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EXPERIMENTAL ARTICLES

Intraspecies Diversity of Dormant Forms of *Mycobacterium smegmatis*

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Abstract—The non-spore-forming gram-positive bacterium *Mycobacterium smegmatis* mc^2 155, related to *M. tuberculosis*, was revealed to be capable of forming different types of dormant forms (DFs) during the life cycle of its cultures. The relationship between the intraspecies diversity of DFs and the cultivation conditions of the mycobacterium was established. The DFs possessed the following common properties: (i) maintenance of viability for a long period of time (5 months), (ii) resistance to deleterious factors such as heat treatment, and (iii) morphological and ultrastructural peculiarities that distinguish DFs from vegetative cells. The diversity of *M. smegmatis* DFs manifested itself in differences in terms of structural organization, conditions required for growth renewal, and capacity to produce antibiotic-resistant variants upon germination on selective media. Well-differentiated cystlike dormant cells (CDCs) were formed in the cultures grown in synthetic SR1 medium with fivefold-decreased nitrogen content. The structural organization of CDCs differed from that of other DF types mainly in the presence of club-shaped cells, thickened lamellar cell walls, coarse cytoplasm texture, and large electron-transparent triacylglyceride inclusion bodies. It was possible to use mycobacterial CDCs as a source of PCR-competent DNA. CDC populations were heterogeneous in cell buoyant density, and the individual fractions , which we isolated, were found to differ in thermal stability and the ability to revert to growth under standard conditions. Coccoid DFs, which retained their colony-forming capacity for a long time but were less heat-resistant than the CDCs, were formed by mycobacteria grown in standard Sauton's medium with initial pH value decreased to 6.2. Poorly differentiated DFs resulted from growing mycobacterial cultures in Sauton's medium with a fivefold-decreased phosphorus content. Upon germination of various DF types, the variants resistant to kanamycin (200 μg/ml) and tetracycline (20 μg/ml) were obtained. CDC suspensions incubated for 5 months demonstrated the highest percentage (1.5%) of antibiotic-resistant clones. The data obtained on the DF diversity of *M. smegmatis*, a fast-growing relative of *M. tuberculosis*, contribute to our understanding of the flexibility of the survival strategy of this bacterium in nature and in the host organism.

Key words: mycobacteria, dormancy, diverse morphological types of dormant forms, cystlike dormant cells, persistence, stress resistance.

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It is presently accepted that latent tuberculosis is due to the persistence of its causative agent, the gram-positive non-spore-forming bacterium *Mycobacterium tuberculosis*. It exists in the host organism in a special physiological state in which the cells do not reproduce, but retain the capacity for reversion to active growth, and, therefore, pose the threat of causing a disease [1–4]. Numerous attempts have been made to develop a model of persistence of *M. tuberculosis* in the host organism and under laboratory conditions. For example, a gradual decrease in oxygen concentration in *M. tuberculosis* cultures resulted in the cells' transition to the state of proliferative dormancy (without cell division). The cells remained able to form colonies and exhibited an elevated antibiotic resistance [5]. During long-term incubation under static conditions without oxygen, *M. tuberculosis* populations assumed a reversible nonculturable state with the formation of rounded cells without detectable respiratory activity. It was suggested that these cells be considered as dormant [6].

The rapidly growing nonpathogenic bacterium *Mycobacterium smegmatis* provides a model for investigating the forms and mechanisms involved in long-

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term persistence of the tuberculosis pathogen. Transition of *M. smegmatis* to a persistent (dormant?) state was demonstrated in response to gradual oxygen depletion in the culture [3, 7] and under carbon, phosphorus, or nitrogen starvation [8]. Nonculturable forms were generated under nonoptimum growth conditions [9]. The above strategies of cell survival during long-term incubation of laboratory cultures are apparently related to different physiological states of mycobacteria: (i) adaptation to stress factors (anaerobiosis or nutrient depletion), (ii) persistence associated with acquisition of antibiotic resistance, and (iii) reversible loss of the colony-forming capacity. It is assumed that the populations of stationary-phase *M. smegmatis* cells are heterogeneous and include metabolically active dividing and nondividing (dormant?) cells [8]. The nonreplication state of aerobic mycobacteria under microaerobic or anaerobic conditions is regarded as an adaptation strategy to cope with a lack of oxygen [3, 5–7]. In many respects, the dormant forms of *M. tuberculosis* and *M. smegmatis* and their properties have not yet been understood, despite numerous attempts to model their survival strategies.

Importantly, the laboratory cultures of *M. tuberculosis* and *M. smegmatis* incubated under the above conditions failed to form morphologically differentiated cystlike forms that have been revealed earlier in a wide variety of gram-negative and gram-positive bacteria [10–13]. According to the works cited, the formation of cystlike dormant cells (CDCs) capable of long-term survival, which is peculiar to most (if not all) nonspore-forming bacteria, occurs under intensified lipid formation and synthesis of reducing equivalents. This is attained by modification of the nutrient medium composition [10–13].

The goal of this work was to reveal dormant *M. smegmatis* cells in cultures developing under various conditions and perform comparative analysis of the properties of these forms promoting species survival, taking into account their capacity to revert to the actively growing stage, stress resistance, and morphological and ultrastructural traits of their cell organization.

MATERIALS AND METHODS

The subject of the study was the gram-positive bacterium *Mycobacterium smegmatis* mc^2 155 (ATCC 700084).

The bacteria were grown on standard Sauton's medium containing the following (g/l): L-asparagine, 4; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 1.4; trisodium citrate, 2; Fe ammonium citrate, 0.05; $ZnSO_4$, 0.001; and glycerol, 60 ml; pH 7.2 (10% NaOH). Sauton's medium was modified by (i) adding 0.2 g/l of $CaCl_2$, (ii) decreasing the L-asparagine content fivefold (to 0.8 g/l), (iii) decreasing the KH_2PO_4 content fivefold (to 0.1 g/l), (iv) creating a C > N imbalance (glycerol, 120 ml/l; sodium citrate, 4 g/l; and L-asparagine, 0.8 g/l), and (v) acidifying the medium (pH 6.2). Mycobacteria were also grown on synthetic SR1 medium containing the following (g/l): glucose, 20; KNO_3, 1, K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.5; and $CaCO_3$, 3; pH 7.2–7.4 [14]. This medium was modified by decreasing the KNO_3 and KH_2PO_4 contents fivefold (to 0.2 and 0.1 g/l, respectively). The culture was homogenized by the addition of 0.05% Tween-80. To obtain the DFs, the bacteria were cultivated in 750-ml flasks with 150 ml of the medium at 30 or 37°C on a shaker (180–190 rpm) for 3 days with subsequent 5-month incubation at 20°C under static conditions. The inoculum was a 3-day culture grown on Sauton's medium at 37°C. The inoculum volume was 200 μl per 100 ml of the medium.

Microscopy. Phase-contrast and epifluorescense observation was carried out using an Eclipse E4000 microscope (Nikon, Japan). Cell suspensions were stained with (i) a Live/Dead Baclight kit (Molecular Probes Inc.) to distinguish between live and dead cells, (ii) propidium iodide (3 μM) to detect damaged and dead cells, (iii) ethidium bromide (5 μM) to detect DNA in the cells, (iv) the tetrazolium dye CTC (1–3 μM) to monitor respiratory activity, and (v) Nile red (4 μg/ml) to detect the lipid bodies. Cell suspensions with dyes were incubated in the dark for 10 min at 37°C. Ultramicroscopy was carried out as described in [12].

Total cell numbers in suspensions were estimated by directly counting cells in a microscope in 20 small squares (2.5×10^{-3} mm^2) of a Goryaev chamber.

The colony-forming capacity of the cells (CFU/ml) was determined by diluting cell suspensions 10^N times, plating them on dishes with agar-containing media (1.5% wt/vol), and incubating for 7–14 days at 37°C. In addition, we determined the number of cells forming microcolonies (d < 0.2 mm) on the agar. The mCFU/ml titer was assayed using a binocular loupe.

Most probable numbers (MPN/ml) of cells capable of growth in liquid media were estimated using dilution cultures inoculated in wells of Corning plates (50 μl of suspension + 450 μl of medium). Viable cell numbers (CFU, mCFU, and MPN) were determined using the following liquid and solid (1.5% agar, wt/vol) media: standard NB medium (Himedia, India), Sauton's medium with 6% glycerol, twofold-diluted Sauton's medium with 0.6% glycerol, and semi-liquid NB medium (0.25% agar, wt/vol).

Heat resistance was assayed as numbers of viable cells (CFU, mCFU, and MPN) and intact cells (by staining with propidium iodide) after heating the cell suspensions (0.3 ml) in a thermostat at temperatures of 55–80°C for 10 min.

Antibiotic-resistant variants were detected on solid NB medium with kanamycin (200 μg/ml) and tetracycline (20 μg/ml).

The homogenous fraction of dormant cells was obtained as follows. Two milliliters of long-term incu-

bated culture were applied on top of sucrose solution (8 ml) with a 1.8–2.1 M gradient in 12-ml glass test tubes, which were centrifuged at 3000 *g* for 40 min. The cell fractions collected from various layers of the liquid were placed in sterile Eppendorf tubes, washed three times to remove sucrose, and resuspended in 1 ml of physiological saline.

DNA templates were obtained by (i) treating the cells with a chaotropic salt solution (a single-stage method) and (ii) additional incubation with proteinase K (100 μg/ml, 2 h) and washing three times to remove the enzyme (a two-stage method) [15, 16].

PCR was carried out in a PTC-200 thermocycler (MJ Research, United States). The incubation mixture (50 μl) contained the PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5–2.0 mM $MgCl_2$), a mixture of the four dNTP (0.2 mM of each), primer oligonucleotides (10 pmol/l of each), 1 μl of a suspension of DNA-containing templates, and a thermostable *Taq*-polymerase (2.5 U). A sample lacking template DNA served as a negative control. Ribosomal 16S RNA gene fragments were amplified using the primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and the 1522r (5'-AAGGAGGTGATCCAGCCGCA-3') [16]. The PCR protocol included denaturation (94°C, 30 s), annealing (60°C, 30 s), and synthesis (72°C, 60 s); 33 cycles were carried out. PCR products were analyzed electrophoretically in 1% agarose gel using the standard Tris-acetate buffer with ethidium bromide.

Statistical treatment of the data was carried out using the Student's test, assuming $P < 0.05$ as a sufficient significance level. The measurements were performed in triplicate, and three independent series of experiments were conducted. The results presented below are the mean values.

RESULTS

The main results of this work are: (i) compelling evidence that the generation of dormant forms (DFs) during the development cycle of *M. smegmatis* cultures results not only from a lack of nutrients, but also from the stress caused by acidification of the medium and (ii) elucidating the intraspecies diversity of the DFs that provides for their formation under various cultivation conditions. A comparative characterization of mycobacterial DFs is given below.

Differentiated cystlike dormant cells (CDCs). The main approach to produce *M. smegmatis* dormant cells that were morphologically well-differentiated included (i) cultivating the bacteria at 30°C on a shaker (180–190 rpm) for 3 days on the synthetic SR1 medium modified by decreasing the nitrogen content fivefold and (ii) subsequent incubation of post-stationary phase cultures at 20°C for 5 months under static conditions. Stored cultures were examined under a phase contrast microscope to detect the cells to be classified as CDCs because of their refractivity, cell wall thickening, and a sporelike head (Fig. 1a). The CDC percentage varied from 21 to 55% of the total cell number, depending on the incubation time.

Mycobacterial CDCs in nitrogen-limited cultures retained viability for a prolonged time, according to data obtained by inoculation on standard agar or liquid NB medium. In 5-month cultures, the CFU titer was 3.7×10^5 CFU/ml and the MPN was 2.2×10^7 cells/ml (Fig. 2a). In contrast to vegetative cells, CDCs were not stainable with CTC, i.e., they possessed no detectable respiratory activity. An important property of CDCs was their increased resistance to deleterious factors (heating). The percentage of CDCs that remained viable (based on the CFU values on standard medium) after incubating at 60, 70, and 80°C for 10 min was higher by two to three orders of magnitude than that of the vegetative cells (Tables 1 and 2). The number of heat-resistant cells in CDC populations increased during the incubation of poststationary phase cultures. The most significant contribution to the pool of thermostable CDC was due to increasing (up to 17%) of the proportion of cells resistant to heating at 60°C (10 min), according to the CFU and MPN values (Fig. 2b; Table 1).

A homogeneous CDC fraction was obtained from a 5-month culture (SR1 medium with nitrogen limitation) by centrifugation in a stepwise (1.8 M and 2.1 M) or linear (0–2.1 M) sucrose gradient. Microscopy revealed that 95–100% (depending on the gradient type) of the lower "heavy" fraction were CDCs, while rod-shaped cells were almost absent. Importantly, not all differentiated forms were located in this gradient zone, which was probably due to cell heterogeneity in buoyant density. The viability of CDC in the homogeneous fraction was 2.6×10^5 and 6×10^7 cells/ml (CFU and MPN, respectively), according to the results of inoculation on standard NB medium. This fraction differed from the total suspension in that it contained a higher percentage of thermostable CDCs, due to the cells that were resistant to heating not only at 60°C (17%), but also at 70°C (13%) (Table 1).

The following are the traits of the ultrastructural organization of differentiated CDCs of *M. smegmatis* distinguishing them from stationary-phase cells (Figs. 1k, 1l): (i) a pronounced outer electron-dense layer and additional electron-dense layers in the thickened cell wall (Fig. 1b), (ii) a clumpy texture of the cytoplasm (Fig. 1c), and (iii) large electron-transparent inclusion bodies (Figs. 1b–1d). According to the results of a test with the specific Nile Red dye, these bodies contained triacylglycerides. A lack of fluorescence in cells stained with propidium iodide, which penetrates only into injured or dead cells, indicated that the membranes were intact and their barrier function was retained. The thickening of CDCs cell walls probably accounts for hindered penetration of ethidium bromide or Live/Dead components into the cells, as was demonstrated by the lack of fluorescence after the standard sample treatment. However, part of the

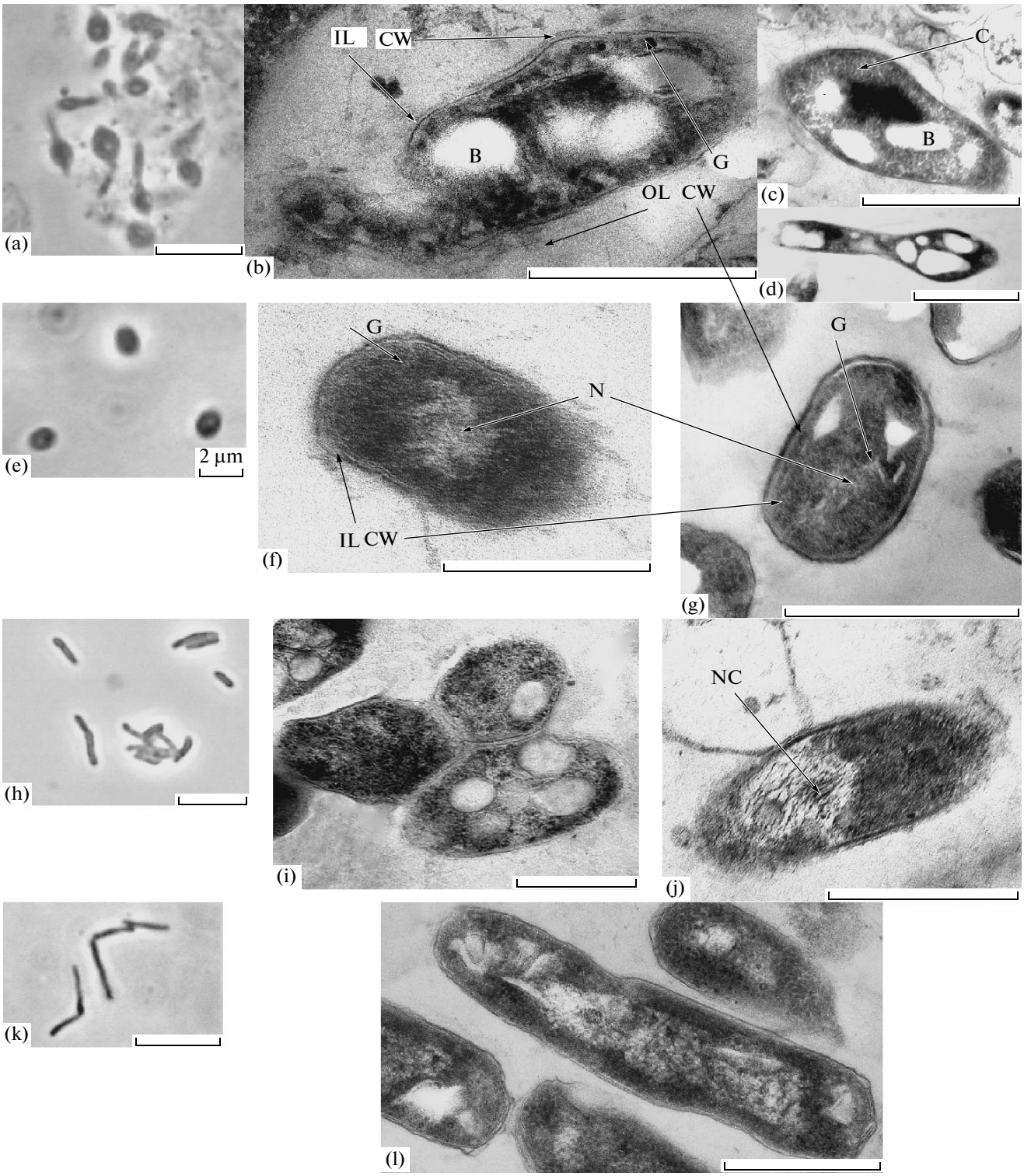

Fig. 1. Phase-contrast microscope images and electron micrographs of *M. smegmatis* cell sections: differentiated CDCs (SR1 medium, N limitation, 5 months) (a–d), coccoid forms (Sauton's medium, P limitation, 5 months) (e–g), poorly differentiated cells (Sauton's medium, P limitation, 5 months) (h–j), and stationary-phase cells (Sauton's medium, 7 days) (k, l). Designations: CW, cell wall; OL, outer CW layer; IL, individual CW layers; N, nucleoid; NC, nucleoid compactization zones (electron-dense DNA patches); G, electron-dense granules; B, electron-transparent bodies; and C, cytoplasm. Bar length: a, h, k, 5 μm; e, 2 μm; b–d, f, g, i, j, l, 1 μm.

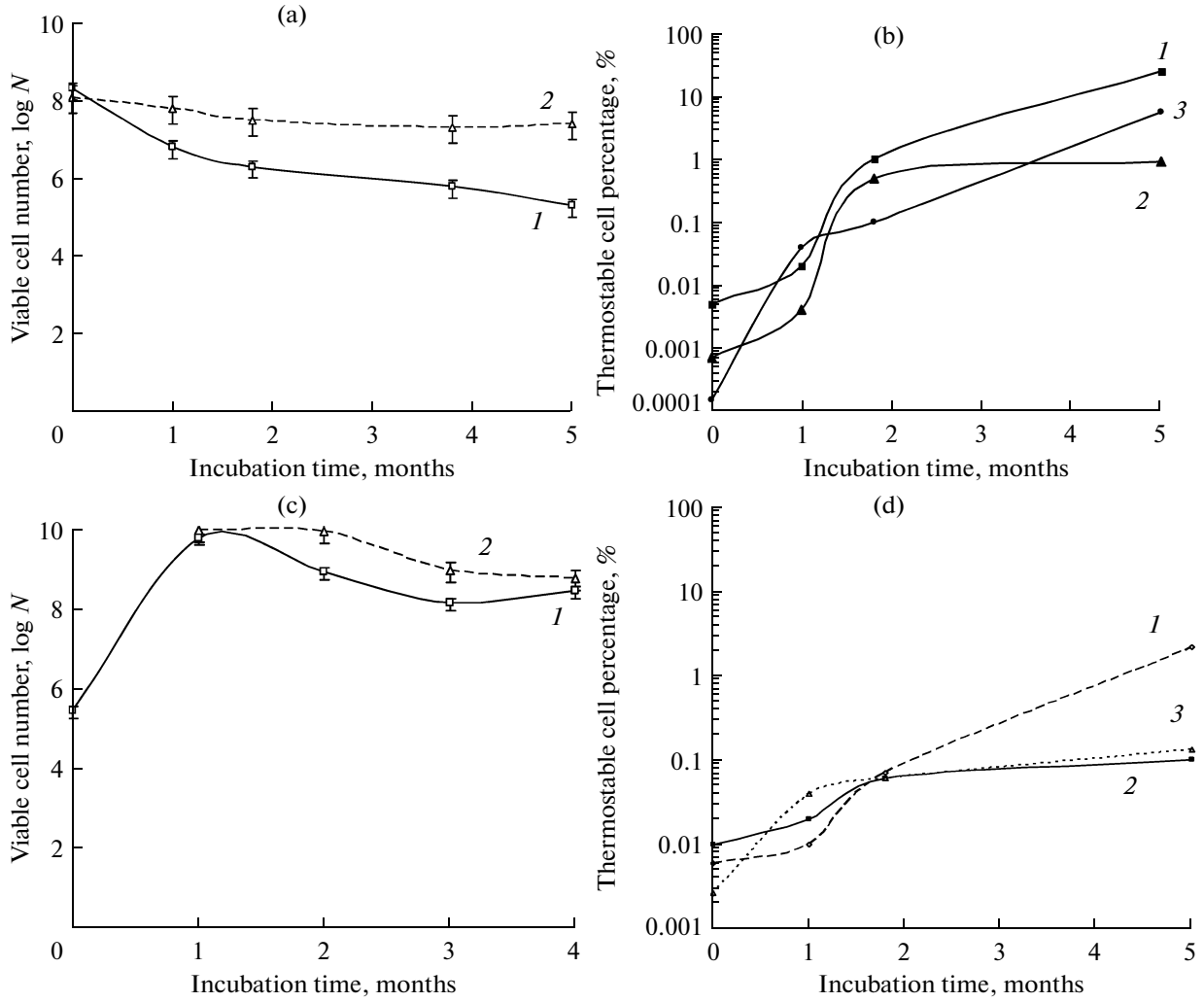

Fig. 2. a, c, Viable cell numbers (log N) in *M. smegmatis* cultures: SR1 medium with N limitation (a) and Sauton's medium (pH 6.2) (c) based on CFU/ml (*1*) and MPN (*2*) values. (b), (d), Changes in DF ratio during long-term incubation of *M. smegmatis* cultures (SR1 medium with N limitation) resistant to heating for 10 min at 60 (*1*), 70 (*2*), and 80°C (*3*). The ratio of heat-resistant cells was determined from the ratio between CFU/ml titers on standard NB agar (b) or maximum viable cell numbers (d) in heated and control DF suspensions.

CDC population displayed red fluorescence after heating at 60°C for 10 min and treating with ethidium bromide, which provided evidence that DNA was intact.

The thickened cell walls of *M. smegmatis* CDCs can hamper the isolation of the DNA template for PCR. Importantly, this is a general problem for working with endospores of bacilli and vegetative cells of some gram-positive bacteria [16–19]. Therefore, special techniques of one- or two-stage pretreatment (see the Materials and Methods section) were used, in order to achieve cell wall permeabilization [15, 16]. Both procedures were efficient in terms of obtaining DNA templates from differentiated CDCs, according to the results of electrophoretic analysis of amplification products (~1500 bp) using PCR with primers for 16S rRNA gene fragments (Fig. 3, *5–7*). In addition, it was established that using undiluted template samples obtained by applying both procedures to mycobacterial vegetative cells did not result in amplification of the target DNA fragments (Fig. 3, *1* and *3*). This might be due to the presence of unknown PCR inhibitors in the cells. A prerequisite for a successful PCR procedure was a tenfold dilution of DNA template samples prior to the procedure (Fig. 3, *2* and *4*). The data obtained suggest that it is in principle feasible to use differentiated mycobacterial CDCs and other dormant forms of microorganisms in studies in the field of molecular diagnostics, provided that the sample preparation technique ensures accessibility of their DNA to PCR amplification tools.

Plating *M. smegmatis* CDCs revealed a phenotypic heterogeneity of growing populations manifested in emergence of antibiotic-resistant variants, which was

Table 1. Numbers of viable and heat-resistant DF in 5-month *M. smegmatis* cultures determined from CFU titers and data obtained using combined assessment methods

DF type	Viable cell number based on CFU titer on the standard NB agar, CFU/ml	Maximum viable cell numbers on the optimum medium, units/ml
Differentiated CDCs (without heating)	$(3.7 \pm 0.6) \times 10^5$	2.2×10^7 MPN/ml, standard liquid NB medium
Differentiated CDCs (heating at 70°C, 10 min)	$(2.4 \pm 0.4) \times 10^3$ (0.64%)	2.2×10^4 MPN/ml, standard liquid NB medium (0.1%)
CDCs in the homogeneous fraction (without heating)*	$(2.6 \pm 0.6) \times 10^5$	6×10^7 MPN/ml, standard liquid NB medium
CDCs in the fraction (heating at 70°C, 10 min)	$(6 \pm 0.8) \times 10^2$ (0.25%)	$(1.0 \pm 0.1) \times 10^7$ mCFU/ml, diluted standard solid Sauton's medium (13.3%)
Poorly differentiated cells (without heating)	$(2.4 \pm 0.5) \times 10^7$	9.2×10^7 cells/ml, liquid Sauton's medium
Poorly differentiated cells (heating at 70°C, 10 min)	$(4.2 \pm 0.7) \times 10^4$ (0.18%)	$(1.3 \pm 0.3) \times 10^5$ mCFUs/ml, complete Sauton's medium (0.14%)

* The total cell number was 7×10^7 (counted in a Goryaev chamber). The ratio of heat-resistant cells is given in parentheses; it was determined from CFU titers (second column) or maximum viable cell numbers (third column) in heated and control DF suspensions.

Table 2. Ratio of heat-resistant cells in *M. smegmatis* dormant cell cultures

DF type	Ratio of cells* (% of the unheated control) resistant to heating (for 10 min) at:		
	60°C	70°C	80°C
Differentiated CDCs** (SR1 medium, N limitation, 5 months of incubation)	17.3	0.64	3.5
Coccoid cells** (Sauton's medium, initial pH 6.2, 4 months of incubation)	0.4	0.11	5.5×10^{-5}
	100% resistance to heating at 55°C		
Poorly differentiated cells ** (Sauton's medium, fivefold P limitation, 5 months of incubation)	0.33	0.18	0.18

Notes: * From the CFU values on the standard medium (NB agar).

** For comparison, the ratio of heat-resistant cells varied within the 0.01–0.06% range in vegetative cultures during the stationary phase.

determined from the ratio between the colony number on selective media with kanamycin (200 μg/ml) and tetracycline (20 μg/ml) and the CFU titer on an antibiotic-free medium. Kanamycin-resistant variants (the KR-Sm type) formed small R type colonies (d < 0.5–0.7 mm) that retained their resistance in transfers on the selective medium and partially (by 45–50%) reverted to the original phenotype on the NB agar. They also exhibited resistance to tetracycline (20 μg/ml of medium); the percentage of antibiotic-resistant TcR-Sm colonies among the KR-Sm variants varied from 12 to 20%.

The pattern of phenotypic variability in populations developing from CDCs on selective media with antibiotics depended on the culture's incubation time (physiological age). For example, inoculating stationary-phase 7-day cultures failed to produce KR-Sm and TcR-Sm variants. However, the aging of CDCs-forming cultures resulted in an increase in both cell number and percentage of the KR-Sm variant from 7 CFU/ml (0.00023%) in a 2-month culture to 3.3×10^4 CFU/ml (1.5%) in a 5-month culture (Fig. 4a).

Coccoid forms. A technique promoting the formation of coccoid dormant cells of *M. smegmatis* (65–70% of the total cell number) involved modification of Sauton's medium (adding 0.05% Tween to homogenize the culture and lowering the initial pH value to 6.2 to create nonoptimum growth conditions), cultivation at 37°C for 7 days on a shaker (180 rpm), and subsequent incubation under static conditions for 2–4 months.

Coccoid forms preserved for a long time the ability to form colonies on standard NB medium. The CFU titer in 2- and 4-month cultures was 9.2×10^8 and 3.0×10^8 CFU/ml, respectively (Fig. 2c). Similarly to CDCs, coccoid cells displayed no experimentally detectable metabolic activity in the CTC test. The cells of this morphotype were more resistant than vegetative

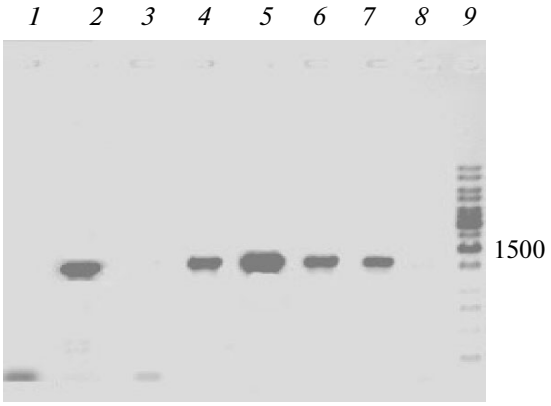

Fig. 3. Electrophoregram of the PCR amplification products of a fragment of the *M. smegmatis* ribosomal RNA gene. DNA-containing cell envelopes obtained by the single- and two-stage method were used as templates (see the Materials and Methods section). Designations: *1–4*, DNA templates from vegetative cells, obtained by the single- (*1, 2*) and two-stage (*3, 4*) method in native (*1, 3*) and tenfold diluted (*2*, *4*) preparations; *5–7*, DNA templates from CDCCDCs (SR1 medium, N limitation, 5 months) obtained by single- (*5*) and two-stage (*6, 7*) method in native (*5, 6*) and tenfold diluted (*7*) preparations; negative control (without adding DNA matrices to the PCR mixture) (*8*); and DNA fragment length markers (*9*).

cells to heating to 55°C, but not to 60–80°C (10 min), as CFU titers on standard medium demonstrated (Table 2).

The following are the traits of the ultrastructural organization of mycobacterial coccoid forms: (i) a round shape, (ii) a thickened cell wall with several layers that differ in their electron density, (iii) a fine-grain cytoplasm texture, (iv) electron-dense granules that are round in shape, and (v) a lack of electron-transparent bodies (Figs. 1e–1g).

Poorly differentiated forms of *M. smegmatis* occurred in a culture grown in Sauton's medium with a fivefold decrease in N or P concentration or an imbalance between C (increased twofold) and N (decreased fivefold), followed by long-term incubation under static conditions for 2–5 months. These cultures contained abundant cell aggregates, while the solitary cell ratio was 5–10%. Coccoid and refractile cells were not detected. The presence of aggregates in the cultures resulted in underestimating the viable cell number. Varying the composition of Sauton's medium influenced the dynamics of the numbers of persisting cells forming colonies on the standard NB agar. The optimum medium for obtaining viable DF populations was phosphorus-limited Sauton's medium. The CFU titer in 5-month cultures was 1×10^8/ml (50% of the initial CFU number in a 7-day, stationary-phase culture).

Populations of poorly differentiated DFs (phosphorus-limited Sauton's medium, 5 months of incubation) were characterized by (i) lack of dividing cells (based on the data of microscopy), (ii) retention of the

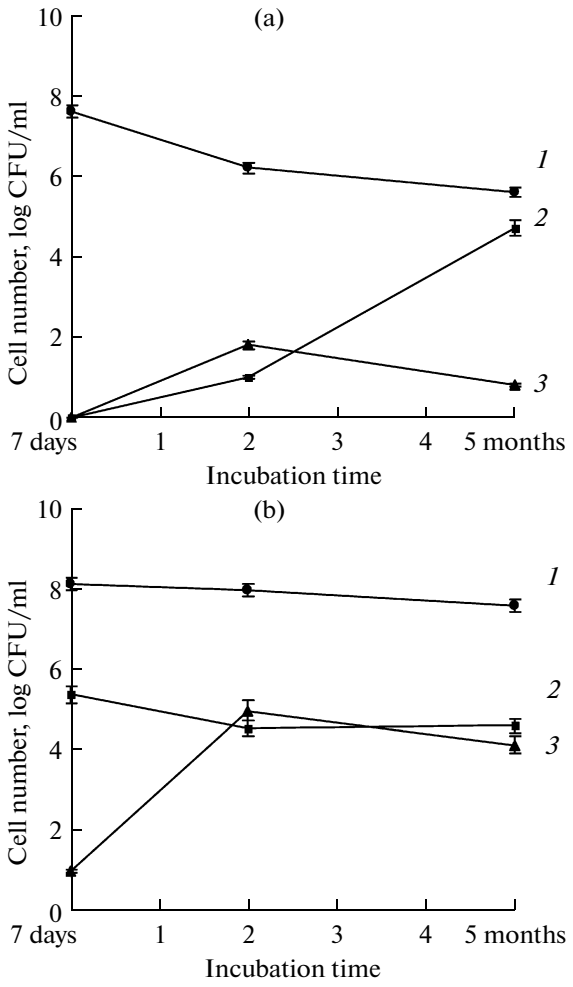

Fig. 4. Viable cell numbers (log CFU/ml) in *M. smegmatis* cultures grown in the (a) SR1 medium with N limitation and (b) Sauton's medium with P limitation during their long-term incubation. Total viable cell number upon inoculation on standard NB agar (*1*), kanamycin-resistant cell number upon inoculation on NB agar with kanamycin (200 μg/ml) (*2*), and tetracycline-resistant cell number upon inoculation on NB agar with tetracycline (20 μg/ml) (*3*).

colony-forming capacity on the standard medium for a long time (for 5 months and above) (Fig. 4b), (iii) lack of respiratory activity (according to the CTC test), and (iv) enhanced heat resistance at 60–80°C (10 min) in comparison to stationary-phase cells, according to the results of plating on standard media (Tables 1 and 2). The traits of the ultrastructural organization of the rodlike forms included (i) lamellar cell walls and (ii) compact nucleoid that contained electron-dense DNA strands (Figs. 1i and 1j).

In contrast to CDC populations, the emergence of antibiotic-resistant variants after inoculating poorly differentiated cells on selective media with kanamycin and tetracycline was more efficient after short-term incubation (for 7 days and 2 months), judging from the high KR-Sm and TcR-Sm colony numbers (around

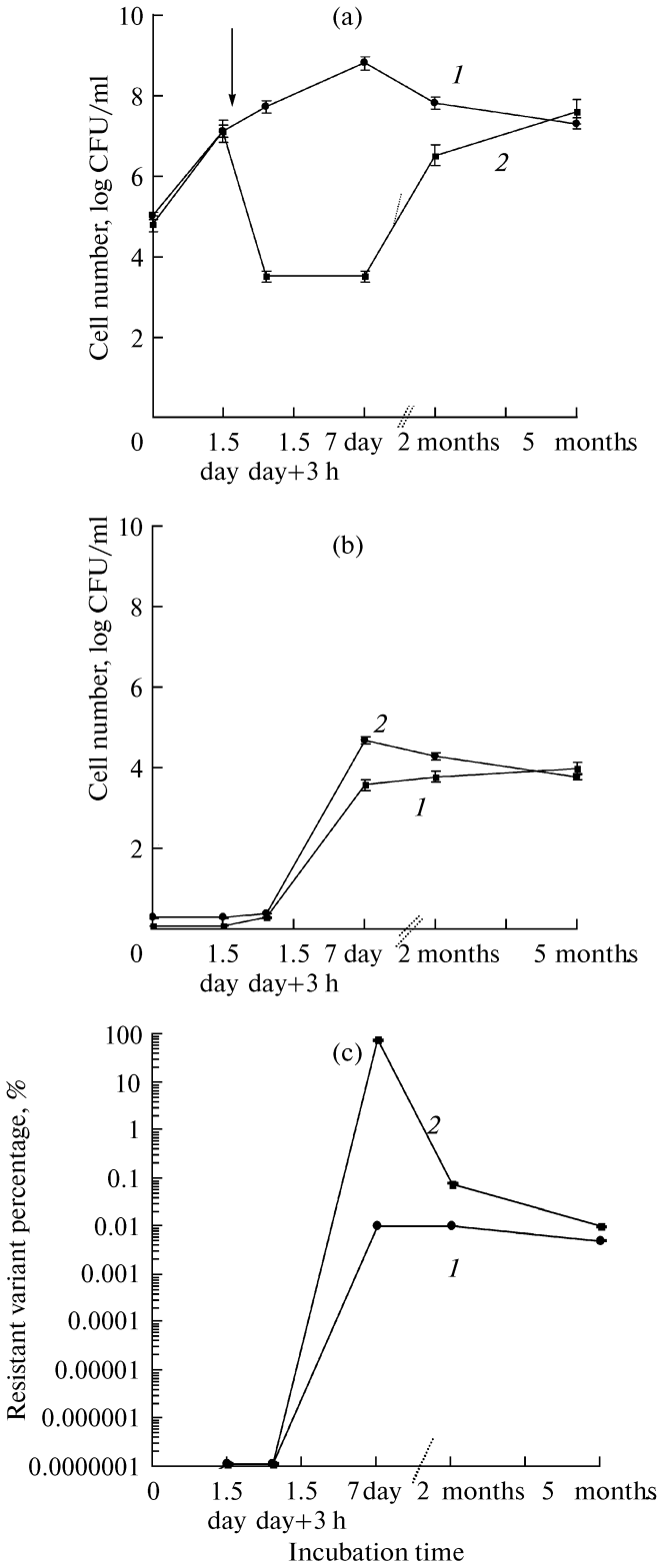

Fig. 5. Cell numbers (log CFU/ml) in *M. smegmatis* cultures incubated for a long time, grown on standard Sauton's medium in the control system (*1*) and after the addition of kanamycin (15 μg/ml) to an exponentially growing culture (*2*). Plating on standard NB agar (a); plating on NB agar with kanamycin (200 μg/ml) (b); and percentage of kanamycin-resistant cells (determined from the a/b ratio) (c). Arrow, time of kanamycin addition.

10^5 CFU/ml) (Fig. 4b). During further incubation (up to 5 months) of populations of poorly differentiated cells, the dynamics of KR-Sm and TcR-Sm colony numbers was identical to that of the total CFU number on the standard medium, whereas the percentage of antibiotic-resistant variants (~0.1%) remained virtually constant (Fig. 4a). Interestingly, the KR-Sm and TcR-Sm variants were characterized by low numbers and frequency in cell populations growing on standard Sauton's medium (0.03%) and on modified media with limited nitrogen concentrations (0.00003%) or a C > N imbalance (0.0025%) (data plots not shown).

Obtaining antibiotic-resistant subpopulations of *M. smegmatis* persistent cells was an additional important issue in terms of simulating the survival strategy of *M. tuberculosis* and other infections during severe stress resulting from antibiotic therapy. Moreover, it is relevant to the relationship between dormancy and persistency that has received much attention (see review [20]). In our studies, persisting cells were obtained by addition of kanamycin (5, 10, and 15 μg/ml) to an exponentially growing *M. smegmatis* culture (1.5 days of cultivation, Sauton's medium). Adding the antibiotic resulted in a decrease in the CFU number from 10^7 to 10^3–10^4 CFU/ml on the standard NB agar (Fig. 5a) and in conversion of the remaining viable cells to the KR-Sm type. The latter formed small colonies on the medium with kanamycin (200 μg/ml), constituting 95% of the surviving cell number (Fig. 5c). Electron microscopic studies of *M. smegmatis* populations after addition of the antibiotic revealed intact cells that resembled poorly differentiated cells. However, research on the properties and structural characteristics of cells in antibiotic-resistant mycobacterial subpopulations requires further investigation.

The subsequent (2–5 month) incubation of the cultures supplemented with kanamycin (15 μg/ml) was followed by an increase in viable cell numbers (determined from CFU numbers on the standard NB agar) to the level of the control culture growing in an antibiotic-free liquid NB medium under the same conditions. The number of cells forming KR-Sm type (with 200 μg/ml kanamycin) and TcR-Sm type (with 20 μg/ml tetracycline) colonies remained constant (Fig. 5b). Similar results were obtained if 5- or 10-μg/ml kanamycin was added to an exponentially growing culture (data plots not shown). Thus, a mycobacterial cell population treated with kanamycin during the active growth stage was restored owing to the antibiotic-resistant phenotype. The antibiotic-resistant cell pool in the control culture (without stress) detected on the NB agar with kanamycin was comparable, in terms of cell numbers (Fig. 5b) but not percentages (Fig. 5c), to the surviving cell pool after the addition of kanamycin (5–15 μg/ml) in an exponentially growing culture in liquid Sauton's medium.

Comparative analysis of viable cell numbers. We used solid and liquid standard NB-based media to

estimate the viability preservation for *M. smegmatis* DF during long-term incubation or after heating. However, using standard conditions and rich media often results in underestimated values of viable cell numbers in a DF population, especially if incubated for a long time or exposed to a high temperature. Therefore, we developed procedures for obtaining more precise values. They involved the use of combined techniques of assessing viable cell numbers based on CFU, mCFU, and MPN titers on various media, including diluted ones (to abolish the effect of substrate-accelerated death).

Well-differentiated CDCs. The maximum viable cell numbers in 5-month CDC cultures and in the CDC fractions were obtained using MPN titers in submerged cultures on the standard liquid NB medium. This value was two orders of magnitude higher than the CFU number on the NB agar (Table 1; Fig. 2a). The highest numbers of heat-resistant CDC (70°C, 10 min) were obtained by determining the MPN titer (on NB medium) for 5-month CDC cultures and by determining the mCFU titer on diluted Sauton's medium for the CDC fraction (Table 1). The tendency toward an increased ratio of heat-resistant cells during long-term incubation of the culture established by CFU counting on standard NB agar (Fig. 2b) was confirmed also upon MPN assays in heated and unheated (control) suspensions (Fig. 2d).

Coccoid and poorly differentiated forms. The results on the numbers of viable coccoid and poorly differentiated DF only insignificantly varied depending on the counting methods and growth media (Fig. 2c; Table 1). The titer of viable cells forming colonies on the standard medium was low in coccoid cell populations heated to 60–80°C for 10 min. This is in contrast to the high number of intact cells obtained by direct microscopic count of the cells stained with propidium iodide. The number of heat-resistant cells (70°C, 10 min) was more efficiently determined in poorly differentiated DF populations by counting mCFUs on rich Sauton's medium (Table 1).

Taken together, these data testify to a heterogeneity of *M. smegmatis* DF populations in terms of (i) their capacity to revert to surface (resulting in macro- or microcolony formation) or submerged growth on various media and (ii) resistance to deleterious factors. This heterogeneity was most prominent in well-differentiated CDC populations, which is in line with the data on the differences among DFs in terms of dormancy profundity and resistance to stress.

DISCUSSION

The main conclusion to be drawn from the data obtained is that the non-spore-forming nonmycelial actinobacterium *M. smegmatis* can generate several morphological types of long-term surviving forms during the development cycle of its cultures. Cystlike, coccoid, and poorly differentiated *M. smegmatis* cells are dormant forms in terms of the following criteria adopted in spore research field [21]: (i) long-term retention of viability (capacity for germination and proliferation), (ii) lack of experimentally detectable respiratory activity, (iii) resistance to deleterious factors, (iv) traits of ultrastructural organization, and (v) their formation during the development cycle of microbial cultures. The formation of dormant forms by mycobacteria developing under diverse conditions lends support to the assumption that dormancy is an inherent developmental stage of microbial cultures.

The second important conclusion stemming from the results of our studies is that *M. smegmatis* DFs exhibited intraspecies morphological and physiological diversity that manifests itself in bacterial cultures grown under various conditions. The diversity is exemplified by the occurrence of the following three DF types: well-differentiated cystlike cells, coccoid forms, and poorly differentiated cells. They differ in their ultrastructural organization, dormancy profundity, and resistance to deleterious factors.

In order of increasing cell structure complexity, the dormant forms may be arranged in the following sequence: poorly differentiated cells > coccoid forms > cystlike cells. The latter two types are characterized by thickened lamellar cell walls, while CDCs also display large electron-transparent bodies (Fig. 1) that contain triacylglycerides (TAGs) [22]. TAGs were also revealed in intracellular inclusions in the stationary-phase *M. smegmatis* cells grown under nitrogen limitation [23] and in nonreplicating *M. tuberculosis* cells isolated from sputum samples [23, 24]. It was suggested that the presence of TAG inclusions is a criterion of persisting (dormant) mycobacterial forms [24]. Comparative analysis of the ultrastructure of the three mycobacterial DF types obtained in this work also revealed other differences from vegetative cells, such as changes in cell wall structure and cytoplasm texture and nucleoid compaction (Fig. 1).

Increasing complexity of the ultrastructural organization of various DF types correlates with an increase in dormancy profundity, as the data on the specific conditions required for the DFs' reversion to vegetative growth demonstrate. In the 5-month cultures of differentiated CDCs and in homogeneous cell fractions, the viable cell titer (MPN) in a liquid medium (86%) was close to the total CDC number (based on microscopy data). It exceeded by two orders of magnitude the CFU value on the standard medium (Fig. 2a; Table 1). These results indicate that more favorable conditions are to be created for the germination of CDCs in a profound dormancy state by decreasing the oxygen amount in the liquid medium in comparison to a surface culture on a solid medium. Nonetheless, special germination conditions were not essential for less differentiated DFs. Their MPN and CFU values were of the same order of magnitude. An increase in the ultrastructural organization complexity of the DFs also results in their enhanced stress resis-

tance. In terms of thermal stability, DFs of different types and of the same age (4–5 months) are to be arranged in the following order: differentiated CDCs > poorly differentiated cells > coccoid forms.

Maintenance of the numbers of colony-forming cells in poststationary phase *M. smegmatis* cultures during long-term starvation is associated, apart from the existence of dormant cells, with the reproduction of a certain cell subpopulation [8, 25]. Obtaining pure fractions of DFs that are metabolically inactive but retain their proliferative potential is, therefore, evidence that they are responsible for the long-term survival of the species. This evidence is presented in this work and in our earlier publication [22]. We obtained the fraction of well-differentiated CDCs homogenous in buoyant density and morphology. However, this fraction was heterogeneous in the capacity of CDCs to revert to active growth under various conditions and of their heat resistance (Table 1 and 2). Retention of the intrapopulation diversity of DFs with regard to their proliferative potential and resistance appears to be an extremely important property of the microorganisms from the standpoint of microbial ecology and symbiogenesis.

Another level of the diversity of *M. smegmatis* DFs manifested itself in their variable capacity for phenotypic dissociation during germination on selective media, as determined by the frequency of the antibiotic-resistant variants. This diversity level depended on the conditions of DFs' formation and their physiological age (Fig. 4). The relation between dormancy and enhanced phenotypic variability was demonstrated for the DFs of representatives of various bacterial taxonomic groups [10]. Phenotypic variability was also demonstrated in starving *M. smegmatis* populations, when up to 75% of the dominant phenotype was replaced by a smooth colonies-forming variant [8]. However, the emergence of antibiotic-resistant phenotypes developing upon plating of the dormant (persistent) forms of this bacterium is described here for the first time and is of importance to practical medicine. The antibiotic resistance phenotype was revealed in a nonstressed *M. smegmatis* culture at the stage of vegetative growth (Fig. 5). These findings are of interest in terms of the survival mechanisms of the bacteria facing "unplanned" drug-induced stress and are apparently related to the persistency phenomenon [20]. On the whole, the phenotypic heterogeneity of DF populations in respect to their resistance to chemical and biological agents provides for a flexible survival strategy of the species involved.

CDC, coccoid, and poorly differentiated DFs of *M. smegmatis* were obtained under conditions that differ from those applied previously to obtain persisting cells in stationary-phase cultures developing under hypoxia [3, 7] and carbon, phosphorus, or nitrogen starvation stress [8] or in the suboptimal Hartmans–de Bont medium [9]. Not all mandatory parameters of prokaryotic dormant forms [21] were determined for these stationary-phase cells. Although the works cited and our data demonstrate that DF traits largely depend on cultivation conditions, they, nevertheless, are primarily determined by the constitutive properties of the genome. For instance, poststationary phase cultures of *M. smegmatis* transposon mutants exhibited a drastic decrease in the CFU number, in contrast to wild-type cultures. It was revealed that these mutants contained impaired genes homologous to the *M. tuberculosis* genes coding for the enzymes of biotin synthesis, polyketide synthase, and a penicillin-binding protein [26]. The formation of coccoid cells was established in the *M. smegmatis* transformant strain with enhanced expression of the *E. coli relA* gene that codes for the synthetase of guanosine tetraphosphate (the intracellular regulator of the stringent response to nitrogen starvation). However, the properties of these cells as dormant forms have not been elucidated [27]. A recent sensational work deals with endospore formation in *Mycobacterium marinum* and presumably in *M. bovis* BSG [28]. However, endospores have not been revealed in *M. tuberculosis* or *M. smegmatis* despite the long-term research on this subject. In conclusion, we emphasize that alternative dormant forms generated under diverse growth conditions, including cystlike and less differentiated cells with different structural and physiological properties, as well as other persisting forms [3, 9, 23], provide for the flexibility of the survival strategy of *M. smegmatis*, a rapidly growing species related to the tuberculosis pathogen.

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