ISSN 0026-2617, Microbiology, 2006, Vol. 75, No. 4, pp. 432–437. © Pleiades Publishing, Inc., 2006.
Original Russian Text © E.G. Salina, G.N. Vostroknutova, M.O. Shleeva, A.S. Kaprelyants, 2006, published in Mikrobiologiya, 2006, Vol. 75, No. 4, pp. 502–508.

CONFERENCE PROCEEDINGS

Cell–Cell Interactions during the Formation and Reactivation of "Nonculturable" Mycobacteria

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Received February 27, 2006

Abstract—To date, the possible existence of "nonculturable" (NC) but potentially viable forms has been shown for some bacteria. NC mycobacteria have attracted particular interest due to the assumption that the latent form of tuberculosis is associated with the conversion of its causative agent, *Mycobacterium tuberculosis,* into the NC state. A number of approaches have been developed to obtain NC forms of mycobacteria, but the mechanisms of transition into or from this state have been insufficiently studied. This review considers cell–cell communications involved in the formation and reactivation of NC forms of the bacteria *M. smegmatis* and *M. tuberculosis.* Special attention has been paid to the secreted Rpf family proteins, which belong to peptidoglycan hydrolases and participate in the resuscitation of NC mycobacteria.

DOI: 10.1134/S0026261706040114

Key words: "nonculturable" bacteria, cell–cell communication, latent tuberculosis

Nowadays, the "nonculturability" of bacteria is a subject of active discussion. Nonculturability is understood as the inability of cells to grow on solid or liquid laboratory media, although quite often the ability to grow in liquid media may be retained in the absence of growth on solid media. The existence of viable but nonculturable (VBNC) cells is widely known for both pathogenic and nonpathogenic species [1, 2]. Cells with the NC phenotype may retain some residual metabolic activity or be dormant with absolutely no metabolism. However, dormant cells in general are not necessarily nonculturable [3–5]. Thus, the terms "dormancy" and "nonculturability" cannot be considered identical.

There is a widespread opinion that the causative agent of tuberculosis—the pathogenic, slowly growing non-spore-forming bacterium *Mycobacterium tuberculosis*, is able to survive in vivo for a long time after the infection in a dormant (or NC) state [6, 7]. Thus, a latent infection arises, which is thought to be related to the formation of dormant cells. Such cells seem to be nonculturable [8].

However, the mechanisms of conversion of bacteria, including mycobacteria, into and from the NC state have been little studied up to now. Some recently obtained data allow us to conclude that cell–cell interactions, which significantly influence the growth and development of bacterial populations, play an important role in the formation and reactivation of mycobacterial NC cells.

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"NONCULTURABILITY" OF MYCOBACTERIA AND LATENCY OF TUBERCULOSIS

According to the World Health Organization, every third man on the Earth is latently infected with the tuberculosis pathogen, *M. tuberculosis*, and such latent infection can be suddenly activated after a long period during which it is not manifested. Khomenko et al. studied different forms of *M. tuberculosis* in tissues of patients and concluded that this organism has special small (filterable) forms, which he supposed to be latent forms of tuberculosis. "Khomenko forms" could not be cultured on standard media, but their introduction into animal organisms in many cases induced the process of tuberculosis, after which the pathogen could be revealed on nutrient media [9].

A number of experimental models employing animals have been developed for the study of latent tuberculosis in vivo, including the so-called model of chronic tuberculosis [10, 11] and the Cornell model of the formation of nonculturable cells upon exposure to antibiotics [12].

For quite a long time, the most popular in vitro model simulating the transition of *M. tuberculosis* cells to dormancy was the Wayne dormancy model [4] consisting in interruption of cell division under gradual oxygen depletion. After a long-term incubation, when the system reaches an anaerobic state at an oxygen level below 0.06%, cell division completely stops and thickening of the bacterial cell wall is observed. The transfer of cells to fresh aerated medium results in their syn-

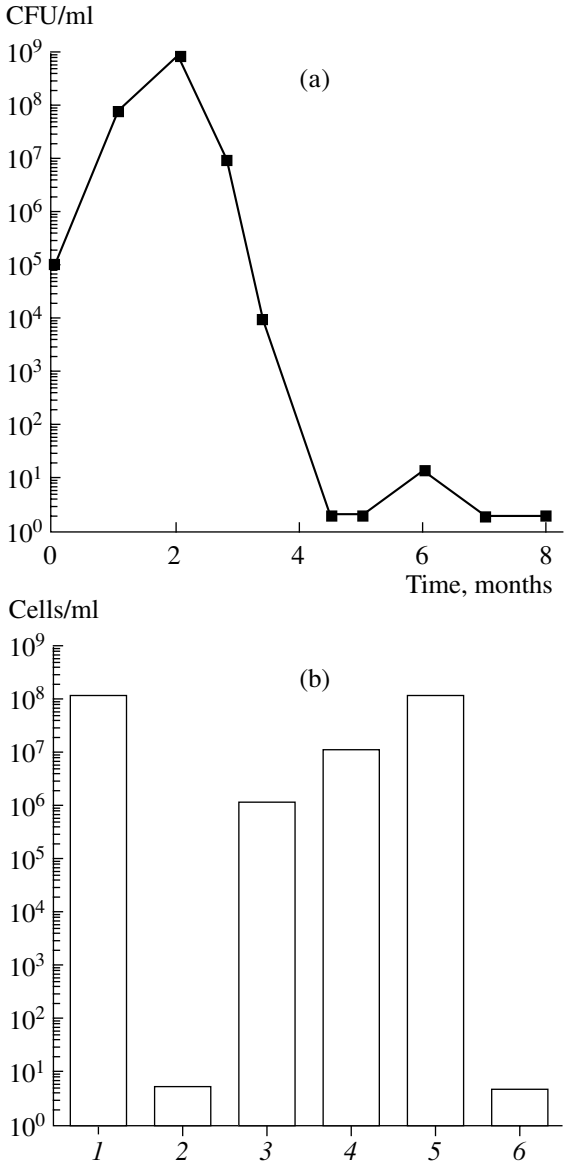

Fig. 1. Formation of NC *M. tuberculosis* cells (a) and their resuscitation (b) in liquid cultivation media: (*1*) total cell number, (*2*) CFU number and cell numbers after resuscitation of wild-type NC cells (*3*) in synthetic Sauton medium in the presence of 0.05% yeast extract, (*4*) in synthetic Sauton medium in the presence of 0.05% yeast extract and recombinant Rpf protein (125 pM), and (*5*) in supernatant from an actively growing *M. tuberculosis* culture used as a resuscitation medium and (*6*) after resuscitation of NC cells of the mutant strain KDT8 lacking three of the five Rpf-encoding genes, in synthetic Sauton medium in the presence of 0.05% yeast extract.

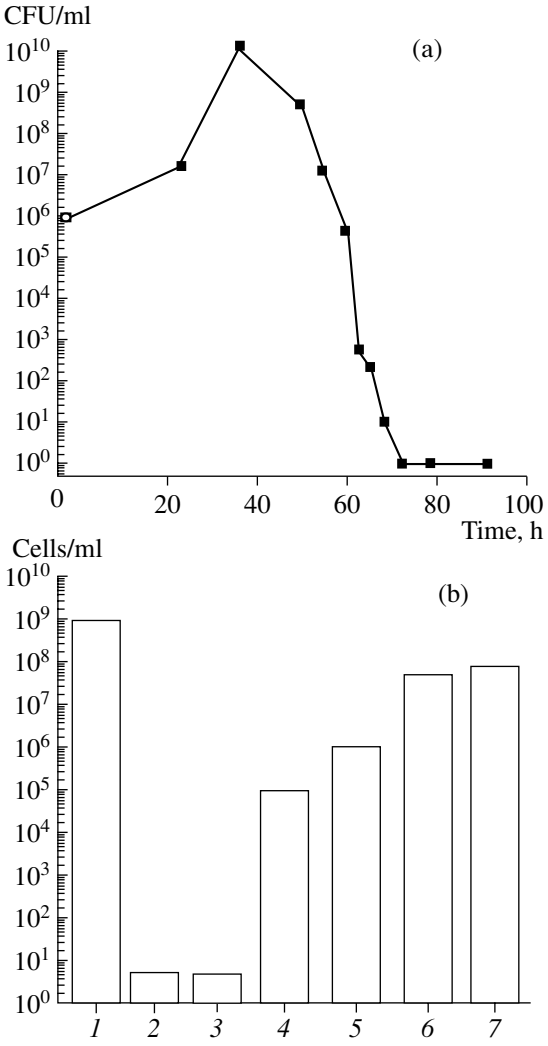

Fig. 2. Formation of NC *M. smegmatis* cells (a) and their resuscitation (b) in liquid cultivation media: (*1*) total cell number, (*2*) CFU number, and cell numbers after resuscitation of wild-type NC cells (*3*) in synthetic Sauton medium in the presence of 0.05% yeast extract, (*4*) in synthetic Sauton medium in the presence of 0.05% yeast extract and recombinant Rpf protein (125 pM), (*5*) in supernatant from an actively growing *M. smegmatis* culture used as a resuscitation medium, and (*6*) in synthetic Sauton medium in the presence of 0.05% yeast extract and Rpf producer (*M. luteus*); and (*7*) after resuscitation of strain AGR (obtained by transformation of *M. smegmatis* with a plasmid bearing the *rpf* gene of *M. luteus*) in synthetic Sauton medium in the presence of 0.05% yeast extract.

chronous division [4, 5]; i.e., dormant cells in the Wayne model remain culturable.

Currently, there also exists a model of obtaining *M. tuberculosis* NC cells by incubation in prolonged stationary phase. After four months of cultivation under gradually decreasing oxygen concentration, such cells, in contrast to the dormant cells in the Wayne model, which retain high viability, completely lose the ability to form colonies on agarized medium [13] but can be reactivated by incubation in a liquid nutrient medium (Fig. 1).

Recently, an in vitro model has also been developed for *M. smegmatis*, a fast-growing nonpathogenic relative of *M. tuberculosis.* In this model, cells cultured on a specially developed potassium-free medium convert to the NC state in the stationary phase under aerobic

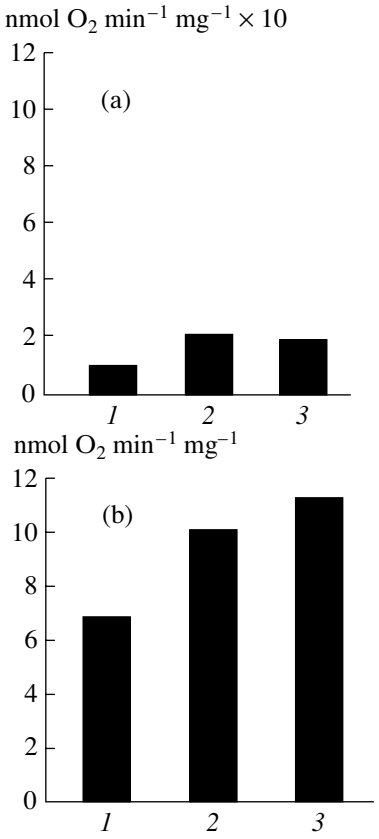

Fig. 3. Respiratory activity of NC (a) and metabolically active (b) *M. smegmatis* cells: (*1*) endogenous respiration and respiration in the presence of (*2*) 5 mM malate or (*3*) 5 mM glucose.

conditions [14] (Fig. 2). The level of endogenous respiration of such cells does not exceed 1–2% of the level of respiration of metabolically active cells (Fig. 3). Such NC cells could be reverted to the active state by a special resuscitation procedure.

CELL–CELL COMMUNICATION AND "NONCULTURABILITY" OF MYCOBACTERIA

The formation of NC *M. smegmatis* cells is accompanied by the accumulation in the growth medium of an inhibitory substance with an antibacterial effect. The supernatant from the culture of NC cells inhibits the growth of active cells of *M. smegmatis* inoculated at a concentration of 10^7 cells/ml. The chemical nature of the inhibitor has not been identified, though it is apparently a low-molecular, sufficiently hydrophilic compound (molecular weight of about 500–700 Da). Probably, increasing concentration of this substance in the culture of multiplying cells induces their conversion into the NC state; then, this phenomenon should be considered as intercellular communication mediated by chemical factors.

Reactivation of NC forms is one of the key issues in the study of nonculturability because it is rather difficult to demonstrate their principal difference from dead cells—potential viability—by other methods. The protein factor Rpf secreted by *Micrococcus luteus* into the culture liquid and showing reactivation ability towards dormant cells of *M. luteus* [15] was also shown to possess resuscitating activity towards NC cells of *M. smegmatis* and *M. tuberculosis* [13, 14]. As is known, chemical communication of gram-positive bacteria is realized via secreted oligopeptides and proteins [16]. In this case, the Rpf protein is obviously such a mediator of bacterial communication.

The attempt of reactivation of *M. smegmatis* NC cells in the presence of the Rpf protein producer, *M. luteus*, showed that this approach allows reactivation of up to 10^7 of NC mycobacterial cells per 1 ml of the culture liquid (Fig. 2), which is somewhat higher than upon reactivation in the presence of an isolated Rpf protein. This result may be explained, for instance, by proteolytic degradation of the protein, which occurs during the prolonged reactivation procedure. It is also possible that the higher values of reactivation during cocultivation of NC cells with the producer are due not only to the presence of fresh Rpf continuously secreted by the producer into reactivation medium, but also to physical contacts of the NC mycobacteria with micrococci during reactivation. It is known that physical cell–cell contacts, in particular the formation of bacterial aggregates during cultivation in liquid media, are quite typical of mycobacteria, nocardias, streptomycetes, and some other bacteria belonging to the high G+C lineage of gram-positive bacteria, even under optimal cultivation conditions; thus, this phenomenon is apparently important for processes of growth and development [16].

Interestingly, NC cells of strain AGR, obtained by transformation of *M. smegmatis* with a plasmid carrying the *rpf* gene of *M. luteus*, show marked capacity for self-reactivation in fresh growth medium in the absence of additional resuscitation factors, which is atypical of the wild-type NC cells. Quantitative assessment of the effectiveness of resuscitation showed that the quantity of reactivated cells of *M. smegmatis* AGR was close to the value obtained upon cocultivation of the wild-type NC cells with *M. luteus* (Fig. 2).

Thus, the important role of protein Rpf in the reactivation of NC forms is evident; however, the mechanism of its activating effect on dormant cells is still not quite clear. The peptidoglycan hydrolase activity of Rpf towards a cell-wall preparation and a model substrate [17], as well as the presence in its C-terminal sequence of the so-called LysM motif, which provides for the binding of proteins with the bacterial cell wall peptidoglycan, suggests that the effect of protein Rpf on nonculturable bacteria may be analogous to the effect of germination-specific lytic enzymes (GSLE) [18] and consist in loosening of the thickened cell wall of dor-

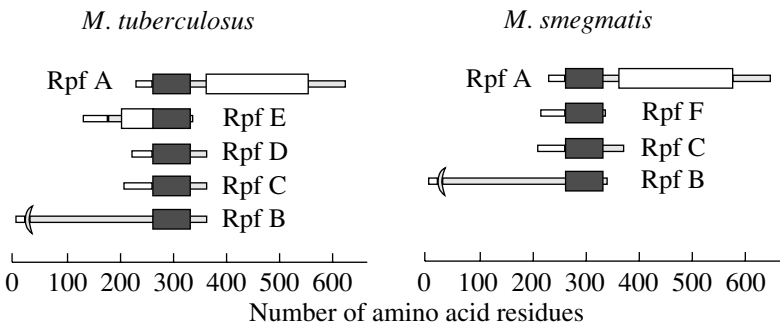

Fig. 4. Hypothetical structure of Rpf proteins of *M. tuberculosis* and *M. smegmatis*: ▭, signal sequence; ▬, conservative Rpf domain; □, a segment with high contents of proline and alanine.

mant bacteria or bacterial spores. As a result, bacterial spores or dormant cells would become more sensitive to external impacts and trophic stimuli (amino acids, ions, reactive oxygen species, etc.), which may initiate viable activity.

The *rpf* gene has been isolated and its nucleotide sequence fully determined. Comparison of the amino acid sequence of the *M. luteus rpf* gene product with the genomes of other organisms has revealed in many bacteria of the high G+C gram-positive lineage, mycobacteria in particular, one or several genes homologous to a certain region of the *rpf* gene of *M. luteus*. Five such genes were found in *M. tuberculosis* and *M. bovis*, four in *M. smegmatis*, and two in *M. leprae.* The supposed structure of Rpf proteins of *M. tuberculosis* and *M. smegmatis* is shown in Fig. 4. It can be seen that three of the five proteins of *M. tuberculosis* (Rpf A, B, and C) exhibit considerable similarity to the corresponding Rpf-like proteins of *M. smegmatis.*

It was demonstrated that, as in case of *M. luteus*, Rpf proteins exhibit stimulatory activity towards the producer, particularly if its growth is disturbed for any reason (e.g., growth stimulation of long-starved *M. bovis* cells); however, the level of Rpf secretion by *M. tuberculosis, M. smegmatis*, and *M. bovis* is very low [19]. For *M. smegmatis*, the maximum accumulation of Rpf proteins was observed in the beginning of the logarithmic growth phase during cultivation on optimal growth medium: it was approximately 50 pg/ml, whereas the level of Rpf protein production by *M. luteus* reaches 1 μg/ml. However, when the supernatant from a *M. smegmatis* culture occurring in the phase of active growth and maximal production of Rpf proteins is used as the reactivation medium, it is possible to reactivate NC cells of this bacterium in a quantity comparable to that achieved upon their cocultivation with *M. luteus* (Fig. 2). Reactivation of NC cells of *M. tuberculosis* in a supernatant of an actively growing culture of the same bacterium (with maximal production of Rpf proteins) allows up to 100% of cells to be converted to the active state. NC cells of the mutant strain *M. tuberculosis* KDT8, which misses three of the five genes encoding Rpf proteins, completely lacked the capacity for self-reactivation in fresh medium (Fig. 1). At the same time, the growth pattern of this strain under normal conditions did not differ from that of the parent strain [20]. It should be noted that mutant strains in which only one of the five genes encoding Rpf proteins were inactivated showed no decrease in the capacity of NC cells for self-reactivation.

Since the reactivation of NC forms is associated with Rpf proteins, it was assumed that the formation of NC cells may also depend on the level of Rpf protein production. It is known that the amount of Rpf protein accumulated in the *M. smegmatis* culture liquid by the stationary phase is beyond the limits of detection by immunological methods. However, as already noted above, these proteins can influence the cells at very low concentrations; therefore, the level of the Rpf protein synthesis at the moment of *M. smegmatis* conversion to the NC state was assessed by reverse polymerase chain reaction that used primers specific to the genes encoding the Rpf proteins A, B, C, and F.

The level of transcription of the *rpf* genes was monitored in the dynamics of conversion to the nonculturable state. In parallel, an actively growing *M. smegmatis* culture was studied. Transcripts of all four genes were revealed in the metabolically active culture; the RpfB-encoding gene was characterized by a decrease in the transcription level in the stationary phase. The culture growing with the formation of nonculturable forms contained no transcripts of the *rpf B* gene even at early growth stages; for other Rpf proteins, transcripts of the corresponding genes were found at the early stages of NC cell formation, but the formation of transcripts declined along with the decrease in the CFU number (Fig. 5). Consequently, the conversion of *M. smegmatis* to the NC state is accompanied by a decrease in the expression level of the Rpf-encoding genes.

CONCLUSIONS

The ability of bacteria of the genus *Mycobacterium* to convert to the NC state under unfavorable growth conditions (absence of K^+ ions in the growth medium for *M. smegmatis* or anaerobic cultivation conditions

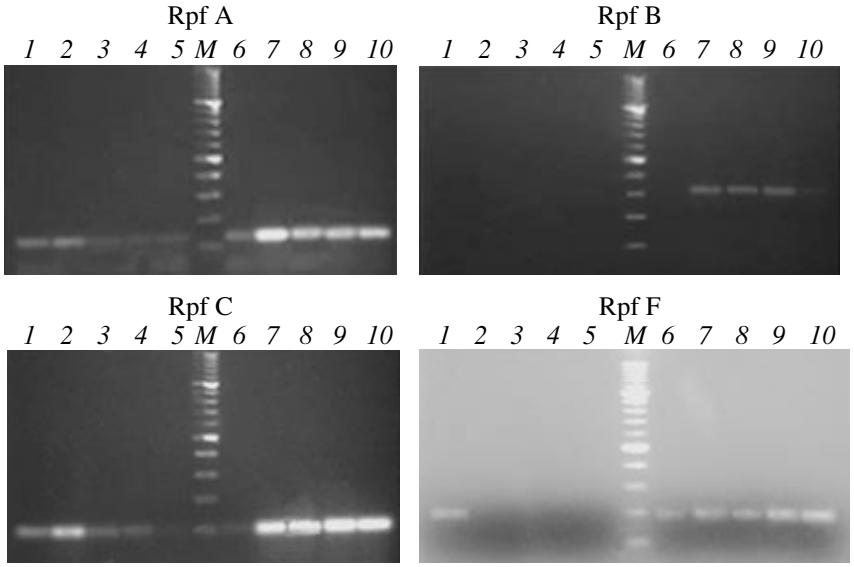

Fig. 5. RT-PCR analysis of the expression level of Rpf-encoding genes of *M. smegmatis* during growth with the formation of NC cells for (*1*) 24 h, (*2*) 48 h, (*3*) 72 h, (*4*) 96 h, and (*5*) 120 h and in a culture actively growing for (*6*) 24 h, (*7*) 48 h, (*8*) 72 h, (*9*) 96 h, and (*10*) 120 h. M, markers of the length of DNA fragments (bp).

for *M. tuberculosis*) seems to underlie the processes that eventually result in latent forms of tuberculosis. However, the reasons for the conversion of the bacteria into the nonculturable state are still unclear. Probably, one reason is the synthesis of some intra- or extracellular factors leading to the cessation of cell division. Our experimental data on the growth-inhibiting metabolite are in good agreement with this assumption. Besides, the inability of cells to divide, particularly marked on solid media, may result from unbalanced metabolism after a stress impact [3].

Conversion from the NC state occurs under the effect of Rpf proteins which are peptidoglycan hydrolases assumed to participate in cell wall modification during resuscitation of NC forms, similarly to the enzymes that activate spore germination. It is necessary to emphasize that the procedure of reactivation under the action of Rpf proteins, which are chemical mediators of bacterial communication, is efficient only in liquid medium, which offers more opportunities for intercellular communication.

It should be noted that NC cells of *M. tuberculosis* have a much more pronounced capacity for spontaneous reactivation as compared to their nonpathogenic relative *M. smegmatis*, whose NC cells can be converted into the active state only in the presence of external reactivating factors. This property of the tuberculosis pathogen is probably due to its virulence and capacity for long persistence in the host organism. The above-described procedure of reactivation of *M. tuberculosis* cells seems to be similar to the process of activation of latent tuberculosis in living organisms.

ACKNOWLEDGMENTS

We are grateful to M. Young, G.V. Mukamolova and V. Mizrahi for providing mutant strains of microorganisms.

This work was supported by the grants of the Program of the Russian Academy of Sciences Presidium "Molecular and Cell Biology", Russian Foundation for Basic Research (no. 06-04-49201), and International Science and Technology Center (project 2201).

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