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EXPERIMENTAL ARTICLES

# The Role of 2-C-Methylerythritol-2,4-Cyclopyrophosphate in the Resuscitation of the "Nonculturable" Forms of *Mycobacterium smegmatis*

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**Abstract**—2-C-Methyl-D-erythritol-2,4-cyclopyrophosphate (MEC), an intermediate of the biosynthesis of isoprenoid compounds in bacteria, was found to be capable of exerting a resuscitating effect on resting *Mycobacterium smegmatis* cells. The introduction of an additional copy of the *ispE* gene encoding cytidyl-methyl-erythritol kinase, an enzyme involved in MEC synthesis in *M. smegmatis*, resulted in the emergence of a capacity for spontaneous reactivation of "nonculturable" *M. smegmatis* cells, which is not characteristic of the wild-type cells of this species. The involvement of MEC in the transition from the "nonculturable" state to the state of active growth is indicative of a previously unknown function of MEC, assumed to consist in regulation of the bacterial genome activity.

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*Key words*: nonculturable cells, MEC, *ispE*, histone-like proteins, genome regulation, *Mycobacterium smegmatis.*

2-C-Methyl-D-erythritol-2,4-cyclopyrophosphate (MEC) is an intermediate of one of the pathways of the biosynthesis of isopentenyl pyrophosphate (IPPP), a key metabolite in the formation of high-polymer isoprenoid compounds in many living organisms. This pathway, called the nonmevalonate pathway, MEP pathway, or Rohmer pathway, was discovered in most of the bacteria studied, in plant plastids, and in malaria plasmodium apicoplasts. In archaea, plants, and animals, the biosynthesis of isoprenoids to the IPPP stage occurs via the so-called mevalonate pathway [1–4], which makes the MEP pathway an attractive target for the search for new antibiotics [5–7]. The publication of data on the influence of MEC on chromatin condensation in *Escherichia coli* overexpressing a chlamydial histone-like protein [8] makes us consider a novel function of MEC in bacterial cells—global regulation of the activity of the bacterial genome at the level of chromatin condensation–decondensation.

The formation of resting ("nonculturable") forms by bacteria, mycobacteria in particular, is of great interest both from the point of view of understanding the nature of the phenomenon and from the practical point of view, connected with medicine and biotechnology. Assuming the involvement of MEC in the resuscitation of resting bacterial forms, we studied the influence of exogenous and endogenous MEC on the formation of "nonculturable" (NC) forms of *M. smegmatis* and on the process of their reactivation.

## MATERIALS AND METHODS

**Subjects of study and cultivation methods.** The following *Mycobacterium smegmatis* strains were used in this work: the wild-type strain mc$^2$ 155; strain AGH, obtained by the transformation of the wild-type strain with plasmid pAG; the experimental strains AG-Ea and AG-Eb, obtained by the transformation of the wild-type strain with plasmids pAG-ispEa and pAG-ispEb, which carry a copy of the *ispE* gene; and the experimental strains AG-DFa and AG-DFb, obtained by the transformation of the wild-type strain with plasmids pAG-ispDFa and pAG-ispDFb, carrying a copy of the *ispDF* gene.

To obtain NC cells, *M. smegmatis* strains were grown for 30–36 h at 37°C on an orbital shaker (250 rpm) in 50-ml flasks containing 20 ml of Nutrient Broth (Himedia) to which 0.05 vol % of Twin-80 was added. This culture was then inoculated in a dose of 1 ml into a 750-ml flask containing 150 ml of modified

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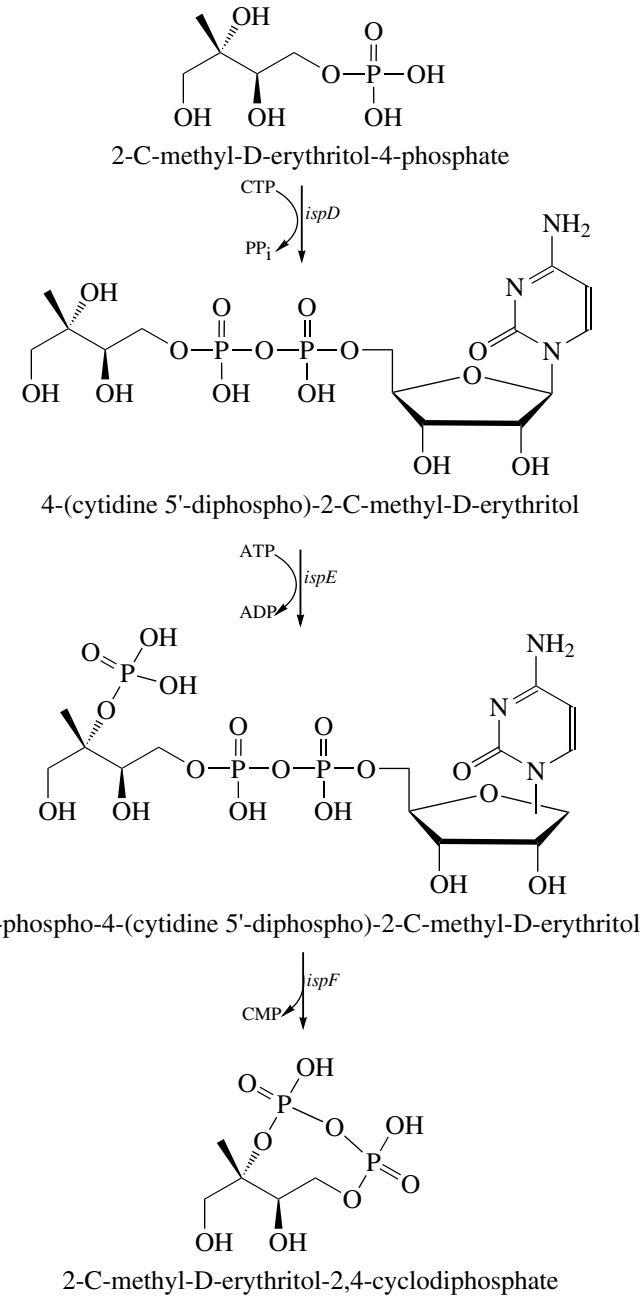


**Fig. 1.** Biosynthesis of MEC.

Hartman's–de Bont medium [9] to which bovine serum albumin (BSA) (Sigma) was added to a concentration of 0.5%. The flasks were incubated at 37°C on a shaker (250 rpm) for three to four days.

**The assessment of cell "nonculturability."** The culture obtained after cultivation for 72 h in modified Hartman's–de Bont medium [9] supplemented with 0.5% bovine serum albumin (Sigma) was serially diluted in a fresh medium, and aliquots of the dilutions were plated onto agarized (1.5%) nutrient broth. The inoculated plates were incubated at 37°C for four to five days, after which the grown colonies were counted to determine the CFU number.

**Resuscitation of *M. smegmatis* NC cells** was carried out in Sauton liquid medium in 48-well plastic plates (Corning). A series of tenfold dilutions was prepared for the resuscitation procedure to obtain a concentration of 10 cells/ml. Aliquots (50 μl) of the dilutions were added to plate wells containing 0.45 ml of Sauton medium with 0.05% yeast extract. The number of wells for each dilution was 3. In the experiments with exogenous MEC, it was added in the $10^{-7}$–$10^{-3}$ g/ml concentration range. The plates were then incubated at 37°C with agitation on a shaker at 150 rpm for six days.

When determining the number of resuscitated cells, wells with visible bacterial growth were taken into account. The value of the most probable number of resuscitated cells in 1 ml was determined using standard statistical tables [10].

When the soil microflora was resuscitated, filter-sterilized MEC preparations were introduced either into melted nutrient agar before pouring it into petri dishes or applied as a drop on the surface of solidified agar in petri dishes, where the MEC drop was mixed with a drop of the tested material and spread over the surface of the plate. The plates were then incubated at 20°C for a week; the number of grown colonies was counted every day. Samples of soil or water from the Moscow River in the region of the Neskuchnyi Garden (Moscow) were used as the tested material. The concentration of MEC after its uniform distribution in the solid medium was 0.02–1 mg in 1 ml of the agarized medium.

**Isolation of MEC.** MEC was obtained from the biomass of the bacterium *Corynebacterium ammoniagenes* ATCC 6872 grown under conditions of oxidative stress (vigorous aeration in the presence of the redox mediator benzyl viologen) as described earlier [11].

**Cloning of the genes involved in MEC biosynthesis.** Strain TG1 of *E. coli* (grown with agitation in LB medium at 37°C) was used. For cloning, the pGEM-T vector (Promega) was used; for expression, plasmid pAG was used, which carries the replication origin sites for *E. coli* and *M. smegmatis*, genes of kanamycin and hygromycin resistance, an amidase promoter with three subsequent open reading frames and an amidase-encoding gene, and a polylinker containing the *Xba*I site (T. Parish). The transformation of bacterial cells was carried out with the electroporation method using the MicroPulser device (Bio-Rad) according to the manufacturer's protocol. Ampicillin (100 μg/ml), kanamycin (50–20 μg/ml), and hygromycin (50 μg/ml) were used for selecting the transformed cells.

The nucleotide sequences of the *ispD*, *ispF*, and *ispE* genes responsible for the sequential reactions of MEC synthesis in *M. smegmatis* (Fig. 1) were identified using the BLAST program and the mapped genome of *Mycobacterium tuberculosis* (NCBI database).

Three genes (*ispDF*, *ispE* (*ispD* and *ispF* are overlapping genes)) with the corresponding ribosome-binding sites (about 70 base pairs before the start codon were taken) were amplified with the following pairs of primers: ispDF, 5'**actagt**aggttttggccgccgcgtcctgatc3' and 5'**actagt**cgacgtgccagcttaccggccctttg3'; ispE, 5'**actagt**-attttcgtcacgccacacatcacgcctcacg3' and 5'**actagt**agtcgtggccgtgcccgcatccgatg3' (the *Spe*I restriction sites, introduced for subsequent recloning, are typed in bold). The amplification products were ligated to the pGEM-T vector and transformed into *E. coli*. The accuracy of the nucleotide sequences cloned was confirmed by the sequencing of both chains. After selection of transformants, the *ispE* and *ispDF* sequences were excised with *Spe*I restriction endonuclease and *Xba*I-ligated to the pAG vector. The ligation products were transformed into *E. coli*. The selected colonies were analyzed for the insert orientation by means of the amplification reaction. The primer 5'gagaatcgcaacgcctacat3', corresponding to the sequence lying between the amidase promoter and the pAG *Xba*I site was used for analysis in addition to the above specific primers. Plasmids pAG-ispEa and pAG-ispDFa, carrying the corresponding genes collinear to the amidase promoter, as well as pAG-ispEb (with reverse *ispE* orientation), pAG-ispDFb (with reverse *ispDF* orientation), and the control initial plasmid pAG, were transformed into *M. smegmatis*.

## RESULTS AND DISCUSSION

When the wild-type strain of *M. smegmatis* was grown in modified Hartman's–de Bont medium with the addition of 0.5% BSA, the formation in the stationary phase of the NC phenotype characterized by a decreased capacity for growth on solid nutrient media was observed. As early as after 36 h of culture growth on solid nutrient medium, the CFU number started to decrease, and after 70–72 h of growth, the bacteria completely lost the capacity for growth on solid media. The control strain AGH, obtained by transformation of the wild-type strain with plasmid pAG, behaved similarly under these conditions.

It was shown earlier that the NC forms of the wild-type *M. smegmatis* could be activated using the resuscitation procedure in Sauton liquid medium in the presence of a number of "resuscitating" factors, including the supernatant fluid obtained from a log-phase culture of *M. smegmatis*, as well as protein Rpf secreted by the bacterium *Micrococcus luteus* in the process of growth [12]. The joint cultivation of the NC forms of the wild-type *M. smegmatis*, as well as of strain AGH, with an Rpf-producing *M. luteus* culture, produced a strong resuscitating effect [9]. An interesting fact is that in *M. tuberculosis* and *M. smegmatis*, the *rpfB* gene, whose product contributes to resuscitation from the nonculturable state [13], is near to, and in the same orientation with, the *ispE* gene [14] involved in the synthesis of the MEC precursor.

It is obvious that two approaches are possible for studying the role of MEC in the process of resuscitation of NC bacteria: (1) overexpression of the MEC synthesis genes by incorporating an additional gene copy and (2) the addition of isolated MEC to the cultivation medium. We attempted to realize both approaches.

**Induction of enhanced MEC synthesis inside the cells.** An enhancement of MEC synthesis was accomplished by the expression of additional recombinant gene copies involved in the synthesis of MEC in vivo.

The experiment used strain AGH, transformed with plasmid pAG (the control strain), and the experimental strains obtained by transformation with plasmids pAG-ispE and pAG-ispDF, carrying the *ispE* and *ispDF* copies, respectively. In plasmids pAG-ispEa and pAG-ispDFa, the cloned genes are collinear to the amidase promoter (strains AG-Ea and AG-DFa); in plasmids pAG-ispEb and pAG-ispDFb, the cloned genes have a reverse orientation (strains AG-Eb and AG-DFb). Acetamide was used as the inducer of the amidase promoter.

Our task was to assess the effect of the overexpression of these genes on the number and the rate of formation of "nonculturable" cells and their resuscitation.

When grown on modified Hartman's–de Bont medium with 0.5% BSA in the presence of 0.05% acetamide as the inducer of the *ispE* expression, the experimental recombinant strains *M. smegmatis* AG-Ea and AG-Eb and the control strain AGH had virtually the same cell density of a culture (an optical density of 0.9–1.1) and an equally high culturability index (more than 50% of the total number of cells in the stationary phase) after 68 h of cultivation, whereas in the absence of acetamide, the number of CFU for the control AGH strain virtually equaled zero after 68 h of cultivation in modified Hartman's–de Bont medium. However, when Sauton medium with 0.05% yeast extract was inoculated (at doses of $10^6$, $10^5$, $10^4$, and $10^3$ cells/ml) with aliquots of 68-h cultures of the experimental and control variants, the AG-Ea cells exhibited signs of growth 48–72 h earlier than the control strain AGH.

On the whole, when growth occurred in the presence of 0.05% acetamide, the formation of the NC phenotype was hampered in both the control strain AGH and the experimental strains AG-Ea and AG-Eb, was reproduced irregularly, and was not observed until 80 h of growth had elapsed, which was a 10-h delay compared to the experimental model in the absence of acetamide. The cells seem to have utilized acetamide (possibly as an additional source of nitrogen), which made the formation of the NC phenotype difficult. We lowered the recommended acetamide concentration to 0.01% to find that, in this case, the culturability of the control strain AGH on solid media completely disappeared after 70–72 h, whereas strains AG-Ea and AG-Eb retained residual culturability at a level of 10 to $10^7$ cells/ml.

In the process of resuscitation of these NC cells, strain AG-Eb, with gene *ispE* in reverse orientation rel-

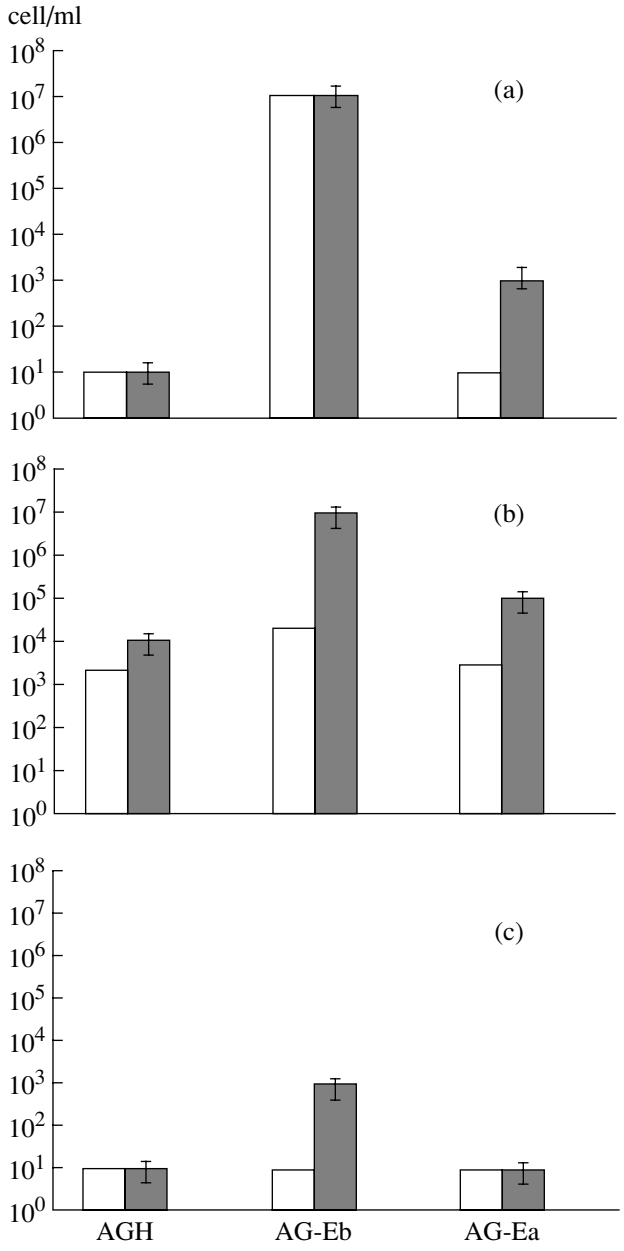


**Fig. 2.** Reactivation of NC cells of recombinant *M. smegmatis* strains. AGH is the control strain, AG-Ea and Ag-Eb are the experimental strains. Empty columns show the residual number of the colony-forming cells in the "nonculturable" culture. Filled columns show the cell number after reactivation determined by the end-point dilution method. (a)–(c) are individual experiments.

ative to the amidase promoter (this strain was used in the experiment as an additional negative control) revealed a unexpected phenotype, namely, a capacity for spontaneous reactivation in the absence of additional resuscitating factors. Strain AG-Ea with the directly oriented *ispE* gene also had such a capacity, but it was much less pronounced. In four of five reactivation experiments, the number of CFU in 1 ml of

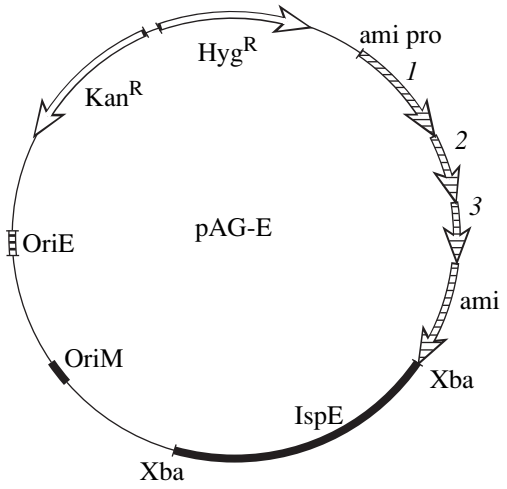


**Fig. 3.** Scheme of plasmid pAG-ispE.

medium increased by one to 2.5 orders of magnitude or more (Fig. 2). It is necessary to point out that the insufficiently good reproducibility of the results observed from experiment to experiment is a common situation for the experiments on the transition to the NC state and reactivation.

Strains with plasmids pAG-ispDFa and pAG-ispDFb did not show any physiological differences from the control strain with plasmid pAG.

The results obtained may be explained by the fact that, when the *ispE* gene was cloned, its promoter region, lying nearby, was recruited, resulting in independence of the gene transcription of the amidase promoter. The efficiency of *ispE* transcription was influenced by the gene orientation in the plasmid, likely due to the joint action of its own promoter and the amidase promoter (Fig. 3).

Thus, it is evident that the overexpression of IspE lengthens the time required to attain the state of "nonculturability" and contributes to the reactivation of NC cells (likely by increasing MEC synthesis). That the overexpression of IspE is implicated with the time of attaining the state of "nonculturability" indicates that the "cell hunger" mechanism is insufficient for understanding the changes occurring in a bacterial cell.

We can propose the following mechanism of the influence of an increased intracellular MEC concentration on the prolongation of the phase of "partial nonculturability" and reactivation of NC cells. It was shown that a resting *M. smegmatis* culture has an increased content of a histone-like protein [15]. Our unpublished data confirm the fact of increased transcription of the *hupB* gene of the *M. smegmatis* histone-like protein with the transition to the NC state. On the other hand, it was found that chromatin condensation in *E. coli* triggered by overexpression of the chlamydial histone-like protein may be abolished by MEC [8]. It seems that one of the processes occurring in the cells losing the capac-

ity for culturability is a change in chromatin due to its binding to histone-like proteins. The structure of MEC includes a macroergic pyrophosphate bond in a tense cycle, and this allows it to bind nonenzymatically to certain groups [16]. By interacting by means of its two negatively charged groups with positively charged histone-like proteins, MEC is capable of neutralizing their binding to DNA, thereby delaying the onset of the state of nonculturability on the one hand, and contributing to the reactivation of NC cells on the other hand.

**Reactivation of "nonculturable" forms of *M. smegmatis* by exogenous MEC.** Since the MEC concentration in a bacterial cell beyond stress conditions is low and it was not possible to assess an increase in the synthesis of MEC by the yield of the product, we attempted to resuscitate the NC cells of *M. smegmatis* by adding exogenous MEC. But in this case, the permeability barrier in the form of the cell wall and cytoplasmic membrane represented an obstacle. We suppose that, in the membranes, there exists a system of transfer or exchange of organic phosphates (this is evidenced by the release of nucleoside triphosphates by mycobacteria [17]), and at a sufficiently high MEC concentration outside the cell, it can be delivered inside.

We used as a "nonculturability" model the wild-type culture of *M. smegmatis*, which loses the capacity for growing on solid nutrient media after 72-h cultivation on modified Hartman's–de Bont medium supplemented with 0.5% BSA. The addition of pure MEC to the resuscitation medium led to restoration of the MEC concentration–dependent capacity for growth (Fig. 4); however, we observed this phenomenon only in two of four experiments, which does not allow us to define explicitly the culture state in which MEC is able to influence the development of bacterial cells. It may be suggested that MEC reveals its resuscitating effect only in the case where the culture still has some residual capacity for growth on solid medium, because we did not observe the resuscitating effect of MEC when the culture survival rate was zero.

In the case where a 65-h culture with a CFU of $2 \times 10^3$ $ml^{-1}$(with a total number of $10^9$ cells in 1 ml) was used in the resuscitation experiment, MEC at a concentration of $10^{-7}$–$10^{-3}$ g/ml was able to exert a resuscitating effect on the NC cells of *M. smegmatis* (Fig. 4).

Can such a high MEC concentration be formed in natural bacterial populations? We cannot rule this out, because the potential capacity of MEC for spontaneous reactions is unlikely to be completely realized in the cell, and under oxidative stress, MEC was shown by us to accumulate in cells in large amounts (up to 0.1 M) [11]. In this connection, it is appropriate to point out that a large group of bacteria isolated from soil by the characteristic of resistance to $Hg^{2+}$ ions is able to accumulate considerable amounts of MEC under normal cultivation conditions (without oxidative stress) [18].

The successful resuscitation of the NC forms of *M. smegmatis* inspired hope that MEC might serve as an exogenous activator of other bacteria as well; however, the straightforward approach to this problem fell short of our expectations.

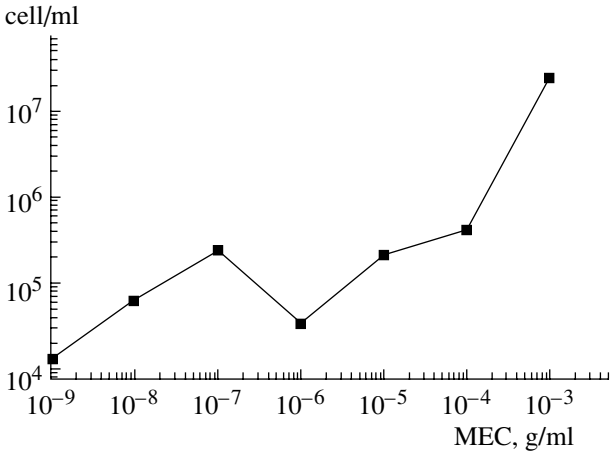


**Fig. 4.** Effect of MEC on the reactivation of "nonculturable" cells of *M. smegmatis.*

It is generally agreed that most bacterial species are in a resting ("nonculturable") state under natural conditions [19, 20]; therefore, one supposition suggests itself: perhaps MEC is one of the regulators of this state, and perhaps it is possible to activate resting cells by simply adding MEC to the cultivation medium?

Soil and water samples were used as tested materials. Under conditions of this experiment, the CFU number in the samples studied did not depend on the presence or absence of MEC in the cultivation medium (up to a concentration of 100 mg/ml, i.e., 330 mM). To increase the permeability of bacterial cells to MEC molecules, which carry two negative charges, we added compounds (the detergents cetyl trimethylammonium bromide and swittergent) that might aid MEC penetration into cells. However, these reagents did not result in the CFU number increase.

Thus, at least under the experimental conditions set by us, no mass resuscitation of resting cells in soil and natural river water occurred under the influence of MEC. To interpret the role played by MEC in the life of soil microflora is an exciting ecological task that will require much effort to solve.

Based on the data presented, it is possible to conclude that MEC, in addition to being an intermediate of isopentenyl pyrophosphate biosynthesis, also prevents the transition of mycobacteria to the state of nonculturability and contributes to their resuscitation. The incorporation of an additional copy of the *ispE* gene involved in MEC synthesis delayed the state of "nonculturability" and contributed to the resuscitation of NC cells. Moreover, it was revealed that exogenous MEC at a concentration of $10^{-7}$–$10^{-3}$ g/ml is capable of exerting a resuscitating effect on the NC cells of *M. smegmatis*.

Despite the ability to resuscitate *M. smegmatis* in the "nonculturability" model described above, MEC did not stimulate the total activation of dormant cells of soil microflora under the conditions of our experiments.

The involvement of MEC in the transition of bacteria from the state of "nonculturabilty" to the state of active growth is indicative of a novel function of MEC, supposedly consisting in the regulation of the activity of the bacterial genome at the level of chromatin condensation–decondensation.

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