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Role of Lipid Components in Formation and Reactivation of *Mycobacterium smegmatis* "Nonculturable" Cells

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Abstract—We have found that transition of actively dividing *Mycobacterium smegmatis* cells into the dormant "nonculturable" state is accompanied by increase in the protein/lipid ratio and disappearance of one of the main lipid components of the mycobacterial cells, trehalose monomycolate. In this case, oleic acid is accumulated in the culture medium due to its secretion by the mycobacterial cells. Addition of lipids of different classes to "nonculturable" *M. smegmatis* cells induces their resuscitation. The lipid reactivating effect is evidently caused by the presence of fatty acids in their composition, because free fatty acids also exhibited reactivation effect. Oleic acid in concentration of 0.05-3 μg/ml exhibited maximal effect, and that allows us to draw a conclusion concerning its signal role in the transition of dormant cells into active state.

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Key words: mycobacteria, "nonculturable" state, oleic acid, lipids, reactivation

One third of the population of our planet is known to be the carrier of a latent tuberculosis form [1] not exhibiting clinical symptoms [2, 3]. It is assumed that this form of disease is due to the existence of *Mycobacterium tuberculosis* in dormant state, when cells are characterized by lowered metabolic activity and absence of replication [4]. Altered shape, thickened cell wall, and "nonculturability" on solid and/or liquid nutrient media are features of these cells. A special reactivation procedure is required to restore the capability of "nonculturable" cells for division [5].

Mechanisms of transition of mycobacteria into the dormant state and recovery from it are still poorly studied, although there are several models proposed for the "nonculturable" state (NC state) of mycobacteria *in vivo* and *in vitro* [6]. Earlier we proposed a model for transition from active into NC state upon incubation of *Mycobacterium smegmatis* cells in potassium-free medium, and the possibility of reactivation of cells after addition of potassium ions and resuscitation promoting factor (Rpf) to the medium was demonstrated [7]. Potassium ions play an essential role in life activities of mycobacteria, and therefore the absence of these ions in the growth medium is a crucial factor for generation of NC forms, for which a decrease of metabolic and respiratory activities was detected [8].

Different classes of lipids are the main components of the mycobacterial cell wall. Lipids of the mycobacterial cell envelope serve as an essential factor providing unique resistance of mycobacteria to negative effects of the environment [9]. Presumably, mycobacterial lipids can be important for stability of their dormant forms securing their long-term viability in conditions of decreased metabolism [10, 11]. However, until now alterations in lipid composition of mycobacteria during transition into dormant state have not been investigated in detail.

Abbreviations: CFU, colony-forming units; DAG, diacylglycerols; FA, fatty acids; MA, mycolic acids; MAG, monoacylglycerols; mH-deB, modified Hartman's–de Bont medium; MPN, most probable number; NC state, "nonculturable" state; TAG, triacylglycerols; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

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The aim of this study is detailed analysis of changes in composition and amount of lipid components in *M. smegmatis* cells and culture medium in active state and during transition into NC state, as well as investigation of resuscitation of NC forms in response to substances of lipid nature.

MATERIALS AND METHODS

Microorganism and media. *Mycobacterium smegmatis* (strain mc^{2}155) was initially grown for 24-30 h at 37°C on an orbital shaker (250 rpm) in 150 ml rich broth E medium (Himedia, India) to which 0.05% (v/v) Tween 80 was added. Then the bacteria were inoculated into modified Hartman's–de Bont (mH-deB) medium [7] containing (in 1 liter) 11.8 g $Na_2HPO_4 \cdot 12H_2O$, 1.1 g citric acid, 20 g $(NH_4)_2SO_4$, 30 ml glycerol, 0.05% Tween 80, 10 ml of microelement solution, and 0.5% BSA (fraction V, Cohn Analog; Sigma, USA). One liter of microelement solution contained 1 g EDTA, 10 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.04 g $CoCl_2 \cdot 6H_2O$, 0.1 g $MnCl_2 \cdot 2H_2O$, 0.02 g $Na_2MoO_4 \cdot 2H_2O$, 0.2 g $ZnSO_4 \cdot 7H_2O$, 0.02 g $CuSO_4 \cdot 5H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, and H_2O till 1 liter. Sauton's medium was used in experiments on investigation of metabolically active *M. smegmatis* cells [12].

Estimation of cell viability. The bacterial cell suspension was diluted in succession in fresh Sauton's medium, and then 100 μl from each dilution was plated onto agarized medium (broth E medium). Inoculated plates were incubated at 37°C. The number of colony-forming units (CFU) was counted 5 days after plating. The limit of CFU detection was $5 \cdot 10^0$ ml^{-1}.

Extraction of cellular proteins and lipids. Cell biomass was separated from cultural liquid by centrifugation (20 min, 12,000*g*). The moist biomass was treated twice by Tris-HCl buffer solution (pH 7.4) with following centrifugation under the same conditions. Then the cells were kept in acetone for 10 min and centrifuged again. The precipitate was weighed to detect mass of dried cells. Proteins were isolated by double extraction with 1% aqueous SDS solution at the ratio of 1 liter of the solution per 2 g dry cells for 20 min with following centrifugation [13]. The protein content in the extract was determined using the Lowry method. Lipids were isolated by the modified Bligh and Dyer technique [8] from the sediment remaining after protein extraction.

Adsorption column chromatography. Lipids were fractionated into different classes such as phospholipids, glycolipids, and neutral lipids using adsorption column chromatography [8].

Thin layer chromatography. Thin layer chromatography was carried out on TLC plates Silica gel 60 F_{254} (Merck, Germany) in the following systems: chloroform–methanol–H_2O (30 : 8 : 1 v/v) (system A), heptane–diethyl ether–acetic acid (55 : 45 : 1 v/v) (system B). The following commercial standards were used: phosphatidylinositol from soybeans, phosphatidylethanolamine from *E. coli*, cardiolipin from bovine heart, trehalose dimycolate and mycolic acid from *Mycobacterium tuberculosis* as well as synthetic mono-, di-, and triacylglycerols. Chromatograms were developed using 60% sulfuric acid and solution of phosphomolybdic acid.

Extraction of lipid substances from culture liquid. Cell biomass was separated from culture liquid by centrifugation and following filtration through a 200 nm pore filter (Millipore, USA). Then the supernatant was lyophilized. The lyophilized solution was extracted by chloroform (1 : 4 v/v, three times, 60 min each). The chloroform extracts containing mixtures of lipidic secretion products were combined, and solvent was removed by evaporation.

Gas-liquid chromatography-mass spectrometry (GLC-MS). To obtain methyl esters of free fatty acids isolated from culture liquid, aliquots of each specimen extract were dissolved in 0.5 ml dehydrated benzene, then 200 μl dehydrated trifluoroacetic anhydride (Supelco, USA) and 200 μl HCl-methanol (Supelco) were added. Reaction was carried out in a hermetically sealed vial at 70-90°C for 1 h. Methyl esters of fatty acids were analyzed on a Shimadzu GS 2010 chromatograph (Shimadzu, Japan) with QP 2010 mass-selective detector in a temperature gradient from 140 to 260°C. The chromatograph parameters are as follows: thermostat, 140°C; injector, 200°C; interface, 210°C; and detector, 200°C. The column is nonpolar, capillary MDN-1 (Supelco), length 30 m, diameter 0.25 mm, layer thickness 0.25 μm. Carrier gas is helium, flow rate 1 ml/min. Flow scale is 1 : 10. The mass recording range is 45-450 m/z. The NIST 05 mass spectral library was used.

Preparative thin-layer chromatography and quantitative determination of free fatty acids. Equal amounts of culture liquid lipid extracts were separated by TLC on silica gel 60 F_{254} (Merck) in the solvent system hexane–diethyl ether–methanol–glacial acetic acid (9 : 2 : 0.2 : 0.3 v/v). Compounds exhibiting chromatographic mobility identical to that of the fatty acid standard were scraped off from chromatographic plate and extracted from the silica gel by chloroform–methanol mixture (3 : 2), centrifuged for 10 min at 12,000*g*, then the supernatant was separated and solvents were evaporated. Extraction was repeated three times. The content of free fatty acids in extract was determined by titration with 0.01 N KOH in absolute ethanol using phenolphthalein as indicator.

Radioactive label incorporations, measurement of radioactivity. To each of three flasks containing 150 ml of 46.5-h-old *M. smegmatis* culture grown in mH-deB, 0.5 ml [^{14}C]aspartic acid (20 mBq, pH 7.0) was added. For radioactive background control the culture from one flask was centrifuged and supernatant was taken. The other flasks were incubated for 24 h at 37°C and 200 rpm. Then supernatants obtained after centrifugation at 12,000*g* were divided into fractions by reverse-phase and

thin-layer chromatography (solvent system chloroform–methanol–ammonia (65 : 35 : 5 v/v)). The resulting TLC spots were scraped off from the plate and placed into 10 ml "toluene reagent" scintillation liquid-8. Radioactivity was measured in a Mark-1 scintillation counter.

Reverse-phase chromatography. Partition chromatography of culture liquid lipid extracts was carried out on modified agarose Octyl-Sepharose CL-4B (Pharmacia, Sweden). Before use, the column (5 ml) was washed in succession by water (one column volume), ethanol (one volume), butanol (two volumes), and in reverse order by ethanol and water. Then the column was equilibrated with 0.025 M Na-phosphate-citrate buffer (pH 7.0). Washing rate was 25 ml/cm^2·h. Stepwise elution after sample application was carried out with water (one volume), ethanol (one volume), and acetonitrile (one volume). Oleic acid was isolated from the ethanolic fraction.

Liposome preparation. Liposomes (200 nm in diameter), obtained by the earlier described technique [14], were passed through a filter with pore diameter 200 nm (Millipore) for sterilization before addition to a cell culture.

Resuscitation of NC *M. smegmatis*. The 48-well plastic plates (Corning, USA) containing 1 ml of twice diluted Sauton's medium supplemented with 0.6% glycerol were used for *M. smegmatis* resuscitation. Some wells contained different amounts of free fatty acids, phospholipid liposomes, and other substances of lipid nature in various concentrations. A 0.05% yeast extract (LabM) was added to all wells. Cell cultures (100 μl) in corresponding serial dilutions were added to each well. Then plates were incubated at 37°C with agitation at 100 rpm for 10-14 days.

For counting the number of reactivated cells, wells with visible bacterial growth were considered. Most probable number (MPN) values were determined using standard statistical tables [15].

RESULTS

Transition of *M. smegmatis* Cells from Active into "Nonculturable" State

Lipid composition of *M. smegmatis* cells growing in optimal conditions and not generating "nonculturable" forms. To study alterations in mycobacterial cell wall during growth in the active state, *M. smegmatis* cells were cultivated in Sauton's medium for 24, 48, and 72 h, which correspond to logarithmic phase, early stationary phase, and late stationary phase, respectively. The growth was registered by determination of CFU/ml. The biomass specimens after their separation from culture liquid by centrifugation were extracted with 1% aqueous SDS solution (protein isolation) with following extraction by the Bligh-Dyer technique (lipid isolation). As follows from Fig. 1, the protein/lipid ratio in cell biomass changes during cell growth: more lipids are accumulated in the cells in early stationary phase and such prevalence of lipids also persists during late stationary phase.

Lipids, extracted from the cells grown under optimal conditions, were separated by adsorption chromatography for classes of neutral lipids, glycolipids, and phospholipids. It was shown that the ratio of neutral lipids and phospholipids in *M. smegmatis* cells does not significantly change during transition from early to late stationary phase, whereas relative glycolipid content decreases 1.7-fold (table).

The detailed study of lipid classes by TLC revealed a similar lipid composition of different age *M. smegmatis*

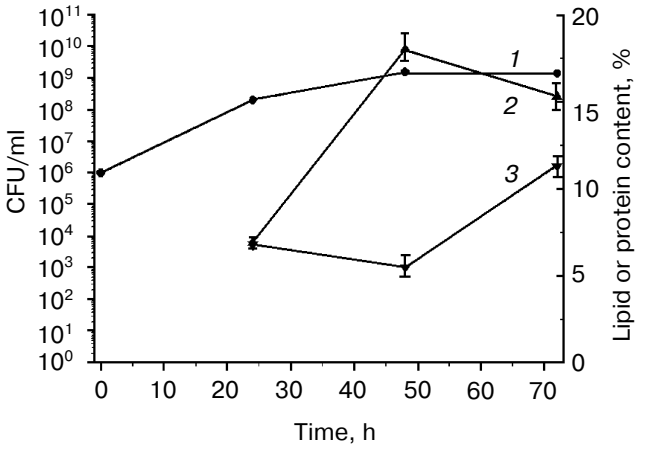

Fig. 1. Dependence of *M. smegmatis* cell growth (*1*) and lipid (*2*) and protein (*3*) content in active state on cultivation time based on dry biomass. The root-mean-square deviation of CFU values is within the range from 10 to 30%.

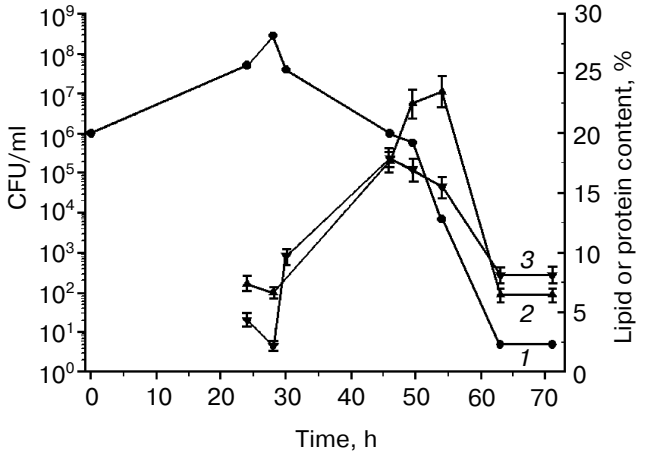

Fig. 2. *Mycobacterium smegmatis* growth in mH-deB medium (*1*), lipid (*2*) and protein (*3*) content in cells during transition into NC state depending on time based on dry biomass. The root-mean-square deviation of CFU values is within the range from 10 to 30%.

Content of three lipid classes in different age *M. smegmatis* cells in active state relative to dry biomass

Culture age, h	Phospholipid content, %	Glycolipid content, %	Neutral lipid content, %
48	44.9 ± 0.3*	16.2 ± 0.3*	38.9 ± 0.3*
72	50.5 ± 0.4*	9.3 ± 0.3*	40.2 ± 0.4*

* Differences are reliable at $P > 0.01$.

cells in active state. In this case, phospholipid composition conventional for mycobacteria was determined: phosphatidylinositol mannosides, cardiolipin, and phosphatidylethanolamine [16]. The presence of fatty acids (FA), mycolic acids (MA), monoacylglycerols (MAG), 1,2-diacylglycerols (DAG), and triacylglycerols (TAG) was found in the neutral lipid class. Data are shown below (Fig. 4).

Thus, investigation of metabolically active *M. smegmatis* cells demonstrated that during transition to stationary growth phase, lipid components are accumulated in the cell along with a stable content of proteins, but during further growth the content of lipids does not change, while content of proteins increases. No significant qualitative changes are observed in the cell lipid composition.

Changes in lipid composition of *M. smegmatis* cells during transition into "nonculturable" state. Changes taking place in the *M. smegmatis* cell lipid composition during transition into the NC state were studied during their cultivation in modified Hartman's–de Bont (mH-deB) medium that primarily differs from traditional medium by the absence of potassium salts. During the first 28 h of cultivation, increase in CFU/ml to maximal level of $2.8 \cdot 10^8$ followed by decrease in this value to 0 during the next 35 h was observed (Fig. 2). It was shown previously that the CFU decline is due mainly to formation of NC cells rather than to the cell death, because the use of special techniques enables resuscitation of NC mycobacteria [7].

Biomass was taken in eight points indicating step-by-step transition into the NC state, separated from culture medium, and extracted as described in the technique section. It was noted that by the 30th hour of cultivation the cell mass decreased by one order of magnitude and then did not change any more. Lipid accumulation occurs until the CFU/ml falls by three orders of magnitude compared to actively dividing cells (53-59 h), then lipid content in NC cells declines to such extent that it becomes lower than that of proteins (Fig. 2). This peculiarity is characteristic of mycobacteria in the NC state and significantly distinguishes them from actively dividing cells, which confirms the previously obtained data concerning the increase in buoyant density in sucrose gradient of mycobacteria in the NC state [17].

TLC analysis made it possible to detect the constant presence during the whole process of transition into NC state of traditional phospholipid components of the mycobacterial cell wall (phosphatidylinositol mannosides, cardiolipin, phosphatidylethanolamine), MAG, 1,2-DAG, TAG, FA, and MA (data are given below in Fig. 4).

Significant changes in mycobacterial lipid composition consist in dramatic decrease in the trehalose monomycolate (TMM) content after the 30th hour of growth, i.e. after achievement of maximal CFU/ml value (Fig. 3). TMM is an inherent component of mycobacterial cell wall along with its derivative, virulence factor, trehalose dimycolate (TDM) [18], and exhibits somewhat lower toxicity for animals compared to TDM [19]. It is interesting that despite the lowering of TMM amount, there are

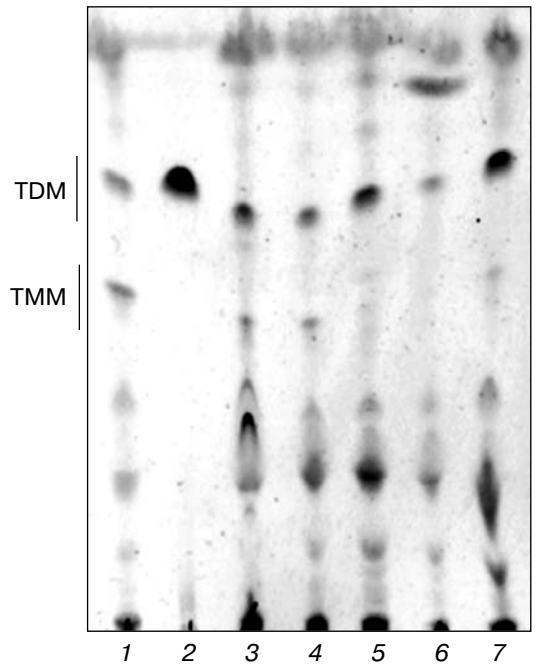

Fig. 3. TLC of lipids in system A. *1*) Lipid extract of *M. smegmatis* cells in active state after 24 h cultivation: *2*) trehalose dimycolate standard; *3-6*) lipid extracts of *M. smegmatis* cells at 24, 30, 46, and 54 h of transition into the NC state, respectively; *7*) lipid extracts of *M. smegmatis* cells in the NC state. Development by 60% sulfuric acid followed by plate heating at 100°C.

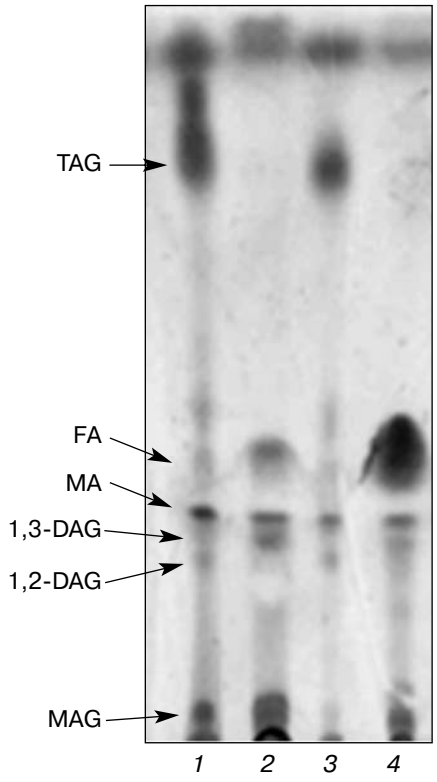

Fig. 4. TLC of lipids in system B. *1*) Lipid extract of *M. smegmatis* cells cultivated for 24 h in Sauton's medium; *2*) lipid extract of culture liquid of *M. smegmatis* cells grown for 24 h in Sauton's medium; *3*) lipid extract of *M. smegmatis* cells cultivated for 24 h in mH-deB medium; *4*) lipid extract of culture liquid after cultivation of *M. smegmatis* cells for 24 h in mH-deB medium. Development by phosphomolybdic acid solution with following plate heating at 100°C.

no significant changes in TDM level during the whole process of NC cells formation. It may be speculated that both in the active and NC states TDM synthesis in mycobacterial cells is maintained at sufficiently high level by means of TMM, because the latter is evidently not synthesized in the NC state after the 30th hour.

Extracellular lipid components generated during transition of *M. smegmatis* cells into NC state. It is known that extracellular free FA are able to define microbial viability since they play the role of signal molecules [20, 21]. Therefore, in parallel with cell biomass studying, culture liquid was analyzed in the involved states to detect the components of lipid nature secreted by *M. smegmatis*. For this purpose culture liquid separated from cells was filtered through a filter with pore diameter 200 nm, lyophilized, and then extracted with chloroform.

The presence of free FA, MA, MAG, and DAG was registered by TLC in culture liquid of all samples (Fig. 4). Qualitative lipid composition of culture liquids did not change during growth in active state and during transition to "nonculturability". In contrast to active cells, one should note the significantly higher amount of FA in extract of culture liquid of cells passing into the NC state. Comparison of lipid extracts of cells and culture liquids reveals the absence of TAG in the latter, which is indicative of certain selectivity of compounds secreted by cells (Fig. 4).

The study of free FA composition of *M. smegmatis* culture liquid by GLC-MS (Fig. 5) has shown that oleic acid is the prevalent component. NMR spectra of fatty acid isolated from culture liquid and of oleic acid standard were identical (data not shown). To identify changes in amount of free FA during active growth and transition into NC state, their amounts were detected after preparative TLC and titration (Fig. 6a). The data confirmed the GLC-MS results concerning tendency to decrease in oleic acid content in culture medium during microbial growth in active state and its increase along the process of formation of NC cells (Figs. 5 and 6 (a, b)).

As it is known, mycobacteria hydrolyze Tween 80 (polyoxyethylene sorbitan monooleate) during growth under laboratory conditions with release of oleic acid incorporated in its composition [22]. Therefore, the presence of oleic acid in early logarithmic phase and its further disappearance from culture liquid during life cycle of mycobacteria in active state can be indicative of active utilization by mycobacteria of oleic acid, produced from Tween 80, as a source of carbon.

For the increase of the oleic acid amount in culture liquid during transition into dormant state, two explanations are possible, either internalization of free fatty acid derived from Tween 80 is hindered, or mycobacteria secrete this fatty acid. To confirm the latter hypothesis, experiments were carried out on radioactive carbon incorporation into mycobacterial lipids as the result of [^{14}C]aspartic acid metabolism. After addition of 0.5 ml non-sterile labeled compound to *M. smegmatis* culture growing in mH-deB medium (culture age 46.5 h), an aliquot was taken immediately for control, while the rest was incubated for 24 h at 37°C. The resulting lipid extracts of culture liquids were separated by reverse-phase and thin-layer chromatography. Measurement of radioactivity of individual spots on the TLC plate showed the radioactivity accumulation in the spot corresponding to oleic acid (12,490 cpm) compared to the control (356 cpm). Thus, it was proved that at least a part of the oleic acid in the medium is secreted, which correlates well with literature data confirming that microorganisms are able to release FA into the environment [21].

Resuscitation of "Nonculturable" Cells

The capability of phospholipids to stimulate growth of *M. tuberculosis* cells in the late stationary phase was mentioned previously [23]. Since FA are components of phospholipids, we supposed that both phospholipids and

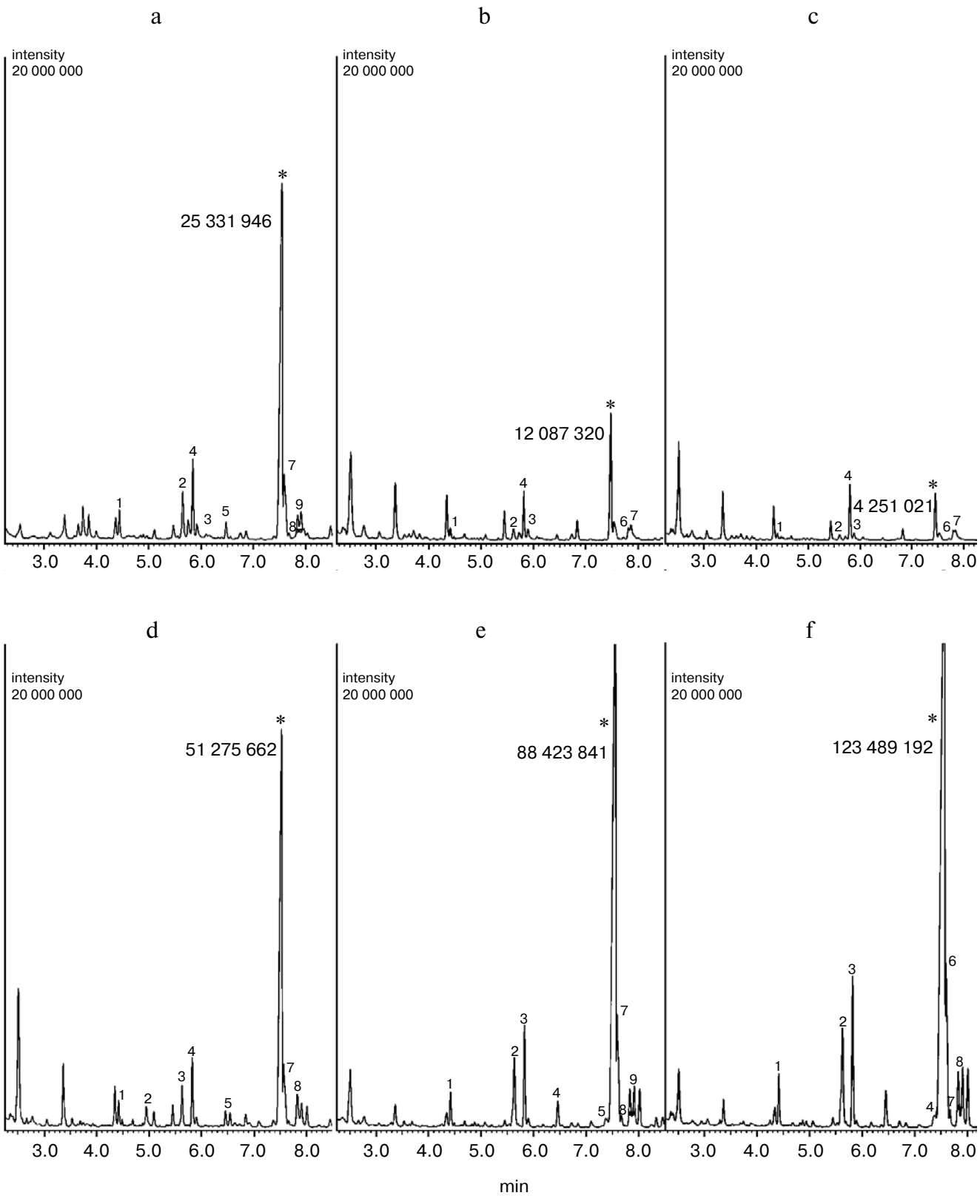

Fig. 5. GLC-MS chromatograms of free fatty acid methyl esters in culture medium of *M. smegmatis*. a, b, c) 24, 48, and 72 h of growth in active state; d, e, f) 24, 48, and 72 h of transition into NC state. * Oleic acid peaks with their areas shown alongside.

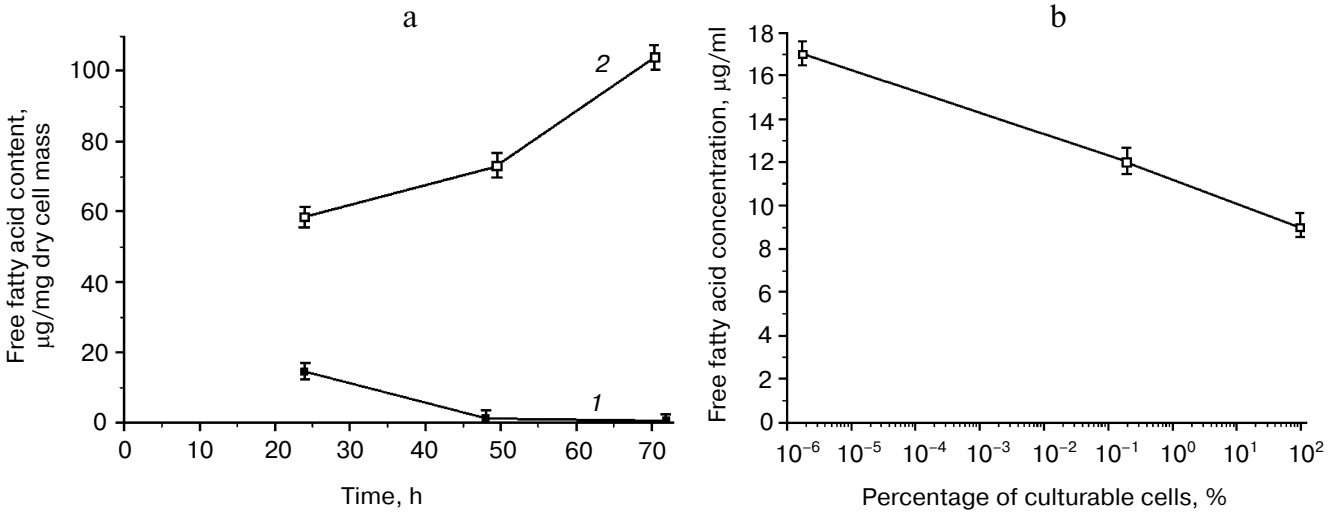

Fig. 6. a) Amount of free fatty acids in culture medium of active cells (*1*) and during transition into "nonculturable" state (*2*) depending on culture age. b) Alteration in free fatty acid contents in cultivation medium during transition of mycobacteria into "nonculturable" state depending on the percentage of culturable cells.

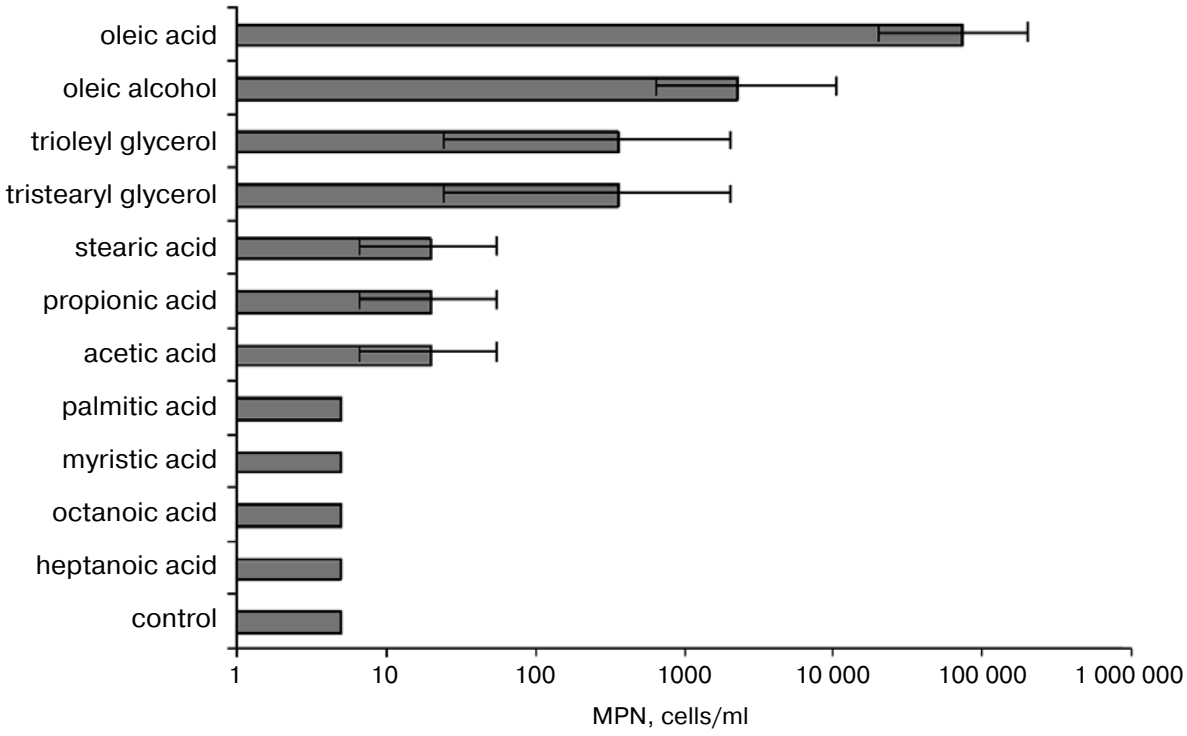

Fig. 7. Effect of FA and substances of lipid nature (in concentration of 1 μg/ml) on resuscitation of *M. smegmatis* NC cells.

FA are able to stimulate reactivation of dormant cells. To study the role of the lipid compounds in resuscitation process, NC cells were transferred into fresh medium containing potassium ions and a certain amount of lipid component, which effect on cell reactivation was estimated by the method of final dilutions.

As it was found different types of lipids are objectively capable to "trigger" the process of resuscitation of NC cells. Addition of oleic acid compared to different length acids, higher alcohols, and TAG in concentration of 1 μg/ml to 10^6 NC cells was the most efficient for reactivation (Fig. 7). The phospholipid and oleic acid

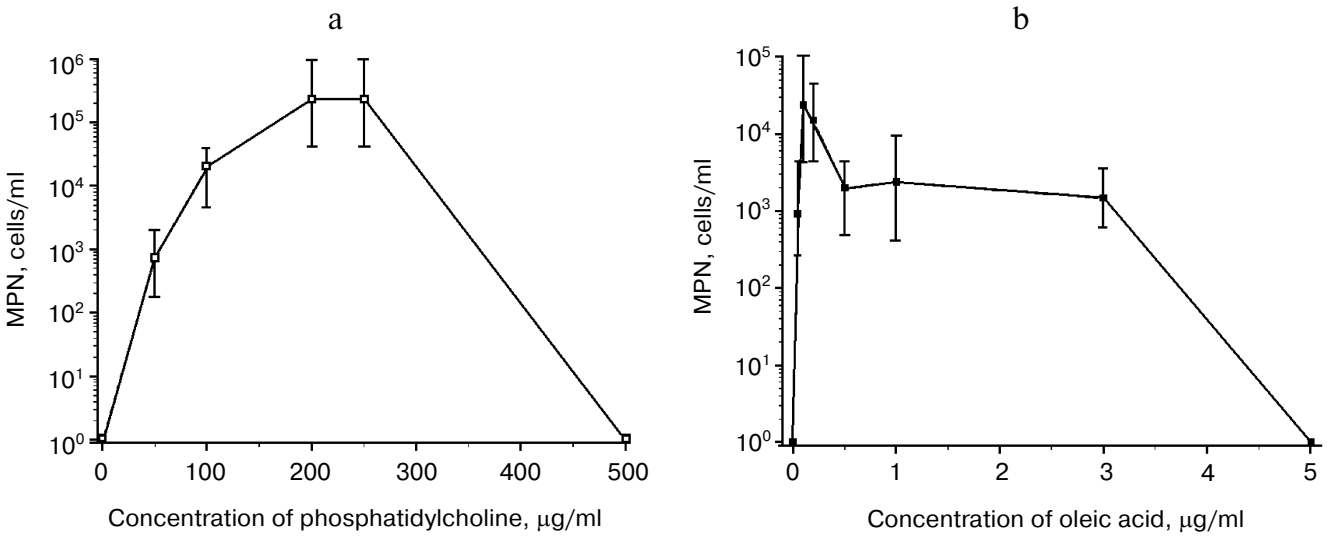

Fig. 8. Effect of phosphatidylcholine (a) and oleic acid (b) in different concentrations on resuscitation of *M. smegmatis* NC cells.

effects on resuscitation were concentration-dependent (Fig. 8). It is important that the oleic acid concentrations (0.05-3 μg/ml) were in significantly lower range compared to phospholipids (50-250 μg/ml) (Fig. 8). This demonstrates that free FA are the substances responsible for reactivation, while phospholipids serve as their sources (due to activity of bacterial phospholipases).

DISCUSSION

Transition into NC state is known to result in strong transformations of various life cycle processes of mycobacterial cells: changes in many enzyme activities, decrease in RNA amount (mRNA, rRNA) and in respiration rate [6]. These data show that dormant cells "minimize" their metabolism, which apparently should affect the content of the main cell components. This study shows the factual drop in cell mass during transition into NC state; especially pronounced is decrease in lipid content, in particular, disappearance of the virulence factor precursor TMM.

Since activity of the most enzymes in dormant cells is decreased [8], they are not able to resist the processes resulting in degradation of their own lipid components, and possibly therefore for prevention of cell wall disruption, mycobacteria have to "minimize" lipid amounts. Thus, some bacteria that use sporogenesis as strategy for adaptation to unfavorable environmental conditions loose a significant part of cell mass [24]. The detected increase in the protein/lipid ratio in mycobacterial cells in the NC state may show that the cell in this form of existence retains only essential proteins. Formally, the detected "delipidation" contradicts the published results on investigation of dormant mycobacterial cells in which TAG accumulation was found [3, 10, 11]. We believe that this discrepancy can be associated with different models of dormancy used in the works. Thus, in our experiments mycobacterial nonculturable cells were used in contrast to nonreplicating ones [3, 10, 11], which represents a deeper level of dormancy in our model [6]. Demonstrated accumulation in culture liquid of free FA (especially oleic acid) is likely to be associated with this loss of lipid components by cells in the process of transition into NC state. It was actually proved in experiment with radioactive ^{14}C(H)aspartic acid incorporation, that oleic acid, present in culture liquid, is secreted.

In this work we have shown for the first time the effect of oleic acid (0.05-3 μg/ml) on mycobacterial NC cells resuscitation. Such low concentration excludes the use of free FA in this process as a nutrient source. FA are known to function as signal substances in a number of important processes in bacterial cells. Thus, arachidonic acid serves as chemoattractant for *Dictyostelium discoideum* [25]. Another example of FA signal function is their secretion into extracellular space by the *Xanthomonas campestris*, plant pathogen, and their following absorption resulting in increased expression of genes associated with cell pathogenicity [26]. So, we have revealed a new, not previously described signal function of oleic acid as an agent stimulating reactivation of NC mycobacterial forms.

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