis* cells into a "nonculturable" state.

In spite of the value of the information obtained from in vivo models on mice, the course of disease in mice and humans is significantly different [31]. For example, aerosol infection with *M. tuberculosis* does not result in formation of a well-formed granuloma in mice; it consists of an aggregation of lymphocytes and macrophages, which is not characteristic of humans. However, the type of immune response in humans and mice infected with *M. tuberculosis* is similar and involves T1 helpers [31].

For imitation of tuberculosis persistence in humans, models of tuberculosis based on rabbits and guinea pigs were developed; the mechanisms of granuloma formation were in these cases closer to this process in humans. In these models, guinea pigs were more resistant to *M. tuberculosis* infection after vaccination with *M. bovis* [31]. Rabbits exhibited higher resistance to *M. tuberculosis* and higher sensitivity to *M. bovis*. Lethality in rabbits infected intravenously with *M. tuberculosis* was low [32].

The model using primates is probably the most suitable for research of latent tuberculosis, although also the most expensive one. Macaques infected by pouring of small numbers of bacteria (approx. 25 CFU) into the bronchi exhibited three scenarios of disease development: (1) fulminant disease for 3 months after infection, (2) its chronic form with decreased appearance of the clinical symptoms, and (3) the variant without visible clinical manifestations [33]. In the lungs of most animals not exhibiting manifestations of the disease, granulomas with dormant forms of *M. tuberculosis* were observed. The granulomas of primates and humans exhibited significant microscopic and immunological similarity [34]. The primate model is therefore the one most successfully reproducing the latent state of tuberculosis infection in humans.

In vitro Models of Formation of Dormant Cells of Mycobacteria

In vitro models are certainly very important for studies of latent tuberculosis; their advantages include greater simplicity and convenience of experimental manipulation. It is, however, unclear whether they can truly reproduce the bacterial persistent state in vivo, since the models do not imitate the immune system of the host, which is believed to play a key part in the regulation of mycobacterial activity in the case of latent infections.

The Wayne model. Wayne [35] demonstrated that, in the static cultures of *M. tuberculosis*, which have not been stirred throughout the period of incubation, two groups of bacteria emerge, dividing rods in the upper, oxygen-rich part of the test tube and nondividing cells in its anaerobic lower part. In the lower part of the test tube, *M. tuberculosis* cells were in a state of gradual oxygen exhaustion, accompanied by cessation of cell division [36]. In this state, the cells of *M. tuberculosis* stopped protein synthesis, which resumed by oxygen supply [37], as was described also for *M. smegmatis* [38] and *M. bovis* [39].

Since heterogeneity of precipitating cells prevents biochemical investigation of these cells, an improved variant of the Wayne model was developed [40]. In this model, closed test tubes were used, which contained magnetic stirring bars to ensure uniform oxygen regime and the homogeneity of the culture. Thus, the equilibrium oxygen concentration in the gas and liquid phases was created, up to complete consumption of oxygen. During the first 70 h of growth, the doubling time was 16.5 h [40]. At an oxygen concentration as low as 1%, most of the cells stopped division and acquired a state associated to the resting one ([^{3}H]-uracil incorporation stopped), with a thickened cell wall. Further incubation at anaerobic conditions (oxy-

gen level below 0.06%) resulted in complete cessation of cell division.

However, unlike the in vivo Cornell model, the dormant cells in the Wayne model were culturable. Transfer to a fresh aerated medium resulted in their synchronous division [41]. This model is presently popular for the imitation of transition of *M. tuberculosis* cells into a dormant state. We believe that the differences in cultivability possibly reflect different degrees of cell dormancy in a nondividing state, depending on the incubation time. The Wayne model probably represents the initial stage of formation of a dormant, "nonculturable" state in *M. tuberculosis*.

However, Brooks et al. [42] demonstrated that metronidazole, which has a bactericidal effect on *M. tuberculosis* cells under anaerobic conditions [36, 40], exerts only a weak effect on the resting cells of *M. tuberculosis* from mice with chronic tuberculosis. This is an indication of their metabolically active state. Therefore, they should not be considered true dormant forms, but are rather similar to the starvation-survival state [43].

It is therefore still not clear whether the cells of *M. tuberculosis* incubated under anaerobic conditions in the Wayne model are resting cells.

The Wayne model, in fact, reflects bacterial adaptation to low oxygen level rather than transition to the dormant state. Moreover, experimental evidence exists that, unlike granulomas in human tissue, in mice tissues *M. tuberculosis* cells do not encounter oxygen limitation [44].

Formation of dormant cells of *M. tuberculosis* as a result of prolonged poststationary incubation. The possibility of *M. tuberculosis* persistence in old cultures in vitro had been demonstrated as early as 1933 [45], when tuberculosis cells stored at 37°C in hermetically sealed vials without stirring were found to retain viability for 12 years.

Nutrient deficiency in the medium is believed to be one of the major reasons for growth cessation in the stationary phase [46]. However, nutrient limitation is not the only unfavorable factor affecting transition to the stationary phase. Apart from starvation by some of the components of the medium, changes in pH, accumulation of toxic compounds, and decreased oxygen concentration occur; some of the stress factors may result from the metabolic activity of bacteria. Numerous morphological, biochemical, and physiological alterations of the cells are observed during the stationary phase [47, 48]. In the stationary phase, microbial cells acquire resistance to a number of lethal stresses; for example, resistance of *M. smegmatis* to osmotic and oxidative stresses was demonstrated [49].

The stationary growth phase may thus be considered a specific type of stress; under such conditions bacteria can apply various strategies to survive unfavorable conditions and utilize various alternative metabolic pathways.

The model of dormant cell formation by *M. smegmatis*, a nonpathogenic relative of *M. tuberculosis*, is promising for investigation of mycobacterial dormant forms. These cells exhibit a "nonculturability" trait in the stationary phase under aerobic conditions [50] and may be considered as a model of latent tuberculosis infection in living organisms.

The cells of *M. tuberculosis* surviving in the stationary phase for a long time were capable of transition to the "nonculturable" state under anaerobiosis [51]. However, in nonhomogenous cultures grown without a detergent, fractionation by serial filtration was required to isolate the fraction of "nonculturable" cells. "Nonculturable" cells in the fractions obtained by filtration of cell suspensions through a 1.5-μm filter were a mixture of ovoid cells 0.8 μm long and small coccoid cells 0.5–0.7 μm in diameter. As was previously reported for *M. luteus* [52], they were smaller than dividing bacterial cells. Both cell types had undamaged membranes (no staining by propidium iodide) and did not exhibit respiratory activity (detected by reduction of the fluorescent CTC tetrazolium). In this model, however, CFU number increased after 4 months of incubation, probably due either to secondary growth of a small fraction of the cells in a heterogeneous population, which retained capacity for reversion to growth, or to spontaneous activation of "nonculturable" cells, or a combination of both processes [51]. In a more homogeneous culture grown in the presence of Tween, formation of "nonculturable" cells was detected after 3–4 months of incubation in the poststationary phase without access of oxygen [53].

While the Wayne model probably imitates the initial stage of dormant cell formation in *M. tuberculosis*, prolonged incubation of *M. tuberculosis* in the poststationary phase with decreased cell "culturability" may be a better representation of the situation in vivo.

Regulation of Gene Expression in Dormant Forms of M. tuberculosis

Transcriptome analysis, the experimental approach involving investigation of the global profile of gene expression in the cells, is widely used in the studies of the dormancy mechanisms in *M. tuberculosis*. Global transcriptome analyses have already been carried out for a number of persisting forms of tuberculosis mycobacteria obtained from in vitro models [54–58]. The profile of expression of a number of *M. tuberculosis* genes was also investigated for the cases of murine macrophage infection [59], from mycobacteria persisting in artificial granulomas [30], and for analysis of the clinical samples from patients with tuberculosis [60].

For mycobacterial cells obtained in the Wayne model (under hypoxia), elevated expression level was revealed for most of the genes of the Dos regulon, including the *dosS* and *dosR* genes, encoding the sen-

sory and the regulatory parts of the two-component regulatory system, respectively; the genes *fdxA* (ferredoxin), *metC* (homocysteine synthase), *lpgS* (lipoprotein of unknown function), *fprB* (probably NADPH reductase), *ahpD* (alkyl peroxidase), and *alkB* (involved in fatty acid metabolism) [57, 58]. Decreased transcription was observed for a number of genes responsible for growth, biosynthesis, and aerobic metabolism of *M. tuberculosis*. In the murine model of artificial granuloma simulating persistence in vivo, enhanced transcription levels of the genes of the Dos operon was also observed [30].

Bacon et al. investigated gene expression in a chemostat culture of *M. tuberculosis* under decreased oxygen content (1% of saturation) [55]. Significantly enhanced expression was reported for 77 genes, of which 33 were parts of the Dos regulon, including *fdxA* (ferredoxin), *pfkB* (phosphofructokinase), *narX* (nitrate reductase), *narK2* (the protein responsible for nitrite transport out of the cell), and *hspX* (α-crystalline), indicating the possible transition of nondividing *M. tuberculosis* cells to the so-called "anaerobic" metabolism. Positive regulation of the genes *accD2* (encoding acetyl/propionyl-CoA carboxylase) and *gltA1* (encoding citrate synthase) was also observed, as well as of the genes the products of which are transcription regulators and participate in mycobactin synthesis.

Transcriptome analysis of stationary-phase *M. tuberculosis* cells incubated under standard conditions [61] revealed activation of some of the Dos regulon genes, including those encoding the two-component regulatory system of stress response, as well as nitrate reductase, nitrite-binding protein, α-crystalline, etc. It may be therefore suggested that expression of the Dos regulon not only acts as a marker of a hypoxia state, but is also responsible for development of the universal cellular stress response. However, recent detailed transcriptome analysis of *M. tuberculosis* under oxygen limitation revealed that enhanced expression of the Dos operon genes under hypoxia was brief and occurred only at the initial stage. A more numerous group of genes was then activated, reflecting the "true" cellular response to hypoxia [62]. The authors therefore concluded that the induction of the Dos regulon under hypoxia was due not to oxygen limitation, but rather to exhaustion of nutrients and accumulation of toxic components in the medium.

In order to reveal the "universal" genes (those exhibiting enhanced expression in all models of *M. tuberculosis* persistence), the profiles of gene expression were compared for the Wayne model of anaerobic persistence [57, 61], the cells under microaerophilic conditions [55], the cells in the extensive stationary phase under aeration [61], and the stationary-phase cells under aerobic conditions [56], i.e., under conditions of limited cell proliferation. Comparative analysis revealed upregulation of a number of genes in all the models, primarily of those of the Dos regulon. For example, for the cells from the aerobic persistence model [56], a significant increase in expression was observed for two genes of the Dos regulon, Rv2629 and Rv2630, although the incubation conditions were characterized by high aeration (50% oxygen saturation). Some coincidences between models were revealed for regulation of the genes outside the Dos regulon, although only two of them exhibited constantly increased transcription levels in persisting bacteria: *acr2* and *rv*0885 [58], encoding Acr2, a chaperon-like protein resembling α-crystalline and similar in its properties to the Acr protein of the Dos regulon, and Rv0885, the function of which is still unknown.

Thus, under various stress factors, including conditions stimulating transition to the stationary phase, independent regulation of the genes of *M. tuberculosis*, which have been traditionally associated with hypoxia, may occur, not necessarily requiring oxygen limitation. In general, the results of analysis demonstrated metabolic modifications in the in vitro models of latent tuberculosis and reflect successful imitation of bacterial persistence in vivo.

Accumulation of sufficient array of data on the global expression profiles in various models of persistence and their subsequent analysis enable identification of potential candidate genes, the expression of which regulates transition to the "nonculturable" (dormant) state and is important for persistence. The products of these genes may be used as targets for development of new antituberculosis medication.

The genes involved in *M. tuberculosis* persistence in vivo are believed to fall into two groups: those responsible for transition to the persisting state and those responsible for its maintenance [63]. For example, the *M. tuberculosis* mutant lacking the *relA* gene (its product in ppGpp, a "strong response" mediator, regulating cellular activity [64]) was capable of normal growth in the macrophages and in vitro with citrate and phospholipids as carbon sources, but did not survive transition into the stationary phase or incubation under anaerobic conditions [65]. The expression of a number of genes in the mutant strain and in the wild type was different, including the genes encoding the reactivating protein: *rpfA* (enhanced expression) and *rpfC* (inhibited expression). These data demonstrate that the *relA* product is required for persistence under starvation, oxygen limitation, and in an extensive stationary phase, as well as in the chronic model and in the artificial granuloma in mice in vivo; it is one of the candidate for participation of the persisting state in humans.

Parish et al. investigated the phenotypic changes resulting from inactivation of *dosR*, the gene encoding the two-component protein of the stress response regulation system in *M. tuberculosis*, and concluded that it was involved in transition of the cells to the persisting state, since the mutant strain exhibited higher virulence than the wild-type cells. The authors suggested that this gene participates in the inhibition of bacterial

division in vivo and thus increases the probability of their disintegration by the immune system of the host [66].

In conclusion, the *M. tuberculosis* strain with mutations in the *rpf*-like genes should be mentioned. For example, the mutants were investigated in which one of the five *rpf*-like genes was individually inactivated [67]. No differences from the wild type were revealed in growth kinetics in vitro and in aerosol infection in vivo. However, the strain with the *rpfB* deletion exhibited slower reactivation in the chronic tuberculosis model on mice in vivo than both the wild type and the strains with inactivation of each of the other individual *rpf*-like genes [68].

Unlike the wild-type cells, the strains in which three out of five *rpf*-like genes were inactivated proved incapable of spontaneous reactivation when NC were transferred to the fresh growth medium [69]. Neither growth nor the dynamics and efficiency of transition to the NC state under anaerobic conditions in vitro differed from those of the wild type. It may be concluded that the Rpf proteins are directly involved in reactivation of persisting bacteria.

It is therefore clear that, in the investigation of genetic regulation of *M. tuberculosis* persistence in vitro, attention was mostly concentrated on the Wayne model of the nonreplicative state under anaerobic conditions. Although widely used in research due to its simplicity and good reproducibility, this model, as was mentioned above, is subject to criticism. As for the "nonculturable" mycobacterial cells, which are believed to participate in the development of latent tuberculosis, genetic regulation of the process of their transition to the "nonculturable" state remains poorly studied due to the absence until recently of in vitro models capable of adequate simulation of this state. Therefore, the newly developed model of formation of dormant cells with the "nonculturability" trait by aerobic poststationary phase cultures of *M. smegmatis*, a rapidly growing nonpathogenic relative of *M. tuberculosis*, seems promising [50].

Restoration of Growth Capacity in "Nonculturable" Forms of Mycobacteria

Analysis of the literature data suggests that dormant cell formation by non-spore-forming bacteria is a specific strategy of microbial response to unfavorable environmental factors. The dormant cells in many cases are "nonculturable," which hampers their detection by conventional techniques. Special procedures are required for reactivation of such cells and reversion to culturability.

Reactivation of NC forms is the key moment in the study of "nonculturable" states. Only resuscitation of NC forms confirms their fundamental difference from dead cells, i.e., viability as potential culturability. Decreased metabolic activity and morphologically intact cells are not sufficient criteria to confirm a *reversible* NC state and may hamper our understanding of the true nature of this phenomenon, especially considering the heterogeneity of the culture, which is a mixture of the cells in various physiological states [29].

The protein similar to the Rpf protein detected previously in *M. luteus* supernatant [70, 71] may be the factor promoting cell reactivation. The *M. tuberculosis* genome indeed contains five genes encoding Rpf-like proteins, which are probably secreted into the medium (since the protein molecule contains a signal peptide) and may act similarly to *M. luteus* Rpf in reactivation of "nonculturable " forms of *M. tuberculosis* [72]. In *M. smegmatis*, self-reactivation of NC forms transformed with the plasmid expressing additional Rpf [50] confirms the role of Rpf proteins in the process of reactivation. In *M. tuberculosis* cells isolated from animals with chronic tuberculosis, one of the Rpf-Rv1884c proteins (RpfC) is the most expressed; this finding is interpreted as indication of the role of this protein in growth maintenance under conditions of persisting infection [64]. The results obtained with *M. tuberculosis* mutants lacking three of the *rpf* genes also suggest that, at least in vitro, this protein family is required for reactivation of the dormant forms [69].

The individual Rpf proteins are probably interchangeable and perform similar functions in this process [67]. This interchangeability, however, is limited, so that a combination of several proteins is required for reactivation, although normal cell growth occurs in the absence of all the *rpf* genes [69, 73]. The mechanism of the activating effect of Rpf on NC cells is still not completely understood. The presence of a LysM motif in its C-terminal sequence, which is responsible for protein binding to peptidoglycan of bacterial cell wall [74] suggests that the effect of these proteins of NC bacterial cells, similarly to that of the germination-specific Lytic enzymes (GSLE) [75], is loosening of the thickened cell wall in dormant forms and bacterial spores. The spores or bacterial dormant forms thus become more sensitive to external factors (including high temperature and reactive oxygen species) and to trophic stimuli (amino acids, ions, etc.), which in turn may provoke activity of the microorganisms.

Microscopy of resuscitating cultures of *M. tuberculosis* revealed that, after 4–5 days, single ovoid and coccoid cells (0.5–0.7 μm) were found in small aggregates (2–6 μm); the size of the cells in these aggregates did not change. Then, approximately after 7 days, the size and shape of the cells changed: apart from aggregated coccoid cells, small (two to five cells) groups of rods appeared. After 15 days of incubation, the culture consisted only of rods, single and in aggregates of different size, typical of viable forms. Thus, changes resulting in cell aggregation occur during transition of *M. tuberculosis* to the culture state [53].

Calculation of the distribution of particle volumes in the population revealed that, although the number of aggregates in resuscitated culture was small com-

pared to the number of single cells, they comprised the main volume of the biomass. This distribution of cell forms is typical of the exponential-phase cultures of *M. tuberculosis* [53]. Formation of cell aggregates of different size and complexity is common among certain bacteria, including those of the high G+C group (nocardia, streptomycetes, and mycobacteria), even in the cultures grown in optimal liquid media [76].

In order to determine whether the rapid increase of CFU resulted from reactivation of "nonculturable" bacteria or from very rapid growth of a small number of surviving culturable cells, growth of *M. tuberculosis* under similar conditions was analyzed at the initial vegetative cell concentration of 3×10^2 cells ml^{-1} [53]. Growth of the culture with initially low cell concentrations of normally growing cells was found to occur at much lower rates than in the variants inoculated with "nonculturable" forms. These results indicate predominance of reactivation of NC over growth of vegetative cells, at least during the first days of cultivation [53].

Publications presenting successful resuscitation of NC cells are presently scarce. Since resuscitating NC cells are believed to combine the traits of differentiated resting forms and of damaged cells, i.e., both exhibit relatively high sensitivity and, similar to germinating spores, require specific activators (germinants), such investigations present some difficulties. Reactivation of "nonculturable" cells (resuscitation) may be difficult due to problems in selecting the reactivation conditions [11, 43]. Zhang et al. reported successful restoration of the colony-forming capacity in *M. tuberculosis* dormant forms on addition of phospholipids or specific peptides to the medium [77]. However, these conditions did not provide for recultivation of "nonculturable" cells obtained by prolonged incubation in the poststationary phase (Shleeva, unpublished data). At least two factors may be responsible for the failure to resuscitate NC forms. Inability to divide developing in the cells after resuscitation results from their unbalanced metabolism after profound stress [78, 79]. For resuscitation of NC dormant cells, poor media are therefore often used, which contain low concentrations of nutrients (including the carbon source) [80]. Synthesis of certain endogenous factors resulting in division arrest is another possible explanation [52]. It was noted that, although the membrane potential and respiratory activity may be restored at initial stages of resuscitation of NC forms, these events do not imply restoration of culturability [81].

Proposed Mechanisms for Transition to a "Nonculturable" State for Asporogenic Bacteria

The reasons for transition of mycobacteria to the "nonculturable" state remain unclear. The main issue is whether the loss of culturability results from a specific cellular program for surviving unfavorable conditions. It is believed that the two-component *devR* system of signal transmission in *M. smegmatis* is required for the regulation of cell survival under stress conditions [82]. In particular, *devR* regulates bacterial adaptation to oxygen limitation. The authors, however, do not exclude the role of *devR* in other variants of starvation. Formation on NC cells by a *devR* mutant under oxygen deficiency probably reflects the role of this regulator in modulation of cell metabolism and viability in the stationary phase. This may result in an NC state, depending on the intensity of unfavorable factors, not necessarily related to the oxygen regime.

One more hypothesis exists concerning the mechanism responsible for development of the general regulatory response, attuning the rates of intracellular metabolic processes with the changing environmental conditions and participating in transition of the cells to the NC state. This mechanism is maintained by the so-called toxin–antitoxin (TA) modules n prokaryotic genomes, where the toxin is a transcriptional or translational inhibitor, controlled by the antitoxin, which is able to inactivate the toxin by forming a complex. The toxin–antitoxin sites were initially characterized as plasmid killer genes [83]. It was, however, demonstrated that similar sites are present in the genomes of free-living prokaryotes [84]. Evidence exists that the TA sites are responsible for the modulation of the global level of translation or transcription under nutrient stress and probably at transition to the dormant state [85].

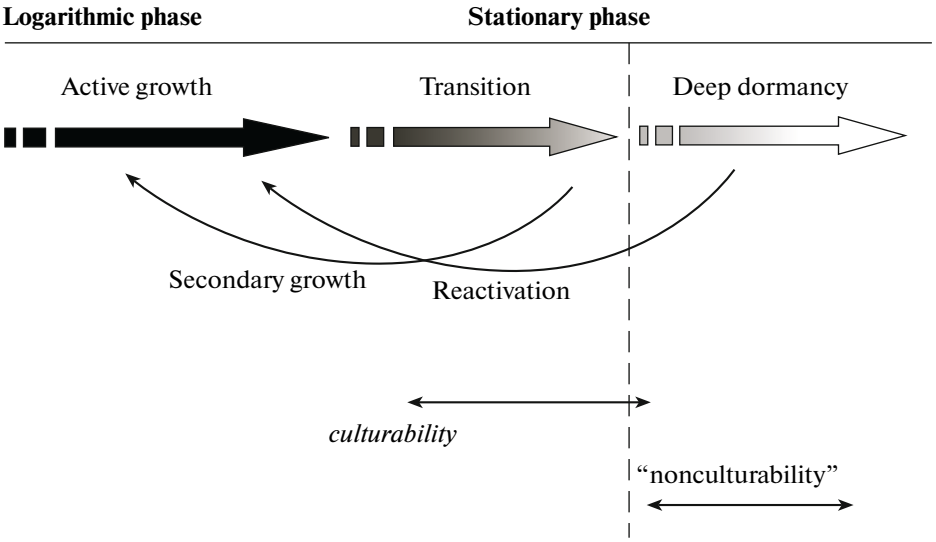
We propose a hypothetical outline of the stationary-phase processes resulting in formation of "nonculturable" cells (figure). Bacterial cells develop through several stages. After the stage of active growth, a transition state occurs in the stationary phase with lowered metabolism but high culturability (this phase probably corresponds to the Wayne model). It is followed by a state of more pronounced suppression of metabolism, with developing "nonculturability," when bacteria cease to form colonies on solid media and, later, to grow in liquid media.

Such forms require reactivation for transition to the culturable state. However, a stationary-phase population is a continuum of cells in different metabolic states and with different degrees of dormancy. This does not rule out formation of morphologically differentiated, persisting cells with increased resistance to external stresses [1, 4].

Thus, we believe that dormancy in non-spore-forming bacteria is not related to formation of one specific cell type, but may be to various degrees characteristic of different cell forms within a population.

CONCLUSIONS

Although the problems of latent tuberculosis and persistency of *M. tuberculosis* have been studied for decades, they are not yet solved. The exact nature of persistent organisms, their characteristics, and localization are extremely difficult to determine. Attempts



A hypothetical outline of the stationary-phase processes during transition of the culture to the "nonculturable" state.

were made to obtain models of bacterial persistency in vivo and in vitro. The results of recent research with such models made a significant contribution to our understanding of the biology of *M. tuberculosis*. It is, however, still not clear, whether the existing models reflect bacterial persistence in humans. Additional information is required in order to verify the existing hypotheses on the characteristics of persistent cells associated with the latent disease.

Modern molecular techniques, including transcriptome analysis, proteome analysis, and real-time PCR, are applied to characterize mycobacteria in various models of persistence and reveal the similarities and differences between them. These data confirm that the Wayne model of persistence reflects rather gradual adaptation of *M. tuberculosis* to hypoxia than transition to the dormant state. While adaptation to hypoxia may be an important factor promoting the formation of a dormant (or at least "nonculturable") state both in vivo and in vitro, other stress factors may also lead to adaptation and production of dormant forms.

From the microbiological point of view, the pivotal question is whether the *M. tuberculosis* dormant forms obtained in vitro are responsible for persistence. Unfortunately, this question still remains unsolved. The alternative hypothesis, relating persistence to the presence of a small population of completely active bacteria within the host, seems doubtful, since these bacteria should have been sensitive to antibiotics and therefore easily inactivated, which is not the case. Some in vitro models, including prolonged incubation in the poststationary phase without oxygen access [51, 77, 86] or under microaerophilic conditions after rifampicin treatment [87] demonstrate that viable cells of *M. tuberculosis* are capable of transition to a "nonculturable" state and that their colony-forming capacity may be restored by special procedures. This is similar to the situation with the Cornell model in vivo and the murine model of chronic tuberculosis, although in vivo a significant part of the cells is probably represented not only by the dormant forms. Taking into consideration the diversity of conditions within a human organism, as well as the heterogeneity of every conditionally homogeneous bacterial culture, we believe that a persisting population is a continuum of various cell types varying in their capacity for resumption of growth, level of metabolic activity, and resistance. Some of them may grow slowly, in spite of the control from the host immune system, while others are in a resting state with very low metabolic activity. Mycobacteria in the organism of the host probably exist in a variety of physiological states, thus necessitating prolonged antibiotic treatment for complete elimination of the infective agent [88]. The strategy of treating latent tuberculosis should be directed against various morphological and physiological forms of these bacteria. Detailed analysis of the patterns and mechanisms of mycobacterial persistence in adequate in vivo and in vitro models is a task for future investigation.

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