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EXPERIMENTAL ARTICLES

Generation of Dormant Forms by *Mycobacterium smegmatis* in the Poststationary Phase during Gradual Acidification of the Medium

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Abstract—Persistent forms of the wild-type strain of *Mycobacterium smegmatis* and its mutants with inactivated *devR* and *hlp* genes were investigated. *devR* encodes the regulatory protein responsible for the formation of nonreplicating mycobacterial forms under hypoxia, and *hlp* codes for a histone-like protein. It has been found that a gradual decrease of pH in *M. smegmatis* wild-type poststationary cultures resulted in the formation of a special type of persisters. They significantly differed from vegetative cells in their properties and were represented by shortened ovoid forms with thickened cell walls. According to atomic force microscopy data, the size of the ovoid forms and vegetative cells was 1.2 × 0.9 μm and 3.7 × 0.8 μm, respectively. The metabolism level was markedly decreased in ovoid cells: the incorporation of [5,6-^{3}H]uracil and thymidine was decreased 200- and 50-fold, respectively. The intracellular ATP content was lowered threefold. The ovoid forms that emerged in poststationary cultures in Sauton's medium when the medium pH value was gradually decreased to 6.0 retained for a long time (9 months) the capacity to resume growth on rich solid and liquid medium. Compared to vegetative cells, the ovoid forms exhibited an elevated resistance to heating (60–80°C) and antibiotics (hygromycin, kanamycin, and tetracycline). The ovoid forms of the *M. smegmatis* wild-type strain were classified as dormant forms based on their survival capacity, resistance to deleterious factors, and structural peculiarities. The ovoid forms generated in poststationary cultures upon decreasing the pH value to 5.0 or below lost the colony-forming capacity. It was established that the capacity to form ovoid cells upon gradual decrease in the pH value to 6.0 was reduced in Δ*devR* and *hlp-0* mutants compared to the wild-type strain (generation of 5–6 and 40% dormant forms, respectively) The amount of *M. smegmatis* dormant cells formed correlated with the acidification degree of the medium. The model developed can be used in tests of new antibacterial preparations that effectively inhibit resuscitating mycobacterial dormant forms that persist in the host organism.

Keywords: mycobacteria, dormancy, ovoid dormant cells, nonculturable forms, stress resistance, medium pH.

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Mycobacterium tuberculosis (MTB) cells can exist in the human organism for a long period of time in a peculiar nonreplicative (dormant) state [1, 2]. Dormant MTB cells, widely believed to be the cause of latent tuberculosis, have not yet been isolated from host tissues because their number in infected organs/tissues may be extremely low [3]. In addition, dormant mycobacterial cells may be in the viable but nonculturable (VBNC) state, in which they are incapable of forming colonies on solid media that normally provide for growth of vegetative cells; therefore, they may be undetectable upon inoculation of clinical samples [4–8]. Despite the long history of in vitro and in vivo studies of MTB, dormancy in mycobacterial cells has not yet been well understood. This is in part due to the lack of in vitro models in which dormant mycobacterial cells could be generated.

It is natural to assume that dormant cells can be obtained in laboratory cultures under conditions that simulate conditions in the host organism. Upon penetrating into the organism, mycobacteria are engulfed by immune cells such as alveolar macrophages. Inside activated macrophages mycobacteria are subject to the influence of reactive oxygen species, nitric oxide, the hydrolytic enzymes of lysosomes, and low pH values. However, this does not result in total destruction of the tuberculosis pathogen [9]. Moreover, mycobacteria can survive in macrophages [10], e.g., at pH values of 6.1–6.5 [11, 12]. Taking into account the fact that vegetative cells of mycobacteria are sensitive to low pH values [13], it seems likely that some phagocyte-engulfed cells can assume the state of low metabolic activity or dormant state. In line with this, MTB pro-

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liferation in macrophages is followed by the transition of MTB cells to the VBNC state, and their resuscitation required liquid medium [4].

Thus, it can be assumed that decrease in the pH value plays a role in the transition of MTB cells to the dormant state. Confirming this suggestion requires in vivo experiments that will model the transition of the mycobacterial cells to the dormant state under such conditions. A question to be addressed in studies on the adaptation of vegetative cells to stress and their transition to the dormant state in poststationary cultures is how quickly unfavorable environmental factors are imposed on bacterial cells. Using the model in which mycobacteria convert to a nonreplicative state under hypoxia (Wayne's model), it was demonstrated that rapid oxygen depletion in the culture resulted in the death of most MTB cells, whereas slowly developing hypoxia promoted cell transition to the dormant state and caused no loss of their culturability [14]. On analogy with the results obtained with Wayne's model, we could expect MTB cells to assume a dormant state upon gradual decrease in the pH of the medium.

The rapidly growing nonpathogenic bacterium *Mycobacterium smegmatis* is a model for studying the forms and mechanisms of long-term persistence of tuberculosis pathogens under unfavorable growth conditions. For this model, cell transition to the dormant state upon slow oxygen depletion [15, 16] was shown, as well as the adaptation of stationary-phase populations to stress caused by carbon, phosphorus, or nitrogen starvation [17].

The goal of the present work was to study the generation of *M. smegmatis* dormant forms during gradual acidification of the medium.

MATERIALS AND METHODS

The subjects of this work were the following strains of the bacterium *Mycobacterium smegmatis* mc^2 155 (ATCC 700084): (i) the wild type, (ii) the hlp-0 mutant with the *hlp* gene inactivated by inserting the kanamycin cassette (provided by Thomas Dick) [18], and (iii) the ΔdevR mutant with inactivated *devR* gene of the two-component *devR* system (provided by Margaret Smeulders) [19].

The cells were initially grown in Nutrient Broth (Himedia, India) at 37°C for 24 h with stirring (220 rpm). The inoculum (1 ml) was added to 100 ml of Sauton's medium containing (g/l) K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 1.4; L-asparagine, 4; ferric ammonium citrate, 0.05; trisubstituted sodium citrate, 2; $ZnSO_4$, 0.001; and glycerol, 60 ml [20]. The initial pH value was 6.0. The culture was grown at 37°C on a shaker (220 rpm) for 16–20 days until a constant pH value of the medium was established. Tween-80 (0.05%) was added to the medium to obtain a homogeneously growing culture. Grown cultures were stored at room temperature in plastic test tubes with screw caps for 1–3 months. The cultures were periodically stirred, and samples were taken for measuring pH value and used as inocula. The mycobacteria were also grown on the synthetic 2AS medium we developed. It contained (g/l) glucose, 40; $MgSO_4 \cdot 7H_2O$, 0.125; NaCl, 1.5; $(NH_4)_2SO_4$ – 2.5; KH_2PO_4, 13.6; histidine (Sigma), 0.44; glutamic acid (Sigma), 4; trace element solution #1 [21], 4 ml; and Tween-80, 0.05%. The pH value was adjusted to 6.0 using NaOH.

Phase-contrast and epifluorescence microscopy was carried out using an Eclipse E4000 microscope (Nicon, Japan). Cell suspensions were stained with (i) propidium iodide (3 μM) to identify impaired or dead cells and (ii) the CTC tetrazolium dye (1–3 μM) to detect respiratory activity. Ultramicroscopic studies were conducted as described in [22]. Atomic force microscopy was performed using a SmartSPM microscope (AIST-NT, Russia). A cell suspension (6–10 μl) was applied to a freshly fractured mica surface and incubated at room temperature for 15 min. Then the excess material was removed by submerging the specimen for 1 min into a drop of distilled water. The washed specimen was dried. The images obtained were processed using FemtoScan software.

The colony-forming capacity (CFU/ml) was determined by plating diluted cell suspensions onto agarized Nutrient Broth (agar, 13 g/l) and Sauton's medium (agar, 1.5 vol %). The plates were incubated for 37°C for 5 days. The most probable number of cells (MPN cells/ml) capable of growing in liquid media was estimated using the serial dilution method; wells on plates were inoculated with 100 μl of the suspension of the relevant dilution and supplemented with 400 μl of twofold-diluted Sauton's medium with 0.6% glycerol.

The total cell number (TCN) in 1 ml of the culture was estimated using a Helber chamber (phase-contrast microscopy; 400x magnification) to determine the cell number in one small square. The mean value was calculated after counting cells in ten small squares; the relative error did not exceed 30%.

The DNA and RNA synthesis level in the cells was determined from the incorporation of hydrogen-labeled uracil or thymidine. The cell suspension was supplemented with 1 μl of [5,6-^{3}H] uracil or thymidine solutions with a radioactivity level of 10 μCi/ml in 50% ethanol and incubated for 4 h at 37°C with agitation. Then the cells were washed with 10% trichloroacetic acid and ethanol on glass filters (Whatman GFC) and the filters were placed in scintillation liquid. The radioactivity level was measured with a Beckman Coulter (United States) LS6500 counter.

To determine the intracellular ATP level, *M. smegmatis* cells precipitated by centrifugation at 3000 *g* were suspended in a small volume of DMSO and disrupted in a BeadBeater disintegrator (BioSpec Products, United States) with zirconium beads. Undestroyed cells were pelleted by centrifugation for 2 min

at 3000 *g*. The ATP content in the cell extract was estimated from luciferin/luciferase bioluminescence intensity determined using a LYUM-1 luminometer. Three replicate measurements were carried out with 0.02 ml of the sample solution and 0.18 ml of the ATP reagent (ApoSENSOR^{TM} ATP Cell Viability Assay Kit, BioVision).

The percentage of antibiotic-resistant cells was determined from the ratio between the CFU/ml values before and after incubating the cells with the antibiotic (kanamycin, tetracycline, or hygromycin) at a concentration of 100 μg/ml for 1 day. After the antibiotic treatment, the cells were washed with 50 mM phosphate buffer (pH 7.0) before inoculation.

Thermal stability was determined by direct microscopic count of the number of cells that remained intact (were not stained with propidium iodide) after heating cell suspensions (0.5 ml) at temperatures of 60–80°C for 10 min.

To obtain dormant cell fractions, 2 ml of a poststationary culture was carefully layered on top of a solution (8 ml) with a linear 1.4–2.2 M sucrose gradient placed in 12-ml glass tubes. The tubes were centrifuged at 3000 *g* for 40 min, and the cell fractions were transferred to sterile microtubes. The cells were washed three times to remove sucrose and resuspended in 1 ml of 50 mM phosphate buffer (pH 7.0).

The ammonium ion contents in the cells and culture liquid were determined by thin-layer chromatography after dansylation of the cell extracts and supernatant as described in [23].

The percentage of ovoid cells in *M. smegmatis* populations was determined from the mean values of direct cell count cells in ten microscope fields (with total number of counted cells no less than 1000).

HPLC was carried out using the Stayer (Aquilon) HPLC system. A 60-day culture supernatant (pH 5.8) was passed through a 0.5-kDa filter, and 5 μl was applied to a column for organic acid separation (Grace Davison Discovery Sciences). The separation was performed in 25 mM phosphate buffer (pH 2.5) at a flow speed of 0.5 ml/min.

The data were statistically processed using the Student's test. The significance level was set at $P < 0.05$.

RESULTS

Generation of Dormant Forms of M. smegmatis Upon Lowering the Medium pH Value in the Poststationary Growth Phase

Mycobacteria are known to be sensitive to changes in pH of the medium. The optimum growth of their cultures occurs on media with neutral pH [13]. While cultivating a wild-type *M. smegmatis* strain on Sauton's medium with a lowered initial pH value (6.0), we observed a significant lag phase (1 day longer than in the culture grown in a medium with initial pH 7.0–7.5). The lag phase was followed by an exponential growth phase characterized by medium alkalization to a pH value of 7.5–8.0 (Fig. 1a). During this growth period, the bacteria utilized asparagine, a substrate contained in Sauton's medium, and converted it to aspartate in a reaction catalyzed be asparaginase (MSMEG_3173). This was accompanied by ammonium ion accumulation and growth medium alkalization. TLC revealed that ammonium ions were accumulated intra- and extracellularly (Fig. 2). The subsequent cultivation of the wild-type strain was characterized by a gradual pH decrease to 5.8–5.7 over days 7–14 . A yellow-colored pigment, which became reddish-brown with the decrease in the pH value, was released by the cells into the culture liquid. These changes in the pH of the medium were apparently due to the active metabolism of vegetative cells.

If Sauton's medium with a lowered initial pH value (6.0) was modified by replacing asparagine with ammonium sulfate, the pH value decreased significantly during culture's active growth due to ammonium uptake by the cells and the accumulation of sulfate anions in the medium. Upon the addition of asparagine to this culture on the 6th day, the pH value drastically increased (Fig. 3a), confirming the relationship between changes of the medium pH and asparagine metabolism. The subsequent acidification of the medium in the poststationary phase was due to the fact that mycobacteria continued active metabolization of glycerol, the carbon substrate, while ammonium ion formation slowed down. It follows from Fig. 3b that glycerol limitation in Sauton's medium resulted in a drastic pH increase after 14 days, when glycerol was depleted.

The pH value of the medium decreased in a similar fashion if glycerol was replaced with glucose. Presumably, this acidification resulted from release of metabolites, e.g., organic acids, from the cells.

HPLC of the culture liquid upon cultivation on Sauton's medium failed to detect the accumulation of TCA cycle acids as well as acetate, pyruvate, and lactate in amounts that could acidify the whole medium volume (data not shown). The citrate detected is likely to represent the residual citrate of Sauton's medium that was not consumed. Further studies should address the issue of detecting metabolites responsible for the acidification of the medium acidification in the stationary phase.

Cells with an altered morphology appeared in wild-type mycobacterial cultures synchronously with acidification of the medium in the poststationary phase (days 7–9). Such cells were ovoid forms with a thickened multilayered cell wall and an electron-dense cytoplasm (Fig. 4). According to atomic force microscopy data (Figs. 4f and 4g), the size of vegetative cells in the exponential phase was 3.7×0.8 μm on an average, which considerably differed from the size of ovoid cells, 1.2×0.9 μm. After reaching the minimum level on the 12th–14th day, the pH value did not change for a long time (6–8 months) if the culture was incubated

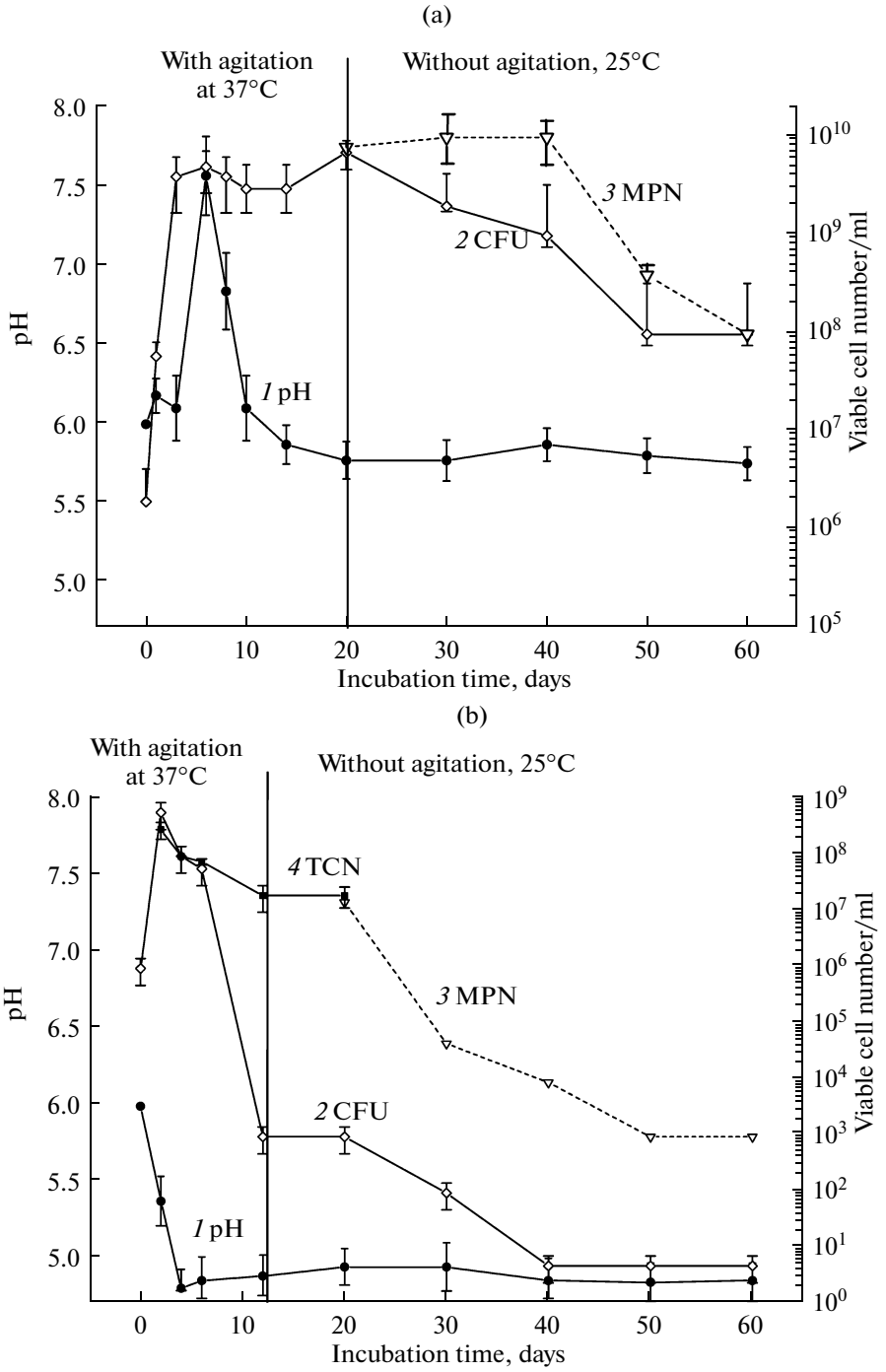

Fig. 1. Changes in pH of the medium (*1*) and viable cell numbers (*2*, *3*, and *4*) in *M. smegmatis* cultures grown on (a) Sauton's medium and (b) the 2AS medium; the initial pH value was 6.0 in both media. The cultures were grown with agitation for 20 days at 37°C and then incubated at room temperature without agitation. Cell viability was estimated from CFU/ml (*2*), MPN cells/ml (*3*), and TCN/ml (*4*) values. Averaged values of the results of three experiments are presented. The mean square deviations are given.

under static conditions (without agitation) at room temperature (Fig. 1a). By the time the pH value reached the minimum level, the wild-type *M. smegmatis* culture was rather heterogeneous. It included three cell types: ovoid, transitional, and rod-shaped forms that accounted for 35, 30, and 35% of the total cell number, respectively (Fig. 4). After storing for 30 days without agitation at room temperature, the ovoid form number increased to 60%, which probably resulted from the transformation of transitional

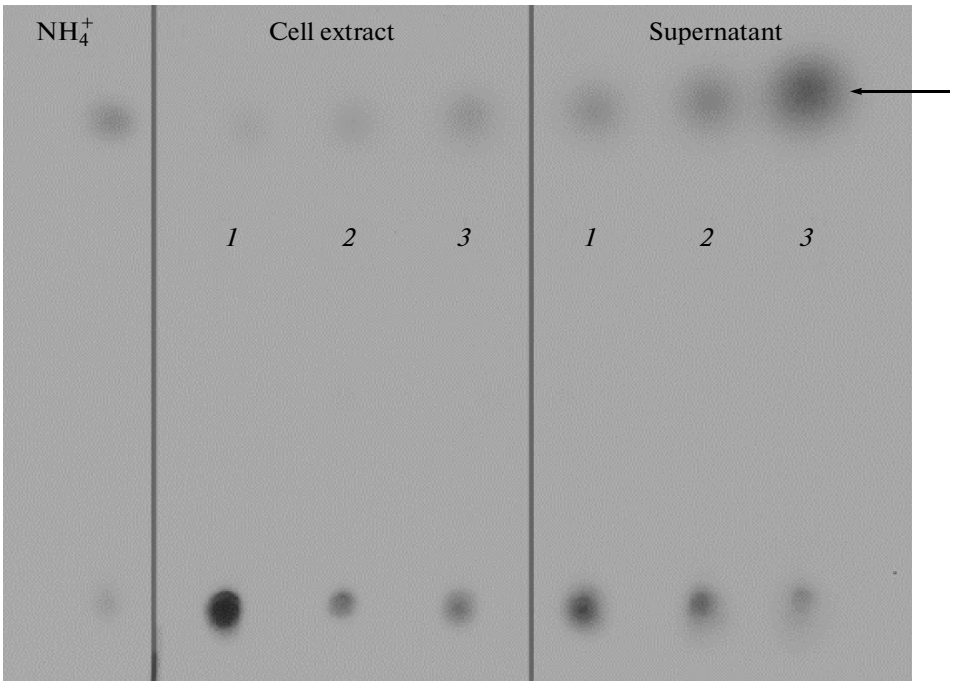

Fig. 2. Thin-layer chromatography of the culture liquid and cell extracts of *M. smegmatis* grown on Sauton's medium (pH 6.0) after dansylation. *1*, exponential-phase culture (2 days); *2*, poststationary culture (20 days); and *3*, ovoid cell-containing culture (60 days). The arrow indicates the position of the NH_4^+ ion.

forms. However, compelling proof of the fact that the morphologically intermediate cells actually represent transitional forms between vegetative and ovoid cells requires further studies.

Isolation of a homogeneous fraction of ovoid cells presented difficulties because the culture included three types of cell forms. After centrifuging poststationary cultures (30–40 days of incubation) in a 1.4–2.2 M sucrose gradient, cells were located within a wide density range (1.4 to 1.9 M). 70% of ovoid cells were in the 1.8 M zone and displayed a higher buoyant density than vegetative cells (1.4–1.5 M).

The cell metabolism level markedly decreased during the pH decrease in cultures grown on Sauton's medium. In the cells of poststationary cultures stored for 20–60 days, the level of incorporation of radioactive RNA and DNA precursors and the extracellular ATP amount were low, although distinct from zero (Table 1). The fact that *M. smegmatis* cultures failed to reduce the CTC dye (based on fluorescence microscopy data) and methylene blue testified to a decelerated or lacking respiratory activity in *M. smegmatis* cultures dominated by ovoid and transitory forms.

The ovoid forms of mycobacteria exhibited higher resistance to elevated temperatures than wild-type cells. After heating the suspensions for 10 min at 60°C, the percentage of intact ovoid cells was 20% higher than that of intact vegetative cells (Table 1), while the colony-forming capacity of ovoid cells was 14 times higher than that of vegetative cells. Ovoid forms were more resistant to antibiotics. After treating *M. smegmatis* cells (10^8 CFU/ml) with 100 μg/ml hygromycin for 24 h, the number of viable ovoid and vegetative forms was 10^7 and 10^2 CFU/ml, respectively (Table 1).

During long-term storage at room temperature of cultures with pH values of 6.1–5.8, CFU/ml numbers decreased to 10^8 (by 1.5 orders of magnitude) by day 60 (Fig. 1a) and to 10^4–10^5 after 1 year of incubation. Serial dilutions and the most probable number method revealed that the viable cell number in 30–60 day *M. smegmatis* populations was 5×10^8–10^{10} cell/ml, which was higher than the CFU titer by one to two orders of magnitude (Fig. 1a).

Thus, the ovoid forms of *M. smegmatis* can be classified as dormant forms according to their properties revealed by us.

To answer the question as to what extent the formation of ovoid cells depends on acidification of the medium and its rate, we added MOPS or MES buffer (10–50 mM, pH 7.5) to stationary-phase cultures. This provided for a lesser pH decrease, which could be made either gradual or abrupt. The pH decreased by 0.5–2 units over 7–14 days of incubation (Table 2). The ovoid form percentage was 45% on standard Sauton's medium with an initial pH value of 6.0 that gradually decreased by 2.2 units by day 14 of cultivation. It was found that, if decreasing molar concentrations of MOPS buffer were added, the pH decrease correlated with the increase in ovoid form number. A high ovoid

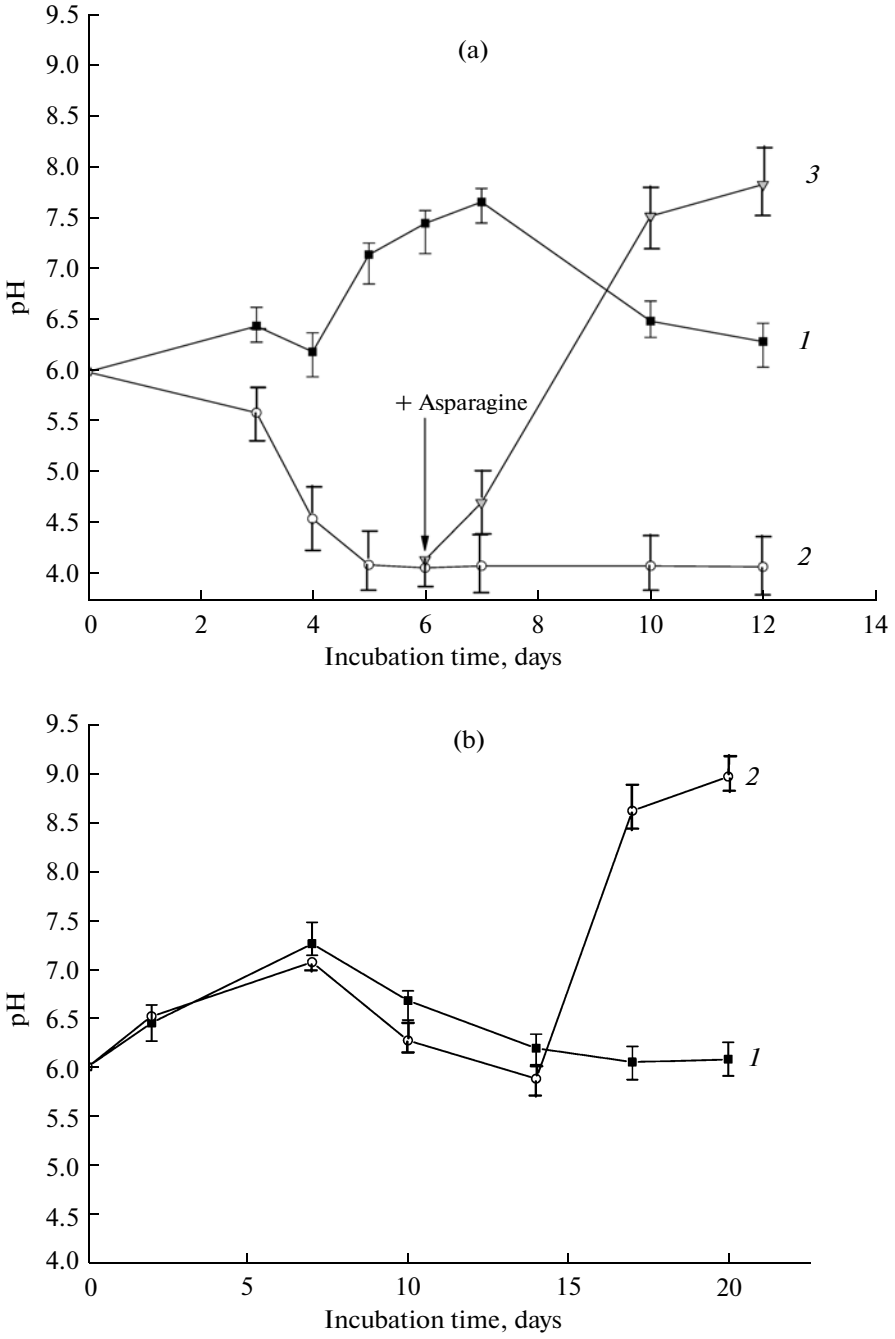

Fig. 3. Dependence of pH changes in *M. smegmatis* cultures on the composition of Sauton's medium. (a) The medium contained *1*, 6% glycerol and asparagine; *2*, 6% glycerol and ammonium sulfate; asparagine was added on the 6th day. (b) The medium contained *1*, 6% glycerol and asparagine; *2*, 2% glycerol and asparagine.

cell yield (35%) occurred in *M. smegmatis* wild-type cultures if the pH value gradually decreased by two units upon the addition of 10 mM MES buffer. It should be noted that the ovoid cell percentage by day 14 of incubation in standard Sauton's medium with an initial pH value of 7.0 was considerably lower (10–15%). In addition, secondary growth started during subsequent incubation, resulting in a pH increase (data not shown). Against this background, the addition of 50 mM MOPS buffer failed to increase the ovoid cell yield. If the medium pH value was decreased by two units abruptly by adding acidic MES buffer (50 mM, pH 3.5) to a 7-day culture, the formation of ovoid cells in *M. smegmatis* did not occur even on day 14 of incubation (data not shown).

Thus, the formation of ovoid cells in *M. smegmatis* is promoted by acidification of the medium in station-

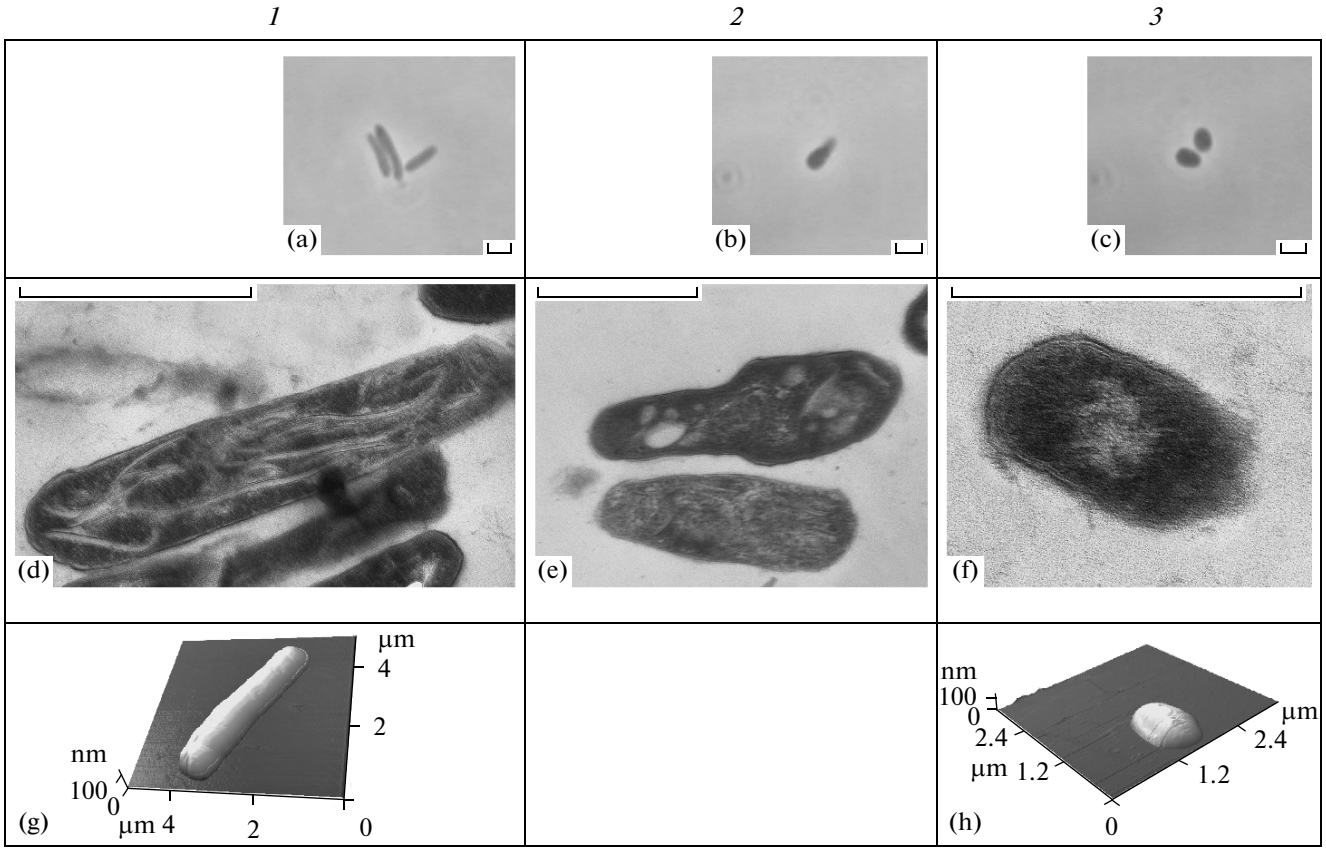

Fig. 4. Phase-contrast microscopy (a–c), electron microscopy of thin sections (d–f), and atomic force microscopy (g, h) of *M. smegmatis* cells. The *M. smegmatis* cultures were grown on Sauton's medium with an initial pH value of 6.0. *1*, vegetative cells (2 days of cultivation); *2*, transitional cell forms (20 days); and *3*, ovoid cells (60 days). Bars, 2 (a–e) and 5 μm (f).

ary cultures to pH values of 6.0–5.7, followed by further incubation.

Formation of "Nonculturable" Forms of M. smegmatis on Glucose-Containing Medium Upon Medium Acidification to pH 4.8 in Poststationary Cultures

It was shown in the above section that a part of the *M. smegmatis* ovoid cell subpopulation formed upon acidification of the medium (pH 5.7–6.0) lost colony-forming capacity. However, these conditions were not sufficient for causing all cells of the persisting mycobacterial population to lose the colony-forming capacity. A more significant pH decrease in stationary-phase cultures occurred on the 2AS synthetic medium (see Materials and Methods). We selected conditions promoting *M. smegmatis* cell proliferation but causing the pH value to start decreasing immediately after the onset of the culture growth. The alkalization phase was lacking, and the pH value became 4.8 by day 5. Under these conditions ovoid cells with significantly decreased colony-forming capacity were formed (Fig. 1b). The results of microscopic studies indicated that the cells remained intact over 40-day incubation in a liquid medium at room temperature, according to the test with propidium iodide. The acidity level (pH 4.8) of stationary-phase cultures was stabilized by the addition of 50 mM acidic MES buffer. During their further cultivation, part of the persister cell subpopulation displayed capacity for cell division upon inoculation into liquid Sauton's medium that was diluted twofold and supplemented with 0.6% glycerol. After incubating such "pH 4.8 cultures" for 40 days, all surviving cells were "nonculturable" if inoculated on solid media (CFU/ml = 0). Nevertheless, 10^4 cells/ml were viable according to the MPN value. The highest culturability restoration level (the MPN value of up to 10^4 cells/ml) was attained with cells of culture incubated for 40–45 days. The MP value decreased to a level of 10^3 cells/ml after 1.5–2 months of further incubation.

Presumably, the development of the nonculturable state during growth of mycobacteria on 2AS medium was promoted by the lower pH value (4.8) in the poststationary phase as compared to Sauton's medium (pH 5.7–6.0) and not by the medium composition. The growth of mycobacteria on the 2AS medium whose pH value in the poststationary phase was maintained within the 6.0–6.2 range (close to the pH of Sauton's medium pH 6.0) resulted in the formation of ovoid cells that were culturable on solid media.

Table 1. Biochemical changes in *M. smegmatis* cells upon transition to the dormant state

Parameters	Exponential phase (2-day) culture cells	Stationary-phase (20-day) culture cells	Ovoid cells (60 days)
Incorporation of the radioactive label (cpm)			
Thymidine [^{3}H]	40196 ± 2354	7560 ± 1524	860 ± 101
Uracil [^{3}H]	93286 ± 3730	12340 ± 1632	408 ± 94
Intracellular ATP concentration in the culture			
[ATP], nmol/ml*	514 ± 41.12	264 ± 15.84	183 ± 9.15
Buogunt density (g/ml)			
	1.182 ± 0.1	1.20 ± 0.1	1.235 ± 0.1
Thermal stability of the cells (% of unheated control)**			
60°C	65 (5.7)	75	85 (0.4)
70°C	9 (0.2)	12	18 (0.11)
80°C	2 (8×10^{-5})	4	10 (5.5×10^{-5})
Cell resistance to antibiotics***			
Kanamycin	0.5×10^{-6}	1×10^{-6}	3×10^{-6}
Hygromycin	1×10^{-6}	1×10^{-5}	1×10^{-1}
Tetracycline	0.5×10^{-2}	1×10^{-2}	1×10^{-1}

Notes: * ATP concentration in 1 ml of a culture containing 1×10^9 CFU/ml

** Percentage of propidium iodide-unstainable cells after heating the cells for 10 min as determined by direct microscopic count. In parentheses are the respective ratios of CFU values determined on standard medium (NB agar).

*** The fraction of cell population resistant to treatment with 100 μg/ml antibiotics for 24 h was determined from the ratios of CFU values after inoculation of standard medium (NB agar) before and after the antibiotic treatment.

Thus, a considerable acidification of the growth medium (to a pH value of 4.8) in the poststationary phase, followed by incubation at this pH value, stimulates the formation of nonculturable ovoid forms of *M. smegmatis*.

Involvement of the hlp and devR Genes in the Adaptation of M. Smegmatis Cells to Growth Medium Acidification

Since the formation of dormant *M. smegmatis* cells is intensified by slow acidification of the medium, it could be anticipated that this process in strains with mutant *hlp* and *devR* genes would differ from the formation of ovoid cells by the wild-type strain. We investigated the *M. smegmatis* Δ*devR(dosR)* mutant with a deletion in the regulatory domain of the DevR–DevS two-component system. This system is involved in controlling the nonreplicative state under microaerophilic conditions (Wayne's model [14]). In another tested strain, *hlp-0*, the *hlp* gene was inactivated. It codes for the histone-like protein Hlp, which secures DNA stabilization in *M. smegmatis* dormant forms generated under nitrogen limitation [26]. Both mutant strains were grown on Sauton's medium with an initial pH value of 6.0, which promoted the generation of viable dormant forms by wild-type cells (Fig. 1a). Under these conditions mutant cells started growing after a prolonged lag phase. They to a lesser extent retained their colony-forming capacity during long-term incubation and contained a lower number of ovoid cells as compared to the wild-type strain. In poststationary cultures incubated at weakly acidic pH values, the ovoid cell percentages on day 20 were 5 and

Table 2. Formation of ovoid cells by *M. smegmatis* as dependent on medium acidification in the poststationary phase (14 days of cultivation)

Cultivation conditions*	ΔpH**	Ovoid cell percentage***	Transitional cell form percentage***
S6	2.2	45	25
S6 + 50 mM MOPS	0.5	5	0
S6 + 25 mM MOPS	1.0	15	5
S6 + 10 mM MOPS	1.5	30	10
S6 + 10 mM MOPS	2.0	35	30
S7	1.2	10–15	5
S7 + 50 mM MOPS	0.4	3	0

Notes: * The buffer was added to Sauton's medium with initial pH 6.0 (S6) or 7.0 (S7) on day 7 of incubation, when the medium pH value reached 7.5, in order to decrease the level of subsequent acidification of the medium;

** ΔpH, the difference between the maximum (after 7 days) and the minimum (after 14 days) pH values of the medium.

*** These data are based on the results of direct microscopic count of ovoid cells and transitional forms, which are ovoid cell precursors (see Fig. 4). The relative error was 5%.

6% of the cell number in the Δ*devR* and the *hlp-0* strain, respectively. This percentage was close to 37% in the wild-type strain (Fig. 5). On day 20 the acidification degree (ΔpH) in poststationary-phase cultures was lower (6.5) in the mutant strains than in the wild-type strain (pH 7.5). Lowering the pH value by one unit on day 20 resulted in the formation of 15–20% of ovoid cells in the wild-type and only and 6% in mutant cultures (Table 2). It should be noted that long-term incubation (for 85 days) of mycobacterial cultures on Sauton's medium with a pH value of 6.0 caused a more rapid decrease in viable cell number in the mutant strains than in the wild-type strain. The mutant strains differed in this respect. The CFU/ml number decreased from 4×10^9 to 2×10^5 in less than two months in the *hlp-0* mutant and from 1×10^{10} to 8×10^8 in the wild-type strain. The cells of the*hlp-0* mutant lost the capacity to resume growth in a liquid medium within this period. All cells lost their integrity based on the results of microscopic studies with propidium iodide. The decrease in the colony-forming cell number was similar in the Δ*devR* and wild-type strains. However, mutant forms, unlike wild-type cells, could not grow in submerged culture.

Thus, the *hlp* and *devR* mutants of *M. smegmatis* fail to generate dormant forms upon acidification of the medium in poststationary cultures.

DISCUSSION

The sensitivity of mycobacterial vegetative cells to pH changes is a well-established fact [13]. We found that in the presence of an amino acid, such a aspartic acid or glutamic acid, in the growth medium with an initial pH value of 6.0, multiplying *M. smegmatis* cells alkalized the culture liquid to a pH level enabling proliferation (Fig. 1a). In the absence of asparagine, the cells acidified the medium; the culture growth decelerated, and proliferation ceased once the pH value decreased to a level below 5.0 (Fig. 1b).

In the present study, it was established that acidification of Sauton's medium (pH 6.0) was related to glycerol metabolism. However, the metabolites responsible for the acidification have not been detected so far. It was noted in earlier works that mycobacteria metabolize carbohydrate substrates as carbon sources and convert them to carbon dioxide and water as the end products [24]. Recently, it has been found that mycobacteria that actively grow on glycerol accumulate large amounts of glyceraldehyde intracellularly [25]. Glyceraldehyde is transformed into phosphoglycerate via the glycolytic pathway. The presence of the glycerate kinase-encoding *MSMEG_2528* gene in the *M. smegmatis* genome suggests that phosphoglycerate can be converted to glyceric acid. It can accumulate in the culture liquid, causing acidification of the medium. An analogous role in the tuberculosis pathogen may be performed by a hypothetical protein encoded by the *Rv2205c* gene. Comparison of the primary sequences gives us grounds for the assumption that the hypothetical protein is the glycerate kinase of *M. tuberculosis*.

If acidic medium pH values were maintained by adding buffer solutions (pH 5.0), cell division was lacking (data not shown). These experiments indicate that vegetative mycobacterial cells are highly sensitive to changes in medium pH. A prerequisite for adjusting the intracellular pH value to a level that allows cell proliferation is the extracellular pH level within a relatively narrow range. Changes in the medium pH can be used as a stress factor that causes arrest of cell division and the generation of dormant forms of mycobacteria. It was shown in this work that these effects were associated with a decrease in culture pH value and resulted in the emergence of ovoid cells in an *M. smegmatis* population (Fig. 4). Similarly to our data on the pH effect on the viability of mycobacteria, rapid oxygen depletion caused the death of most cells in Wayne's model. In contrast, gradually developing hypoxia resulted in cell transition to a dormant state, so that they remained culturable [16].

The formation of dormant *M. smegmatis* and *M. tuberculosis* cells, including nonculturable forms, was documented earlier [27, 28]. However, these forms did not display the ovoid morphotype and their percentage in the population was low. In the model described in the present work (gradual medium acidification in the poststationary phase) (Fig. 1a), the ovoid cell percentage in 14-day cultures was 45%. Subsequently, this percentage increased, which was probably due to the transformation of transitional forms (Table 2). The ovoid cell yield was 60% by day 20 of incubation on the 2AS medium we developed (Fig. 1b).

M. smegmatis ovoid cells formed in poststationary cultures upon a gradual decrease in the pH value to 6.0 can be classified as dormant bacterial forms according to the following features: (i) viability retention during a long period of time (6–9 months), (ii) low level of metabolism (Table 1), (iii) lack of experimentally detectable respiratory activity, (iv) enhanced resistance to deleterious factors such as high temperatures and antibiotics (Table 1), and (v) peculiarities of morphological and ultrastructural organization (Fig. 4). Similarly to other dormancy models studied earlier [28, 29], the ovoid cells of *M. smegmatis* assumed the nonculturable state during long-term incubation in an acidified medium with a pH value of 4.8.

It should be noted that the generation of dormant forms of bacteria, including those resulting from medium acidification, is an active process involving various cell-metabolism stages. Based on the data on the expression levels of diverse genes in tuberculosis pathogen cells upon their transition to the dormant state, the involvement of at least 200 genes in this process was suggested [30]. In the present work, we have directly demonstrated the important role of at least two genes, *hlp* and *devR*, in the efficient transition to the dormant state. It was assumed earlier that protein

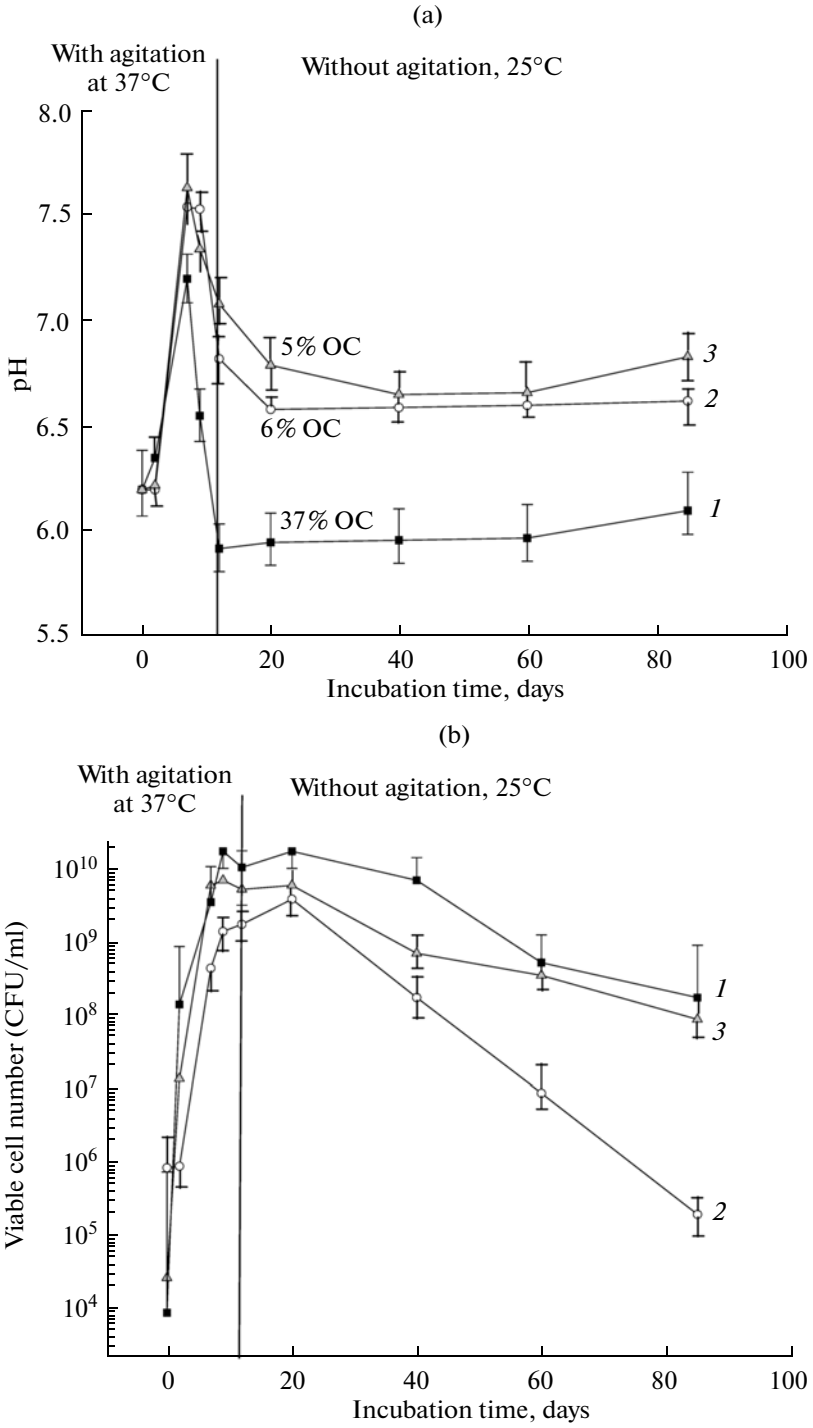

Fig. 5. Changes in pH of the medium (a) and percentage of ovoid cell (OC) and viable cell (CFU) number (b) during the cultivation of *M. smegmatis* wild-type strain (*1*) and of mutants with inactivated *hlp* (*2*) and *devR* (*3*) genes. Cultivation was on Sauton's medium with initial pH 6.0. Averaged values of the results of three experiments are presented. The mean-square deviations are given.

DevR regulates the adaptation of bacteria to oxygen deficiency [14]. However, in the authors' opinion, the products of the *devR* gene may also be implicated in other kinds of stress responses [31]. From the results of the present work, it follows that the *devR* gene and, plausibly, the "Dos-regulon" cluster controlled by it perform essential functions in the adaptation of vegetative mycobacterial cells to acidification stress.

We showed earlier that histone-like protein Hlp encoded by the other gene, *hlp*, is involved in the

enhanced resistance of dormant mycobacterial cells to high temperatures, UV irradiation, and antibiotics, which is apparently due to Hlp-dependent nucleotide compactization [26]. During acidification of poststationary cultures of the mutant strain of *M. smegmatis*, cells with an inactivated *hlp* gene lost their viability and did not generate ovoid dormant forms. In all likelihood the *hlp* and *devR* genes form part of a global system of cell metabolism regulation and adaptation that enables mycobacteria to generate dormant forms and survive unfavorable growth conditions in a state of dormancy.

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REFERENCES

1. Amberson, J.B., The Significance of Latent Forms of Tuberculosis, *N. Engl. J. Med.*, 1938, vol. 219, pp. 572–576.
2. Flynn, J.L. and Chan, J., Tuberculosis: Latency and Reactivation, *Infect. Immun.*, 2001, vol. 69, pp. 4195–4201.
3. Hernndez-Pando, R., Jeyanathan, M., Mengistu, G., Aguilar, D., Orozco, H., Harboe, M., Rook, G.A.W., and Bjune, G., Persistence of DNA from *Mycobacterium tuberculosis* in Superficially Normal Lung Tissue during Latent Infection, *Lancet,* 2000, vol. 356, no. 9248, pp. 2133–2138.
4. Biketov, S., Mukamolova, G.V., Potapov, V., Gilenkov, E., Vostroknutova, G., Kell. D.B., Young, M., Kaprelyants, A.S., Culturability of *Mycobacterium tuberculosis* Cells Isolated from Murine Macrophages: a Bacterial Growth Factor Promotes Recovery, *FEMS Immunol. Med. Microbiol*, 2000, vol. 29, no. 4, pp. 233–240.
5. McCune, R.M., Tompsett, R., and McDermott, W., The Fate of *Mycobacterium tuberculosis* in Mouse Tissues as Determined by the Microbial Enumeration Technique. II. The Conversion of Tuberculous Infection to the Latent State by Administration of Pyrazinamide and a Companion Drug, *J. Exp. Med.*, 1956, vol. 104, no. 5, pp. 763–802.
6. de Wit, D., Wootton, M., Dhillon, J., and Mitchison, D.A., The Bacterial DNA Content of Mouse Organs in the Cornell Model of Dormant Tuberculosis, *Tuber. Lung. Dis.,* 1995, vol. 76, no. 6, pp. 555–562.
7. Dhillon, J., Lowrie, D.B., and Mitchison, D.A., *Mycobacterium tuberculosis* from Chronic Murine Infections That Grows in Liquid but Not on Solid Medium, *BMC Infect. Dis.*, 2004, vol. 4, p. 51.
8. Khomenko, A.G. and Golyshevskaya, V.I., Filterable Forms of *Mycobacterium tuberculosis, Z. Erkran. Atm.-Orgt*, 1984, vol. 162, pp. 147–154.
9. Rook, G.A.W., Seah, G., and Ustianowski, A.M., Tuberculosis: Immunology and Vaccination, *Eur. Respir. J.*, 2001, vol. 17, pp. 537–557.
10. Deretic, V, and Fratti, R.A., *Mycobacterium tuberculosis* phagosome, *Mol. Microbiol.*, 1999, vol. 31, no. 6, pp. 1603–1609.
11. Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J., and Russell, D.G., Lack of Acidification in *Mycobacterium* Phagosomes Produced by Exclusion of the Vesicular Proton-ATPase, *Science*, 1994, vol. 263, no. 5147, pp. 678–681.
12. Xu, S., Cooper, A., Sturgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P., and Russell, D.G., Intracellular Trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-Infected Macrophages, *J. Immunol.*, 1994, vol. 153, no. 6, pp. 2568–2578.
13. Rao, M., Streur, T.L., Aldwell, F.E., and Cook, G.M., Intracellular pH Regulation by *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG, *Microbiology*, 2001, vol. 147, no. 4, pp. 1017–1024.
14. Wayne, L.G. and Lin, K-Y., Glyoxylate Metabolism and Adaptation of *Mycobacterium tuberculosis* to Survival under Anaerobic Conditions, *Infect. Immun.,* 1982, vol. 37, no. 3, pp. 1042–1049.
15. Cunningham, A.F. and Spreadbury, C.L., Mycobacterial Stationary Phase Induced by Low Oxygen Tension: Cell Wall Thickening and Localization of the 16-Kilodalton Crystalline Homolog, *J. Bacteriol.*, 1998, vol. 180, no. 4, pp. 801–808.
16. Dick, T., Lee, B.H., and Murugasu-Oei, B., Oxygen Depletion Induced Dormancy in *Mycobacterium smegmatis, FEMS Microbiol. Lett.,* 1998, vol. 163, no. 2, pp. 159–163.
17. Smeulders, M.J., Kee R J., Speight R.A., and Williams, H.D., Adaptation of *Mycobacterium smegmatis* to Stationary Phase, *J. Bacteriol.,* 1999, vol. 181, no. 1, pp. 270–283.
18. Lee, B.H., Murugasu-Oei, B., and Dick, T., Upregulation of a Histone-Like Protein in Dormant *Mycobacterium smegmatis, Mol. Gen. Genet.*, 1998, vol. 260, no. 5, pp. 475–479.
19. Keer, J., Smeulders, M.J., and Williams, H.D., 2001. A *purF* Mutant of *Mycobacterium smegmatis* Has Impaired Survival during Oxygen-Starved Stationary Phase, *Microbiology,* 2001, vol. 147, no. 2, pp. 473–481.
20. Connell, N.D., *Mycobacterium*: Isolation, Maintenance, Transformation, and Mutant Selection, *Methods Cell Biol.,* 1994, vol. 45, pp. 107–125.
21. Smeulders, M.J., Keer, J., Speight, R.A.., and Williams, H.D., Adaptation of *Mycobacterium smegmatis* to Stationary Phase, *J. Bacteriol.*, 1999, vol. 181, no. 1, pp. 270–283.
22. Suzina, N.E., Mulyukin, A.L., Kozlova, A.N., Shorokhova, A.P., Dmitriev, V.V., Barinova, E.S.,

Mokhova, O.N., El'-Registan, G.I., and Duda, V.I., Ultrastructure of Resting Cells of Some Non-Spore-Forming Bacteria, *Mikrobiologiya*, 2004, vol. 73, no. 4, pp. 516–529 [*Microbiology* (Engl. Transl.), vol. 73, no. 4, pp. 435–447].

23. Tkachenko, A.G. and Chudinov, A.A., Exchange of Polyamines between the Cell and Medium as One of the Factors Determining Development of Fed-Batch Cultures of *Escherichia coli*, *Mikrobiologiya*, 1989, vol. 58, no. 4, pp. 584–590.
24. Merrill, M.H., Studies on Carbon Metabolism of Organisms of the Genus *Mycobacterium*: III. End Products of Carbohydrate Utilization as Determined in Synthetic Media Cultures, *J. Bacteriol.,* 1931, vol. 21, no. 5, pp. 375–81.
25. Carvalho, L.P., Fischer, S.M., Marrero, J., Nathan, C., Ehrt, S., and Rhee, K.Y., Metabolomics of *Mycobacterium tuberculosis* Reveals Compartmentalized Co-Catabolism of Carbon Substrates, *Cell*, 2010, vol. 17, no. 10, pp. 1122–1131.
26. Anuchin, A.M., Goncharenko, A.V., Demina, G.R., Mulyukin, A.L., Ostrovsky, D.N., and Kaprelyants, A.S., The Role of Histone-Like Protein, Hlp, in *Mycobacterium smegmatis* Dormancy, *FEMS Microbiol. Lett.,* 2010, vol. 308, no. 2, pp. 101–107.
27. Anuchin, A.M., Mulyukin, A.L., Suzina, N.E., Duda, V.I., El-Registan, G.I., and Kaprelyants, A.S., Dormant Forms of *Mycobacterium smegmatis* with Distinct Morphology, *Microbiology*, 2009, vol. 155, no. 4, pp. 1071–1079.
28. Shleeva, M.O., Mukamolova, G.V., Telkov, M.V., Berezinskaya, T.L., Syroeshkin, A.V., Biketov, S.F., and Kaprel'yants, A.S., Formation of Nonculturable Cells of Mycobacterium tuberculosis and Their Resuscitation, *Mikrobiologiya*, 2003, vol. 72, no. 1, pp. 76–83 [*Microbiology* (Engl. Transl.), vol. 72, no. 1, pp. 64–70].
29. Shleeva, M., Mukamolova, G.V., Young, M., Williams, H.D., and Kaprelyants, A.S., Formation of "Non Culturable" Cells of *Mycobacterium smegmatis* in Stationary Phase in Response to Growth under Sub Optimal Conditions and Their Rpf Mediated Resuscitation, *Microbiology* (UK), 2004, vol. 150, no. 6, pp. 1687–1697.
30. Salina, E.G., Mollenkopf, H.J., Kaufmann, S.H.E., and Kaprelyants, A.S., *M. tuberculosis* Gene Expression during Transition to the "Non-Culturable" State, *Acta Naturae,* 2009, vol. 2, pp. 73–77.
31. O'Toole, R., Smeulders, M.J., Blokpoel, M.C., Kay, E.J., Lougheed, K., and Williams, H.D., A Two Component Regulator of Universal Stress Protein Expression and Adaptation to Oxygen Starvation in *Mycobacterium smegmatis, J. Bacteriol.*, 2003, vol. 185, no. 5, pp. 1543–1554.