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EXPERIMENTAL ARTICLES

Formation of Nonculturable Cells of *Mycobacterium tuberculosis* and Their Resuscitation

M. O. Shleeva*, G. V. Mukamolova*, M. V. Telkov*, T. L. Berezinskaya, A. V. Syroeshkin**, S. F. Biketov***, and A. S. Kaprel'yants***

**Bach Institute of Biochemistry, Russian Academy of Sciences, Leninskii pr. 33, Moscow, 117071 Russia*

***Laboratory of Applied Hydrochemistry and Analytical Chemistry, State Institute of Oceanography, Russian State Committee for Hydrometeorology, Moscow, Russia*

****State Research Center for Applied Microbiology, Obolensk, Moscow oblast, Russia*

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Abstract—Nonculturable cells were found to occur in populations of *Mycobacterium tuberculosis* cells during the long poststationary phase. These cells were small (0.6–0.8 μm) ovoid and coccoid forms with intact cell walls and negligible respiratory activity, which allows them to be regarded as dormant cells. Nonculturable cells were characterized by low viability after plating onto solid medium; a minor part of the population of these cells could be cultivated in liquid medium. Cell-free culture liquid of an exponential-phase *Mycobacterium tuberculosis* culture or the bacterial growth factor Rpf exerted a resuscitating effect, increasing substantially the growth capacity of the nonculturable cells in liquid medium. During resuscitation of nonculturable cells, a transition from ovoid to rodlike cell shape occurred. At early stages of resuscitation, ovoid cells formed small aggregates. The recovery of culturability was associated with the formation of rod-shaped cells in the culture. The data obtained demonstrate the in vitro formation of dormant cells of *Mycobacterium tuberculosis*, which do not grow on solid media but can be resuscitated in liquid medium under the effect of substance(s) secreted by actively growing cells.

Key words: nonculturable cells, *Mycobacterium tuberculosis*, resuscitation, protein Rpf.

It is widely accepted that slowly growing pathogenic mycobacteria (e.g., *Mycobacterium tuberculosis* and *Mycobacterium leprae*) can persist for a long time in a dormant state in vivo after the onset of the infection process [1, 2]. Presumably, such dormant cells of *M. tuberculosis* can exist for many years in the host (latent infection). Subsequently, they can revert to the active state, which results in an active infection process. Until recently, no compelling evidence for the existence of mycobacteria in a dormant state in vitro or in vivo was available. The transition to a dormant state was documented in experimental studies with a number of other non-spore-forming bacteria [3].

We have established that the gram-positive non-spore-forming bacterium *Micrococcus luteus* can persist in a dormant state for a long time in a nutrient-depleted culture liquid after the onset of the stationary phase [4]. These dormant cells were characterized by a loss of the capacity to grow on solid and liquid media. However, the addition of cell-free culture liquid from a logarithmic-phase culture of *M. luteus* resulted in the induction of cell division and conversion of dormant cells to normal colony-forming bacteria [4, 5]. It was also established that *M. luteus* cells produce a factor (Rpf) that is released into the medium. It enables dormant cells of micrococci to divide [4]. Subsequently, Rpf was shown to be a protein with a molecular weight of 16–17 kDa, which exhibited its activity at picomolar concentrations [6, 7]. This protein promotes the resuscitation of nonculturable cells and reduces the apparent lag phase of an active *M. luteus* culture if a low dose of inoculum is used. The gene encoding protein Rpf was cloned and sequenced, and the protein was expressed in *Escherichia coli* and purified to homogeneity. Genes homologous to *rpf* were detected in a large number of microorganisms belonging to the group of G+C-rich gram-positive bacteria. According to the information available in databases, homologous genes are contained in bacteria of the genus *Mycobacterium*. *M. tuberculosis* has five such genes [6]. It seems likely, therefore, that the products of *rpf*-similar genes perform analogous functions, i.e., are involved in the transition from the dormant to the active state in mycobacteria.

The goal of this work was to develop a model of in vitro transition of *M. tuberculosis* to the dormant state and to investigate the behavior of dormant cells during their resuscitation.

MATERIALS AND METHODS

Mycobacterium tuberculosis (strain Akademiya) was obtained from the Phthisiopulmonology Center (Mos-

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cow). The bacteria were routinely cultivated without stirring at 37°C in 14-ml glass tubes with plastic screw caps; the tubes contained 2 ml of Saton medium supplemented with bovine serum albumin (5th fraction), glucose, and NaCl; this combination of supplements will be referred to hereinafter as ADC [8]. The culture was incubated in the poststationary state at 37°C for 8 months. To obtain solitary cells, the culture was passed through 50- and 20-μm glass filters and thereupon through five Whatman nitrocellulose filters with pore sizes of 4, 2, 1.5, 0.8, and 0.45 μm. Relatively homogeneous cultures of *M. tuberculosis* were obtained by growing the bacterium in 200 ml of the above medium in the presence of 0.05% Tween-80 with stirring (150–200 rpm) in a 750-ml flask with a tight rubber stopper. Syringes were used for anaerobic sampling.

Assessment of cell viability. A suspension of bacterial cells was serially diluted with fresh medium, and, after each dilution step, 100-μl aliquots were plated onto agarized Saton medium supplemented with AGS. The inoculated plates were incubated at 37°C. The resulting colonies were counted 2 months after inoculation. The CFU detection threshold was 5×10^0 ml^{-1}.

To resuscitate dormant cells, the serial dilution method (with subsequent cultivation in liquid medium) was used. After each dilution step, 200-μl aliquots were transferred to ten 14-ml tubes containing 2 ml of Saton medium supplemented with AGS. Five of these tubes also contained the protein Rpf at a concentration of 100 pM. The medium in five additional tubes was ADC-supplemented cell-free culture liquid obtained from a logarithmic-phase (1-month-old) *M. tuberculosis* culture by passing it through a 0.22-μm filter (Whatman). Thus, 15 tubes were used at each dilution step. The tubes were incubated at 37°C for 2 months without stirring.

The total cell number per milliliter of culture was determined in a Helber chamber using the formula $n/5 \times 10^8$, where n is the average number of cells in one small square.

Preparation of recombinant factor Rpf. Recombinant protein Rpf was obtained as described by Mukamolova *et al.* [6].

Cell distribution in terms of size and shape was assessed using a Malvern 3600Ec device, which determines particle size based on laser light diffraction data. A low-capacity He–Ne laser emitted a monochromatic light beam (λ_{max} = 633 nm) that passed through the experimental cell. Using Fourier lenses, the diffraction pattern was focused on a multielement photoelectric detector. The detector was directly connected to a computer that processed the data, starting from the integration of the set of diffraction patterns that reflect the instantaneous size distribution of the particles. All particles in the experimental sample moved through the illuminated zone due to continuous stirring. To assess the size distribution of cells, the formula $n_i = f(r_i)$was used, where n_i is the quantitative contribution of the size group r_i to the total cell population [9].

Table 1. Viability of cells of various size in a *M. tuberculosis* culture upon long-term storage

Viability (CFU/TCN)	Size of filter pores, μm
0.00000	0.45
0.00000	1.5
0.00001	2.0
0.00020	4.0
0.20000	10.0

Note: A 4-month-old *M. tuberculosis* culture grown in tubes with screw caps in ADC-supplemented Saton medium without Tween was consecutively passed through filters with pore sizes of 50, 10, 4, 2, 1.5, and 0.45 μm. TCN signifies total cell number as determined by direct microscopic count.

Fluorescent microscopy. The respiratory activity of bacterial cells was estimated using the fluorescent dye CTC (5-cyano-2,3-ditolyltetrazolium chloride, Eppelheim, Germany). The cells were incubated with 4 mM CTC (dissolved in 10 mM Na phosphate buffer, pH 7.0, immediately before the measurement) for 30 min at 37°C. Fluorescence was monitored with a Nikon fluorescent microscope at a wavelength of the

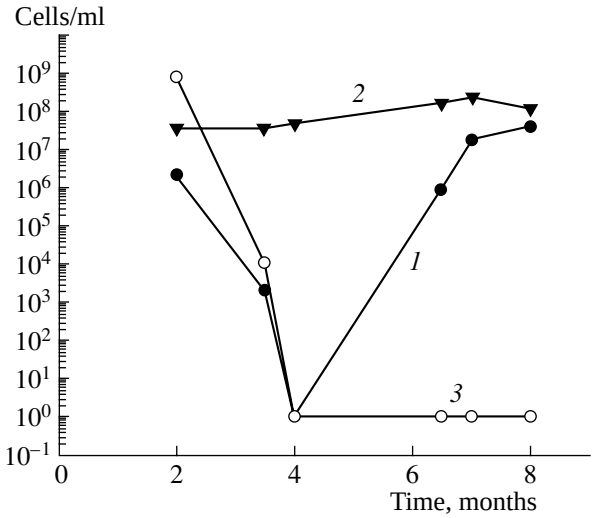

Fig. 1. Formation of nonculturable cells of *M. tuberculosis* during long-term incubation in the stationary phase. Logarithmic-phase cells of *M. tuberculosis* were inoculated on ADC-supplemented Saton medium. The culture was incubated at 37°C without stirring for 8 months in tubes with screw caps. Samples taken at regular intervals were passed through a filter with a pore size of 1.5 mm. CFU (*1*) and total cell number (*2*) were determined in the filtrates by plating and microscopic count, respectively. Another *M. tuberculosis* culture was grown in the same medium but supplemented with Tween-80 for 8 months at 37°C with stirring at 200 rpm. Samples taken at regular intervals were inoculated to determine the CFU numbers (*3*).

Table 2. Resuscitation of nonculturable cells of *M. tuberculosis*

Method of determination of cell number	Cells/ml
Microscopic count	3.8×10^7
CFU count	<5
MPN	1.1×10^5
MPN + Rpf	1.4×10^6
MPN + supernatant	5.7×10^7

Note: MPN signifies most probable number method (serial dilution followed by cultivation in liquid medium). To resuscitate nonculturable cells, we used the fraction obtained upon filtration of a *M. tuberculosis* culture through a 1.5-μm filter. Resuscitation was performed in ADC-supplemented Saton medium with or without recombinant Rpf protein (125 pM) or the supernatant obtained by centrifugation of a logarithmic-phase culture (3–4 weeks of cultivation) of *M. tuberculosis*.

excitatory light of 530–550 nm. The degree of membrane degradation was estimated by staining the cells with 4 μM propidium iodide in phosphate buffer. Rhodamine 123 was used to detect the generation of transmembrane potential [10]; fluorescence was recorded using a fluorescent microscope (the wavelengths of the excitatory and the emitted light were 510–560 and 590 nm for propidium iodide and 450–490 and 520 nm for Rhodamine 123).

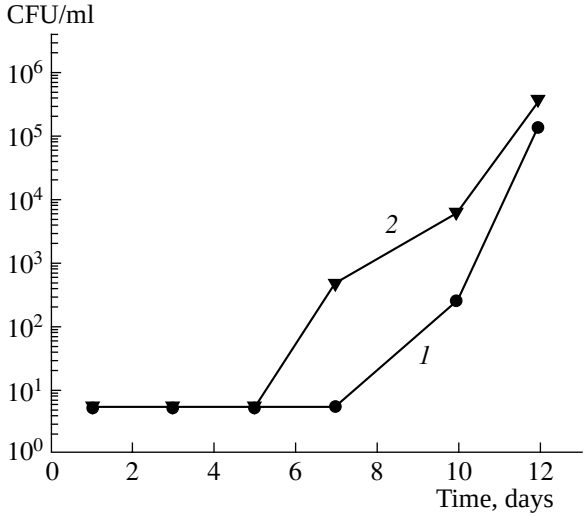

Fig. 2. Emergence of colony-forming cells during the resuscitation of nonculturable *M. tuberculosis* cells in liquid medium (the Tween-containing model). Four-month-old nonculturable cells of *M. tuberculosis* grown in Saton medium with Tween-80 were transfered at a concentration of 10^7 cells/ml to a fresh medium with (*1*) or without (*2*) 0.05% Tween-80. Cultivation was carried out at 37°C with stirring at 150 rpm in 100-ml flasks. Samples were taken at regular intervals to determine CFU numbers.

RESULTS

Transition of *M. tuberculosis* cells to nonculturable state. *M. tuberculosis* (strain Akademiya) was grown on ADC-supplemented Saton medium without stirring in 14-ml screw-cap tubes. Samples were taken 1 month after inoculation (at the end of the logarithmic phase) and, thereafter, every 2 weeks for 8 months. The number of viable cells was estimated by plating culture aliquots onto solid medium. Since the cells were grown without detergent, they showed considerable aggregation. To obtain solitary cells, the culture was passed through a series of filters with gradually decreasing pore size (20 to 0.45 μm). Based on our earlier data for *Micrococcus luteus* [11], we expected that nonculturable cells would be smaller in size than normal cells. The 1.5-μm filtrate lacked cells capable of forming colonies on agar-containing medium (Table 1), even though a large number of cells could be observed microscopically. The total cell number estimated by a microscopic count was 4×10^7 cells/ml (Fig. 1, curve *2*).

Nonculturable cells in the 1.5-μm filtrate were represented by a mixture of ovoid cells with a length of about 1.1 μm and smaller coccoid cells with a diameter of 0.5–0.7 μm. Both cell types had intact membranes (the cells did not stain with the fluorescent dye propidium iodide). They did not exhibit any respiratory activity in tests with CTC. The time dependence of the degree of nonculturability of cells of the 1.5-μm filtrate is plotted in Fig. 1 (curve *1*). The minimum viability level was reached 4 months after inoculation. Subsequently, the number of viable cells (determined as the CFU number) gradually increased and, after 8 months, approached the microscopically counted total cell number, which did not change during the whole experiment.

To obtain a culture with nonaggregated cells, *M. tuberculosis* was grown with a detergent (Tween-80). The culture was grown with stirring (200 rpm) in a flask with a tight rubber stopper on Saton medium supplemented with ADC and 0.05% Tween-80. Under these conditions, the CFU number decreased at the same rate as in the 1.5-μm filtrate systems (Fig. 1, curves *3* and *1*, respectively). The culture contained a large number of typical ovoid and coccoid cells after 4 months of cultivation (data not shown). These cells formed no colonies when plated onto agar medium. The state of nonculturability persisted until the end of the experiment (8 months after inoculation), in contrast to the static culture grown without Tween-80.

Transition of nonculturable *M. tuberculosis* cells to the active state. It was established by us earlier that resuscitation of dormant *M. luteus* cells was successful if carried out in liquid medium [3]. We used the serial dilution method with subsequent cultivation in liquid medium; this made it possible to simultaneously resuscitate cells in liquid medium and estimate the number of viable bacteria in the original culture [12]. The transition of ovoid and coccoid cells contained in the 1.5-μm filtrate of the 4-month-old culture of *M. tuber-*

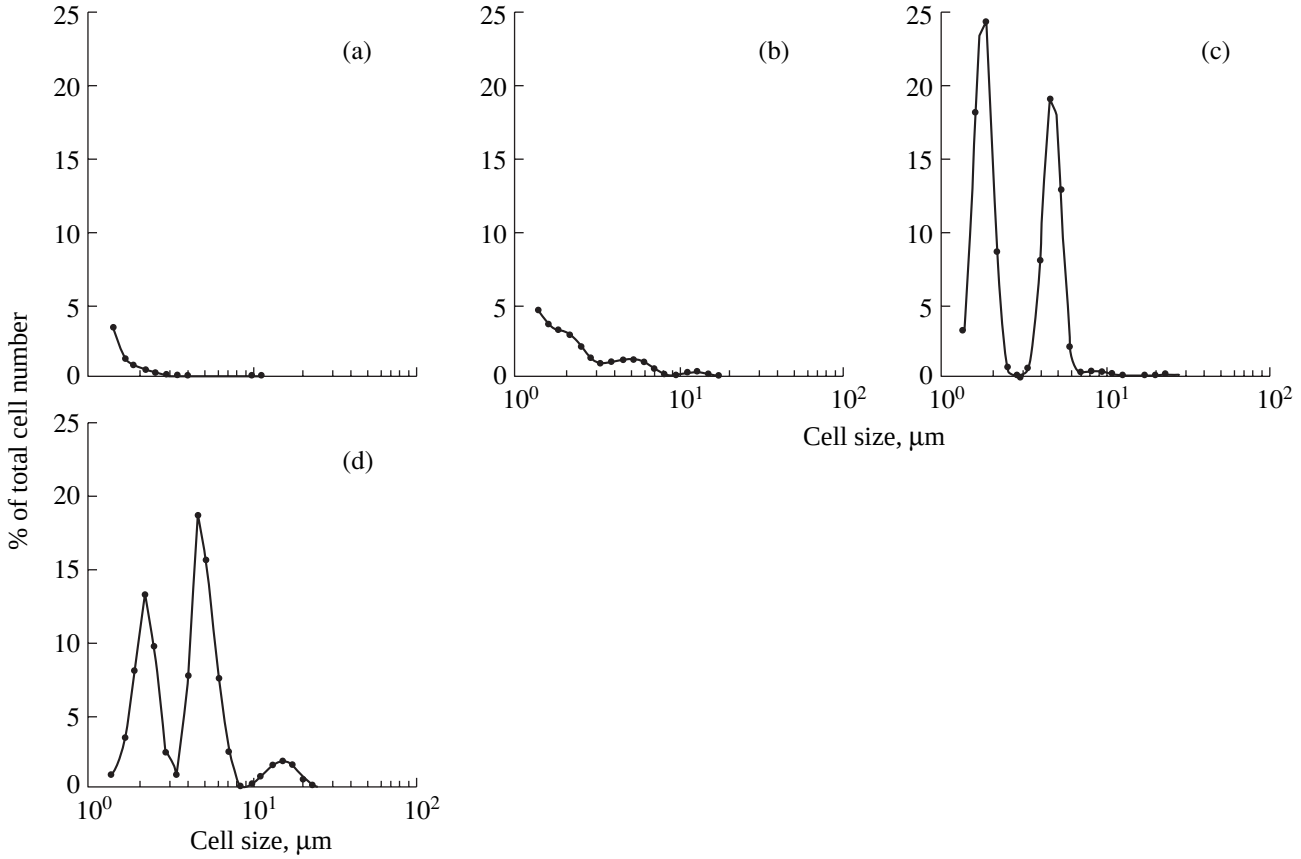

Fig. 3. Cell distribution in terms of size in the process of resuscitation of nonculturable cells of *M. tuberculosis* (the Tween-containing model). Samples were taken during the resuscitation of cells obtained using the Tween-containing system (see legend to Fig. 2), and the particle size in the culture was assessed using the diffraction method (a) immediately upon inoculation, (b) after 3 days, (c) after 5 days, and (d) after 7 days of cultivation.

culosis to the division-competent state was demonstrated by the serial dilution method followed by cultivation in liquid medium. In contrast to our previous experiments with *M. luteus*, estimation of cell viability by this method yielded values 5 orders of magnitude higher than values determined from the CFU number (Table 2). The presence of picomolar concentrations of recombinant protein Rpf resulted in an increase in the number of viable cells by 1–2 additional orders of magnitude. The use of cell-free culture liquid from a logarithmic-phase culture of *M. tuberculosis* for serial dilutions and further cultivation resulted in a still more significant increase in the number of viable cells (Table 2), which attained the microscopically counted total cell number in some experiments. However, cells that passed through the 0.45-μm filter did not convert to dividing forms in any of the systems described above (data not shown).

Nonculturable *M. tuberculosis* cells that were grown and stored for a long time anaerobically with Tween could convert to dividing forms in a fresh medium. Resuscitation of 5- and 6-month-old cells that had completely lost the capacity to form colonies on solid medium yielded results that were similar to those obtained with filtrates of heterogeneous cultures. The cell number estimate based on the serial dilutions and further cultivation in liquid medium was several orders of magnitude higher than the CFU number.

To monitor cell changes during the transition from the nonculturable state to the active growth state, resuscitation was performed in 100-ml flasks with 30 ml of medium (3-ml inoculum) with stirring. The cell distribution in the population in terms of size was assessed every 3 days by the laser diffraction method. Immediately after the transfer to a fresh medium, nonculturable cells pregrown with Tween were characterized by the following parameters: CFU, less than 5 cells/ml; microscopic count, 10^7 cells/ml. The CFU number started to increase 7 days after inoculation (Fig. 2, curve *2*) and amounted to 3.1×10^5 cells/ml by day 14 of cultivation. Changes in the composition of the cell population occurred as early as 3 days after inoculation (Fig. 3b). Based on the diffraction data, the cell size was 0.6–0.7 μm upon inoculation (Fig. 3a). After 3 days of cultivation, forms with a size of 1–2 μm also appeared; in addition, a small number of aggregates sized from 4 to 6 μm (Fig. 3b) occurred. The cells with a size of 0.6 μm disappeared after 5 days, and larger particles (2, 4–

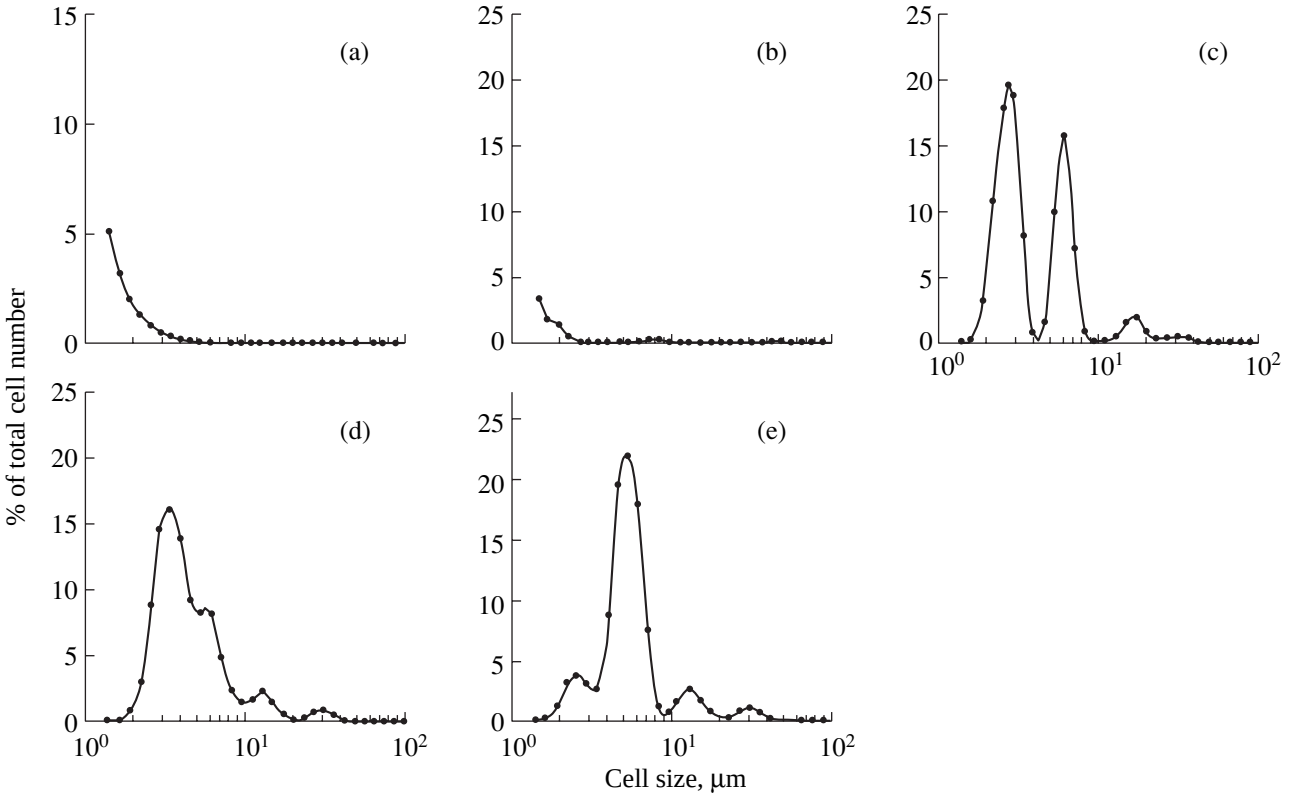

Fig. 4. Cell distribution in terms of size in the process of resuscitation of nonculturable cells of *M. tuberculosis* (the model without Tween). Samples were taken during the resuscitation of cells obtained using the Tween-lacking system (see legend to Fig. 1), and the particle size in the culture was assessed using the diffraction method (a) immediately upon inoculation, (b) after 3 days, (c) after 7 days, (d) after 9 days, and (e) after 15 days of cultivation.

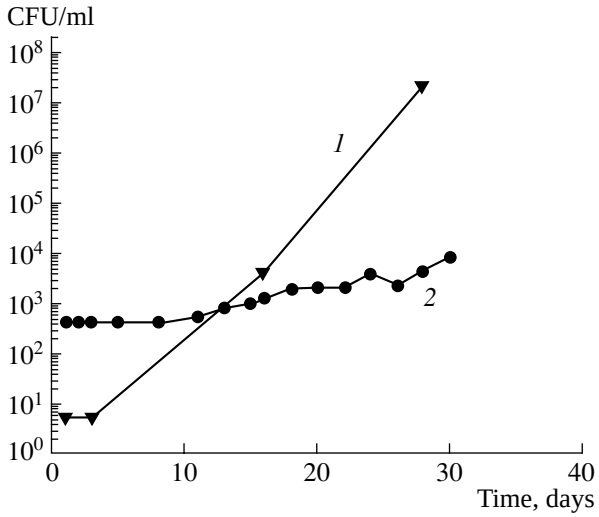

Fig. 5. Time dependence of the resuscitation of nonculturable cells of *M. tuberculosis* (the Tween-lacking model). Nonculturable cells of *M. tuberculosis* obtained using the Tween-lacking model were transferred to a fresh medium at a concentration of 10^7 cells/ml. Samples were taken at regular intervals to determine CFU numbers (*1*). Logarithmic phase cells of *M. tuberculosis* (3×10^2 cells/ml) were inoculated into Saton medium supplemented with ADC and grown under the same conditions as the nonculturable cells. Samples were taken at regular intervals to determine the CFU numbers (*2*).

6, and 10 μm in size) were detected. One week after inoculation, the population of *M. tuberculosis* cells was composed of particles with the same size (2, 4–6, and 10 μm) (Fig. 3c), but the ratio between the subpopulations was different (Fig. 3d). Analysis of the cell distribution in the population in terms of the particle volume showed that the main contribution to the total cell mass was made by cell aggregates, even though the number of aggregates appearing during the resuscitation process was insignificant in comparison with the number of solitary cells. This pattern of distribution of cells is characteristic of *M. tuberculosis* cultures during the logarithmic growth phase.

An analogous study was conducted with the cells from the 1.5-μm filtrate obtained from the culture grown in tubes (Fig. 4). In this system, we also observed an increase in the CFU number after 9 days of resuscitation, and, after 15 days, it was 2×10^7 cells/ml (Fig. 5). In order to find out whether the rapid increase in the CFU number was due to the resuscitation of nonculturable bacteria or to the logarithmic growth of the few culturable cells still remaining in the culture, we incubated the cells of an active *M. tuberculosis* culture (initial concentration, 3×10^2 cells/ml) under the same conditions (Fig. 5). It was established that a culture with such a low number of normally proliferating cells

in the inoculum grows much slower than the culture with nonculturable forms as the inoculum. If the resuscitation medium contained Tween, the morphological changes in the cells were not so drastic, and an increase in the CFU number occurred after 10 days of incubation (Fig. 2, curve *1*).

We conducted microscopic studies of the cultures during their resuscitation. Solitary ovoid and coccoid cells (0.5–0.7 μm) formed small aggregates (2–6 μm) after 4–5 days, and the cell size in the aggregates did not change. Changes in cell size and shape occurred after approximately 7 days. Small aggregates consisting of 2–5 rod-shaped cells were detected, in addition to aggregated coccoid cells. After 15 days of incubation, the whole culture was composed of rod-shaped cells, either solitary or occurring in aggregates of different size. Rod-shaped form is typical of viable cells of *M. tuberculosis.* Thus, prior to the transition to the culturable state, *M. tuberculosis* cells undergo changes resulting in their aggregation.

DISCUSSION

Although the formation of nonculturable forms was documented for a relatively large number of bacteria, virtually no evidence was presented for the reversibility of this process, i.e., of the ability of the nonculturable forms to revert to the active, division-competent state [13]; this diminishes the microbiological and medical relevance of such findings. It was earlier established that, during long-term storage, *M. luteus* cultures contained a large number of nonculturable cells that remained dormant for several months [3]. Evidence that these cells can regain the capacity to divide was obtained using techniques of resuscitating them in liquid media.

This study used similar methods to obtain and subsequently resuscitate nonculturable cells of *M. tuberculosis.* Like *M. luteus*, it belongs to the G+C-rich bacteria. *M. tuberculosis* cells incubated for a long time in the growth medium assumed the nonculturable state. However, isolation of the nonculturable cell fraction required a series of filtration procedures due to the heterogeneity of cultures grown in the absence of a detergent.

The increase in the CFU number after 4 months of incubation in the growth medium (Fig. 1, curve *1*) may result from secondary development of a minor admixture of viable cells remaining in the population of nonculturable cells, from spontaneous resuscitation of nonculturable cells, or from a combination of these two processes in the heterogeneous population. A more homogeneous culture grown with Tween failed to yield colonies during the long test period (Fig. 1, curve *3*).

There is a substantial difference between the nonculturable cells of *M. luteus* and *M. tuberculosis* with respect to the resuscitation process. A prerequisite for the resuscitation of nonculturable cells of *M. luteus* is the addition of cell-free culture liquid from an actively growing culture or of protein Rpf [6], whereas the number of viable cells of *M. tuberculosis* increased up to 6 orders of magnitude after cultivation in fresh Saton medium without specific additions (Table 2). Cell-free culture liquid or protein Rpf additionally enhanced the resuscitation efficiency against the background of fresh medium per se (Table 2).

Our research on the distribution of cells in terms of their size during the resuscitation process revealed that aggregates formed within the first 3–5 days of cultivation. Interestingly, colony-forming (normal) cells appeared significantly later. Possibly, the formation of intercellular contacts is prerequisite for the resuscitation of nonculturable cells. The implications of this finding are yet uncertain, and additional studies are necessary.

Our data clearly demonstrate that the term "nonculturability" has an operational meaning (nonculturability under the given conditions). Biketov *et al.* [14] showed that *M. tuberculosis* cells that failed to form colonies on an agar-containing medium grew in liquid medium after the addition of protein Rpf [14].

Presumably, a protein similar to the protein Rpf of *M. luteus* is the active factor in the *M. tuberculosis* cell-free culture liquid, although the stimulatory activity of Rpf was lower than that of the culture liquid (Table 2). Indeed, the *M. tuberculosis* genome contains five genes encoding Rpf-similar proteins, which may be released into the environment and may function like Rpf while resuscitating nonculturable cells of *M. tuberculosis.*

The nonculturable cells of *M. tuberculosis* can be considered dormant forms, based on a number of their properties (e.g., decreased metabolic activity). Wayne's model, in which cell suspensions of *M. tuberculosis* were gradually depleted of oxygen, is the best known model concerned with the transition of *M. tuberculosis* to the dormant state in vitro [15]. Presumably, protein synthesis is arrested in these dormant cells, to be resumed once oxygen is supplied to the culture [16]. A similar phenomenon was recently revealed in *M. smegmatis* [17] and *M. bovis* [18]. Unlike the cells in our system, the mycobacterial cells in Wayne's system retained high viability and developed metronidazole sensitivity, using anaerobic metabolic pathways [19]. This indicates that the cells remained metabolically active. Therefore, they cannot be regarded as true dormant forms and rather resemble cells in the starvation-survival state [4]. We suggest that the differences in the degree of culturability possibly relate to a different dormancy level of nondividing *M. tuberculosis* cells, due to the different duration of the culture storage periods (cultures were stored for several days in Wayne's study and for several months in this work). Long-term incubation of poststationary cells of *M. tuberculosis* may more precisely approximate the in vivo situation. Presumably, Wayne's model represents an initial stage in the process of the formation of a true dormant state of

M. tuberculosis. The fact that anaerobiosis was prerequisite for obtaining nonculturable cells of *M. tuberculosis* in our studies may point to a relationship between Wayne's model and the model used in this work.

We consider it probable that the capacity of *M. tuberculosis* to form dormant cells in vitro revealed in this work underlies the processes that are involved in the pathogenesis of latent forms of tuberculosis. Thus, the data obtained in this work may be of practical value in respect to the development of drug preparations for the treatment and prophylaxis of latent tuberculosis.

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