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EXPERIMENTAL ARTICLES

Dormant Forms of *Micrococcus luteus* and *Arthrobacter globiformis* Not Platable on Standard Media

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Abstract—The colony-forming ability of long (3–9 months) incubated cystlike resting cells (CRC) of the non-spore-forming gram-positive bacteria *Micrococcus luteus* and *Arthrobacter globiformis* was studied in this work. The preservation of the CRC proliferative potential as assayed by plating on standard LB agar was shown to depend on the conditions of the formation of the dormant cells. In aged post-stationary cultures of micrococci and arthrobacters grown under carbon and phosphorus limitation the number of colony-forming units (CFU/ml) of CRC decreased in the course of 3–9 month incubation to the level of 10^6–10^7 CFU/ml. However, *M. luteus* CRC obtained under carbon and nitrogen limitation and *A. globiformis* CRC obtained under nitrogen limitation and starvation completely lost their ability to form colonies on standard solid medium after 4–6 months of incubation and turned into a 'non-culturable' (non-platable) state. In this case, the ratio of live cells in the population of *M. luteus* and *A. globiformis* 'non-culturable' CRCs (determined by the Live/Dead staining test) was 10–44% of the total cell number. To study the possible preservation of proliferative potential in non-platable CRCs, various methods of their reactivation were applied. Although preincubation of CRC suspensions in a buffer solution of 0.1 M K_2HPO_4 (pH 7.4) or in the presence of lysozyme (1 or 10 μg/ml) resulted in increased numbers of live cells (determined by the Live/Dead test) or in disruption of the cell conglomerates, it did not increase considerably the CFU titer on LB medium. Variations in the medium composition, such as addition of sodium pyruvate as an antioxidant or dilution of the medium, promoted the formation of macrocolonies by a small portion of nonplateable CRC of *M. luteus* (50–80 CFU/ml), whereas the number of the cells capable of microcolony formation (mCFU) was $1.8–6.8 \times 10^5$ mCFU/ml, exceeding the CFU titers by four orders of magnitude. The application of semisolid agar and the most probable number (MPN) method was the most efficient for determination of the mCFU titer, and an almost complete reversion of 'non-culturable' micrococcal CRCs to microcolony formation was observed (up to 2.3×10^7 mCFU/ml). The usefulness of diluted complete media for the restoration of the colony-forming ability of the dormant forms was confirmed in experiments with 'non-culturable' CRCs of *A. globiformis*. The development of special procedures and methods for determining actively proliferating cells not detected by ordinary methods is of great importance for advanced monitoring studies.

Key words: dormancy, cystlike cells, 'non-culturable' state, reactivation, microcolonies, the MPN method

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Non-spore-forming actinobacteria of the genera *Micrococcus* and *Arthrobacter* are widespread in natural habitats and were revealed in large amounts in ancient permafrost sediments [1–4] and buried paleosoils, which are characterized by the absence of available water and nutrient flow [5]. Survival of non-spore-forming bacteria in the ecosystems, where conditions unfavorable for growth prevail, is probably due to the formation of cystlike resting cells (CRC) in a state of anabiosis (cryptobiosis). The CRC occurrence and prevalence in microbial populations was revealed in situ by electron microscopy of permafrost samples [6, 7] and was suggested in buried paleosoils. However, the number of microbial cells capable of colony formation on standard media was lower by at least two orders of magnitude than the total cell number determined by direct microscopic examinations of permafrost sediments and subsoil sediments [2, 4], as well as of modern soils and other natural ecosystems [8]. This discrepancy can be explained by the heterogeneity of the populations of dormant forms both in their proliferative potential and in ability to grow on standard media. A state, in which microorganisms retain their viability but

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cannot be cultivated under standard conditions, is considered as a form of a dormant state [8–10].

Actinobacteria *Micrococcus luteus* and *Arthrobacter globiformis* were chosen as the objects of this study for the following reasons: (1) The inverse transition of *M. luteus* cells into a nonculturable state with a loss of colony-forming ability by the major part of the aging population (stored for 6 months) is well studied [9–11). The revival (resuscitation) of 'non-culturable'cells occurs in the presence of either the culture liquid of exponentially grown cultures [10] or the extracellular Rpf protein [11, 12]. (2) Both species have constitutively dormant cystous forms, which differ structurally from the vegetative cells and, in accordance with the criteria accepted in spore research, possess essential characteristics of resting forms of prokaryotes, including long-term preservation of the colony-forming ability [7, 13, 14]. The study of CRC of other non-spore-forming bacteria revealed that the number of the cells capable of colony formation depended on the conditions of the formation of the dormant cell [15]. No such investigations were performed with CRCs of *M. luteus* and *A. globiformis*. Thus, heterogeneity of the dormant population of actinobacterial in their ability to germinate on standard media as well as the dependence of this process on conditions of CRC formation remain unclear.

The aim of this study was to elucidate the conditions determining the transition of dormant forms of micrococci and arthrobacters into a 'non-culturable' state and to develop the procedures for their revival that will allow more complete detection of potentially proliferating cells in CRC populations.

MATERIALS AND METHODS

Non-spore-forming bacteria *Micrococcus luteus* NCIMB 13267 and *Arthrobacter globiformis* B-66^T (VKM) were used in this work. *M. luteus* was grown in the basal synthetic medium containing the following (g/l): lithium lactate, 5; KH_2PO_4, 4; NH_4Cl, 4; $CaCl_2$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; thiamine, 0.04; methionine, 0.02; trace elements (mg/l): $FeSO_4 \cdot 7H_2O$, 20; $MnCl_2 \cdot 4H_2O$, 20; $ZnSO_4$, 0.4; $B(OH)_3$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.5; $Na_2MoO_4 \cdot 2H_2O$, 0.2; and pH 7.25. *A. globiformis* was grown in unlimited medium containing the following (g/l): glucose, 10; $K_2HPO_4 \cdot 3H_2O$, 1; KH_2PO_4, 0.1; $(NH_4)_2SO_4$, 0.2; $CaCl_2$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; and pH 7.25. The bacteria were cultivated at 28°C in 250-ml flasks with 50 ml of the medium on a shaker (140–160 rpm). The media were inoculated with the stationary-phase cultures at the initial optical density (OD_{600}) of 0.2 units, which was determined on a Specord spectrophotometer in 10-mm cuvettes.

CRC of the studied strains were obtained in post-stationary-phase cultures grown in modified unbalanced media and then incubated for 2–9 months under static conditions at 18–20°C. To obtain CRC of micrococci, bacteria were grown in media with a twofold decreased content of lithium lactate (2.5 g/l) and tenfold decreased amounts of KH_2PO_4 or NH_4Cl (0.4 g/l each). The CRC of arthrobacteria were obtained by cultivation in the medium with a twofold decreased content of $(NH_4)_2SO_4$ (0.1 g/l) or by transfer of the stationary-phase cultures grown in unlimited medium into 0.09% solution of sodium silicate $Na_2SiO_3 \cdot 9H_2O$ (pH 7.4) in a ratio of 1 : 10 (vol/vol).

The growth of bacterial cultures was monitored by measuring OD of the cell suspensions and by calculation of the maximal specific growth rate (μ_{max}) and biomass yield (Y_{max}). The mass of dry cells (MDC) was determined after biomass drying at 105°C.

The total cell number (N) was determined by direct cell counting under a Zetopan microscope (Reichert, Austria) in at least 20 fields of vision (550 μm^2) in 20-μl samples covered with 18 × 18 mm cover slips. To distinguish dead and live cells, the specimens were stained with a two-component dye, Live/Dead Baclight® kit L-13152 (Molecular Probes Inc.) and incubated in the dark for 10 min according to the manufacturer's instructions. Stained specimens were examined under a MBI-15 U42 fluorescence microscope (Russia) or under an Axioplan microscope (Carl Zeiss, Germany). The live cells showed green and yellow fluorescence, whereas dead cells were red-colored.

Thin-sectioning electron microscopy examinations were carried out as described earlier [7].

The number of colony-forming cells (CFU/ml) was determined by plating 10^N-fold diluted bacterial cultures or cell suspensions (100 μl) on petri dishes with agarized (1.5%) media. After inoculation, the dishes were incubated at 28°C for 3–14 days. In the cell dilutions resulting in no more than 100 CFU/dish, the colonies different from the dominant type were counted; their stability was determined by successive stab-inoculations on solid media.

The number of microcolony-forming cells (mCFU/ml) was determined by direct counting of microcolonies on (1) 20 randomly chosen squares (10 × 10 mm) on the surface of agarized media in petri dishes; (2) the replicas obtained by placing sterile 18 × 18 mm cover slips on the agar surface and subsequent fixation with 30% ethanol; and (3) 20-μl aliquots of semisolid medium (0.25% agar) inoculated with cell suspensions diluted in the ratios of 1 : 10, 1 : 100, and 1 : 1000 (vol/vol) and incubated at 28°C for 3 days. A ×2.5–5 binocular loupe was used for the first approach, and conventional microscopy, for the latter two.

The number of viable cells (capable of proliferation) was estimated by the most probable number (MPN) method. Aliquots of the cultures or cell suspensions diluted 10^N-fold were inoculated into test tubes (1.8 ml of medium + 0.2 ml of cell suspension) or in 48-well plates (450 μl of medium + 50 μl of cell suspension) and incubated at 28°C for 3–14 days. The most

probable number of viable cells was calculated using conventional tables.

To study growth reversion, prior to plating on solid media, the CRC suspensions were treated by the following methods: (A) Cells were washed twice for 2 h with a tenfold volume of 0.1 M K_2HPO_4 (pH 7.4) with subsequent centrifugation (5000 *g*); (B) The cell suspensions diluted tenfold with 0.1 M K_2HPO_4 (pH 7.4) were incubated in the presence of lysozyme (Sigma, United States) in concentrations of 1 or 10 μg/ml at 28°C for 45 min or (C) the same suspensions were incubated in the presence of a IX-F egg phosphatidylcholine (Sigma, United States) in concentrations of 0.8, 8.0, and 80 μg/ml (ethanol concentration was 5%, vol/vol) at 28°C for 1.5 h; (D) The cell suspensions (0.3 ml) were supplemented with the solution (0.6 ml) of the extracellular reactivating factor (RF) isolated from the culture liquid of *Luteococcus casei* [16] and incubated at 28°C for 3 h prior to plating on solid media; in several experiments, RF was added into the wells with the medium in a 1 : 1 ratio (vol/vol) either at the moment of CRC inoculation or 2, 4, and 6 h after inoculation; (E) Sodium pyruvate solution (0.15 ml) was added onto a surface of the solid medium (up to the final concentration of 0.1%, wt/vol) as an antioxidant according to [17]. The number of viable cells was determined with the use of liquid and solid (1.5% agar) media based on the standard Luria-Bertani (LB) broth (Difco), LB broth diluted two- and fivefold, basal synthetic medium with 10 g/l lactate, and semisolid LB-based media (0.25% agar).

Measurement replications were fivefold when OD, CFU, and MPN were analyzed and 20-fold when the total number of cells (N) and mCFU titer were determined. The presented results show the averaged values and standard deviations. The MPN values are given with a confidence interval estimated for $P < 0.05$. The results were statistically processed by the Student method.

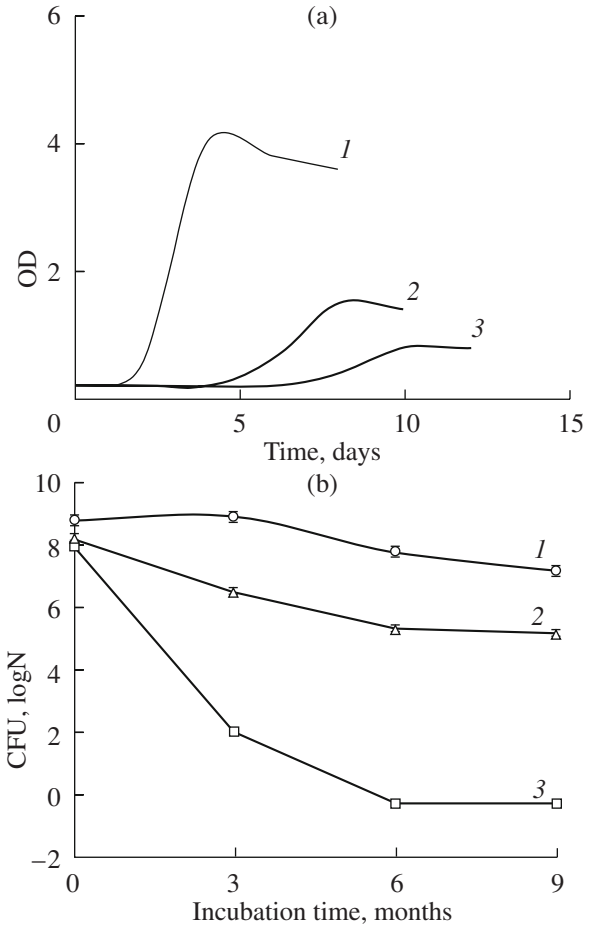

Fig. 1. (a) Growth of *M. luteus* and (b) the number of colony-forming cells (CFU/ml) in long-term incubated cultures in: basal unlimited medium (*1*), carbon- and phosphorus-limited medium (*2*), and carbon- and nitrogen-limited medium (*3*). Standard deviations (10%) for (a) are not shown.

RESULTS

To obtain the CRC populations of *M. luteus* and *A. globiformis* and to study their properties, such as the maintenance of colony-forming ability and transition to a "non-culturable" state, we used media deficient in carbon and nitrogen or in carbon and phosphorus. It is carbon limitation that distinguished these modified media from those earlier applied for obtaining the CRC of micrococci and arthrobacters, which preserved for a long time their ability to form colonies on solid media [13, 14].

Dormant forms of *M. luteus*. The growth of *M. luteus* under double limitations in media with a twofold decreased content of lactate and tenfold decreased amounts of phosphorus (C + P) or nitrogen (C + N) was characterized by a longer lag-phase, a later transition to the stationary phase, and lower values of specific growth rate and biomass yield than that in basal unlimited medium. Growth retardation was more pronounced in the medium deficient in carbon and nitrogen (C + N) (Fig. 1a, Table 1). Under double limitations (C + P) or (C + N), the cell cycle of *M. luteus* was culminated by formation of refractive CRCs (Figs. 2b and 2c), which differed in size and in the level of refractivity from the vegetative cells (Fig. 2a) and comprised 35 and 67% of the total cell number, respectively. Small cells prevailed in the cultures grown under limitation by carbon and nitrogen (C + N) (d = 0.6–0.7 μm, Fig. 2b) or by carbon and phosphorus (C + P) (d = 0.7–0.8 μm, Fig. 2c), while the cells grown in the basal unlimited medium were bigger (d = 1.0–1.2 μm, Fig. 2a). At the late stages of development in unbalanced media, cell aggregates containing both small (8–12 cells) and large (up to 80 cells) conglomerates were formed; this process intensified under long-term incubation (3–9 months) and was most pronounced in nitrogen-limited cultures (Fig. 2d). The content of live cells (determined by the Live/Dead test)

Table 1. Characteristics of *M. luteus* growth on complete and limited media

Medium	Lag-phase, days	Specific growth rate (μ_{max}), h^{-1}	Onset of the stationary phase, days	Biomass yield (Y_{max}), gMDC/ml	Number of viable cells*, CFU/ml
Basal medium	1.5	0.15	3.5	1.6	6.4×10^8
Medium limited in carbon and phosphorus	4	0.05	8	0.43	1.6×10^8
Medium limited in carbon and nitrogen	7	0.02	12	0.32	1.2×10^8

Note: *Determined by plating of the stationary-phase cultures on standard medium, LB agar.

in small and large conglomerates varied from 25 to 75% and from 8 to 17%, respectively. The occurrence of conglomerates led to certain difficulties in counting the number of viable cells since it is unclear whether the colony is formed by a single cell or by a conglomerate of several cells. The viability numbers as determined by CFU, mCFU, and MPN may therefore be underestimated.

The preservation of the CRC ability to form colonies on LB agar after long-term incubation (up to 9 months) depended on the conditions of CRC formation. Thus, in the micrococcal cultures grown under carbon and phosphorus limitation (C + P), the CFU titer decreased by two orders of magnitude during 6 months and then (by the 9th month) stabilized at the level of $(1.7 \pm 0.2) \times 10^5$ CFU/ml (Fig. 1b). At the same time, the CFU titer of CRC incubated for 9 months determined on LB agar supplemented with 0.1% pyruvate increased to $(1.0 \pm 0.1) \times 10^6$ CFU/ml; in the case of the CRC pre-incubation with lysozyme (1 μg/ml, 45 min), the CRC titer on standard LB agar increased to $(6.2 \pm 0.2) \times 10^5$ CFU/ml. However, treatment of the CRC with a higher lysozyme concentration (10 μg/ml) resulted in cell destruction that was indicated by the absence of colony formation and by the cell lysis observed by microscopic examination. CRC of micrococci incubated under (C + P) limitation for 6 and 9 months, when plated on LB agar formed numerous (up to 62–71% of the total number) small colonies ($d < 1$ mm) (Fig. 2e). In the case of stab inoculation on solid medium, small colonies completely reversed to the initial dominant type which formed colonies of 5–7 mm in diameter.

Under (C + N) limitation, the titer of CFU on LB agar in the populations of micrococcal dormant cells decreased by six orders of magnitude (to 2.1×10^2 CFU/ml) during 3 months of incubation (Fig. 1b). The formation of small colonies was also observed in 3-month old CRC that exhibited low but detectable CFU titer. However, no colony-forming cells were observed in the CRC populations incubated for 6–9 months (0.5 CFU/ml; 1 colony per 20 dishes); the most probable number of viable cells determined in liquid standard medium (LB broth) was extremely low (11 cells/ml). According to direct microscopic examinations, the total number of intact cells in the population of non-growing CRC was 2.6×10^8 cells/ml, whereas the ratio of live cells (emitting green and yellow fluorescence) determined by the Live/Dead test was 10% (Table 2). Thus, the lack of the ability of almost all cells in the population to form colonies on solid media or to grow in liquid standard media cannot be explained by their death under long-term incubation but is due to their transition of CRC to a "non-culturable" state.

Dormant forms of *A. globiformis*. The dependence of the CRC ability to germinate in standard media on the conditions of their formation and duration of incubation was also demonstrated for *A. globiformis*. When grown both in balanced synthetic unlimited medium and under nitrogen limitation, *A. globiformis* B-66 formed CRCs that rarely occurred in LB cultures. However, the dynamics of preservation of the colony-forming ability was different in the dormant cells formed under different conditions. In arthrobacters grown on unlimited synthetic medium, the number of CRCs capable of growth on LB agar remained within an order of magnitude (10^9 CFU/ml) for 2 months of incubation and decreased to 2×10^5 CFU/ml during subsequent incubation for up to 4 months (Fig, 3a). In the CRC population formed under nitrogen limitation, the CFU titer decreased by an order of magnitude after 2 months of incubation and to single CFUs/ml by the 4th month of incubation (Fig. 3a). In another variant, CRCs of arthrobacters were formed when the stationary-phase cells were transferred into the solution of $Na_2SiO_3 \cdot 9H_2O$ (at the same cell density) and incubated for 4 months at 18–20°C. In 2-month arthrobacter suspensions starving in the silica solution, the number of cells capable of colony formation on standard solid medium sharply decreased to zero (graph is not shown).

In the arthrobacter CRC populations formed under nitrogen limitation and incapable of growth under standard conditions after 3.5 months of incubation, the number of live cells (determined by the Live/Dead test) was 1.6×10^8 cells/ml (44% of the total cell number). High concentration of live cells (about 10^7 cells/ml) was also observed in starving cell suspensions. The observed discrepancy between the total cell number and virtually zero CFU number indicates that the standard media (LB agar) and conditions are unsuitable to determine the titer of viable resting cells of arthro-

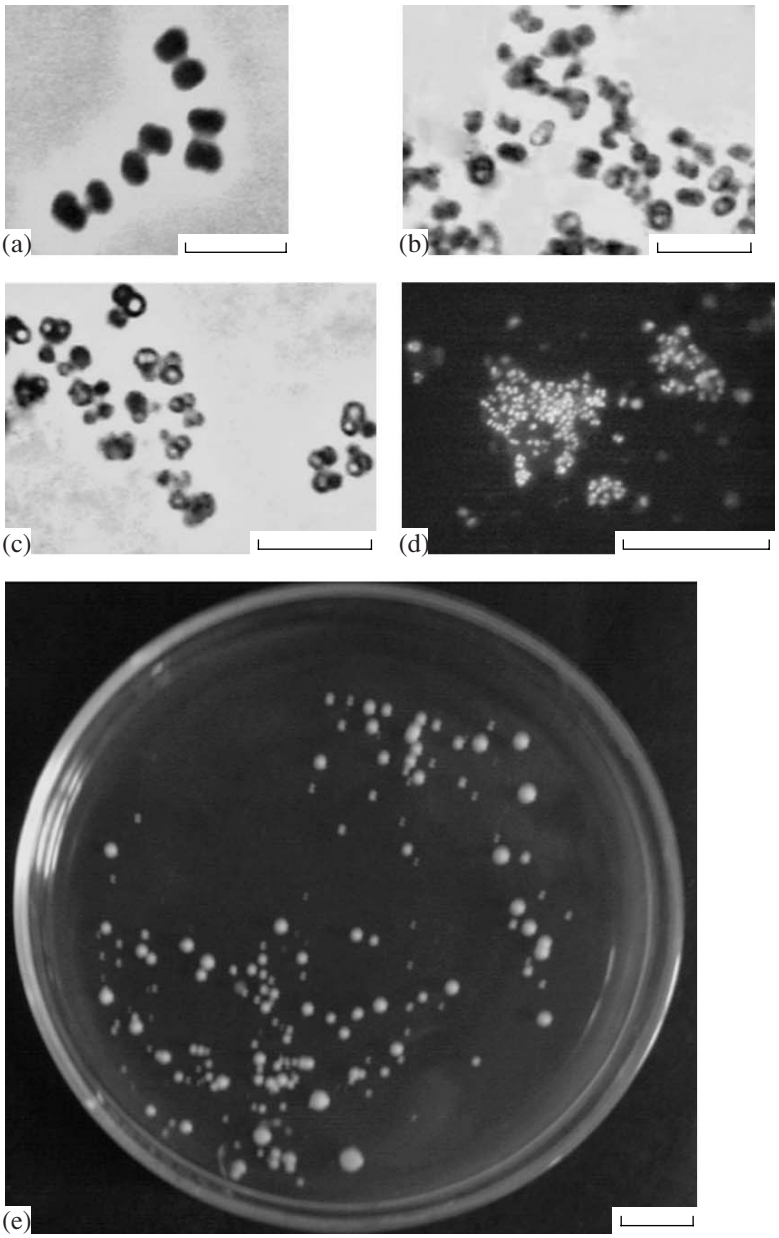

Fig. 2. Vegetative cells (a), CRC of *M. luteus* (b–c), and large conglomerates (d). Dormant forms were obtained from cultures grown on media: limited by carbon and phosphorus (b) and limited by carbon and nitrogen (incubation for 9 months) (c, d). Small colonies developed after plating of 6-month cultures of micrococcal CRC on the medium limited by carbon and phosphorus (e). The bars represent 5 μm (a–c); 25 μm (d); 10 mm (e).

bacters, similar to the above "non-culturable" forms of micrococci. Since rich media can invoke the effect of substrate-accelerated death in the cells potentially capable of growth, we used complete but diluted media to determine the titer of actively proliferating (viable) cells in the dormant and starving populations of "non-culturable" *A. globiformis* CRCs. This approach was supported by the fact that in the case of arthrobacters, the CRCs incapable of growth on LB agar were obtained under starvation conditions or on the media with more pronounced nitrogen deficiency than those used for obtaining CRCs of micrococci (concentrations of $(NH_4)_2SO_4$ were 0.1 and 0.4 g/l, respectively).

The number of colonies grown on twofold diluted LB agar from the *Arthrobacter* CRC incubated for 4 months under nitrogen limitation was 3.3×10^6 CFU/ml (Fig. 3b), i.e., 2% of the total number of live cells determined microscopically with the Live/Dead test. The arthrobacter CRC from the cell suspensions incubated for 2 months under starvation (in a solution of $Na_2SiO_3 \cdot 9H_2O$) showed the titer of 1.2×10^6 CFU/ml on diluted LB agar that corresponded to

Table 2. Effect of the preincubation methods on the revival of "non-culturable" CRCs of *M. luteus* on standard media (LB agar or LB broth)

Method of preincubation*	Cell number		Observed effect
	CFU/ml (on LB medium)	MPN, cells/ml	
Control	0.5	11 (3–33)	
Total cell number (N) including:			$(2.6 \pm 0.7) \times 10^8$ cells/ml
"live" cells with green and yellow fluorescence:			$(2.6 \pm 0.4) \times 10^7$ cells/ml (10%)
"dead" cells with red fluorescence:			$(2.33 \pm 0.4) \times 10^8$ cells/ml (90%)
(A) Cell washing with buffer solution	0	0	2.5-fold increase in the level of cells with green and yellow fluorescence (up to 25.4%)
(B) Preincubation with lysozyme			
1 μg/ml	20	ND***	Disruption of cell conglomerates
10 μg/ml	10		
(C) Preincubation with phosphatidylcholine	0	ND***	Not detected
(D) Addition of reactivating protein from *L. casei*	0	82.5** (24.2–264)	See Table 3

Notes: * (A–D) designations as in "Materials and Methods";
** the addition after 6 h of incubation.
***ND, not determined.

10% of the total cell number (Fig. 3b). Thus, plating of dormant "non-culturable" cells of *A. globiformis* on diluted medium provided for a significant, although not complete reversion of their colony-forming ability.

Methods of resuscitation of non-platable CRCs of *M. luteus*. Various methods for CRC resuscitation and enumeration of the cells potentially capable of proliferation were tested on non-culturable CRCs of *M. luteus* obtained by carbon and nitrogen limitation (C + N) and incubation for 9 months. (A) The first method of resuscitation involved washing of unculturable cells with a slightly alkaline 0.1 M K_2HPO_4 solution (twice for 2 h). This procedure was aimed at the extraction of anabiosis autoinducers (AA), removing the metabolic block and providing for K^+ transport into the cells [18]. No cells capable of proliferation were revealed by plating of washed CRCs on LB agar (CFU titer) or in LB broth (MPN count). However, the Live/Dead test showed a considerable increase in the content of live cells with green and yellow fluorescence (up to 25.4% of the total cell number) accompanied by a decreased level (to 74.6%) of the cells with red fluorescence, i.e., of dead cells or those with an impaired barrier function of the membranes (Table 2). (B) Preincubation of non-platable micrococcal CRCs with lysozyme (1 and 10 μg/ml, 45 min) aimed at partial hydrolysis of peptidoglycan of the surface structures [7] resulted in only partial reversion of the colony-forming ability of their CRC (20 and 10 CFU/ml, respectively). However, direct microscopic examinations of the cell populations treated with lysozyme revealed disruption of 25–35% of large cell aggregates into small ones, as well as a lack of refractivity typical of dormant forms in 30–40% of cells. The CRC treated with lysozyme (1 μg/ml) differed from the control (Fig. 4a) in loosening of the outer layers of the cell wall (CW), which lacked its lamination and in the destruction of the layer common for the cells of the conglomerate (Fig. 4b). (C) Pretreatment of the cells with phosphatidylcholine (0.8–80 μg/ml, 1.5 h) was not effective for the restoration of the colony-forming ability of CRC. (D) Pre-incubation of suspensions of "non-culturable" CRCs of *M. luteus* for 3 h in the solution of the reactivating factor (RF) from the culture liquid of *L. casei* [16] (in a ratio of 1 : 2) did not promote colony formation on LB agar. However, the MPN method revealed that the addition of the RF not earlier than 6 h after inoculation with non-platable micrococci resulted in a 7.5-fold increase in the number of cells capable of growth in LB broth relative to the control (Table 2).

Media for CFU enumeration. Since "non-culturable" CRCs were unable to restore their colony-forming ability but could respond to the above procedures (except for preincubation with phosphatidylcholine), thus demonstrating their potential viability, it was suggested that standard media and cultivation conditions were unsuitable for the germination and proliferation of "non-culturable" cells. The following variants of media were therefore used to determine the cells potentially capable of proliferation: (a) complex organic media that were diluted fivefold; (b) poor synthetic media to remove the effect of substrate-accelerated death; and

(c) media supplemented with sodium pyruvate to scavenge peroxide forms of oxygen [17, 19]. Plating of "non-culturable" CRC of micrococci without preliminary treatment, or washing on LB agar with 0.1% pyruvate, or on a fivefold diluted LB agar resulted in an increase in the number of colonies by 160 and 100 times, respectively, as compared with the CFU titer on standard LB agar (Table 3).

Enumeration of mCFU. It should be emphasized that prolonged incubation of unculturable CRCs (10–14 days) resulted in the formation of very small colonies (microcolonies) ≤0.1–0.2 mm in diameter, which at a certain angle of illumination were visible as a rough coating. Enumeration of mCFU (method 1) by examining 10 squares on the agar surface under a binocular loupe (Fig. 5a) revealed that the titer of microcolonies grown from CRCs of 9-month micrococcal cultures was about 10^4 mCFU/ml. This method had certain limitations since it did not permit enumeration of smaller colonies and their differentiation from foreign particles. Therefore, we used method 2 for mCFU counting by direct microscopic examination of ethanol-fixed replicas of the agar surface after plating aliquots (5 μl) of diluted CRC suspensions. It was necessary to distinguish the microcolonies from cell aggregates (Fig. 2d) in the populations of "non-culturable" CRCs; therefore, only the microcolonies, which were larger than aggregates and contained dividing cells, were taken into account (Fig. 5b). By method 2, the titer of viable CRC of micrococci at the level of 10^5 mCFU/ml could be determined (Table 3).

Comparative analysis by method 2 of the mCFU titer of micrococcal CRC germinating without pretreatment on different media revealed that synthetic medium with lactate was the most suitable for microcolony formation. Medium supplementation with sodium pyruvate had no effect on formation of microcolonies, unlike ordinary colonies. However, when unculturable CRCs were incubated in the presence of RF from *L. casei*, the highest titer of mCFU (6.8×10^5 mCFU/ml) was observed on LB agar with pyruvate; it exceeded threefold the value obtained in the variants without reactivation. It was found that RF increased by an order of magnitude the number of microcolonies obtained on fivefold-diluted LB agar with pyruvate, although this medium was not optimal for cell growth in the control variant. On the whole, the mCFU titers were higher by more than four orders of magnitude than the corresponding CFU titers, but lower than the total numbers of live cells determined microscopically by the Live/Dead test (Table 3).

Cell counting in semisolid media. Stimulatory effect of sodium pyruvate added to LB agar on the colony formation observed in our experiments is in agreement with earlier results [17, 19] and can be explained by its antioxidant activity that promotes the revival of cells with increased sensitivity to reactive oxygen species (ROS). Another approach to cell protection against

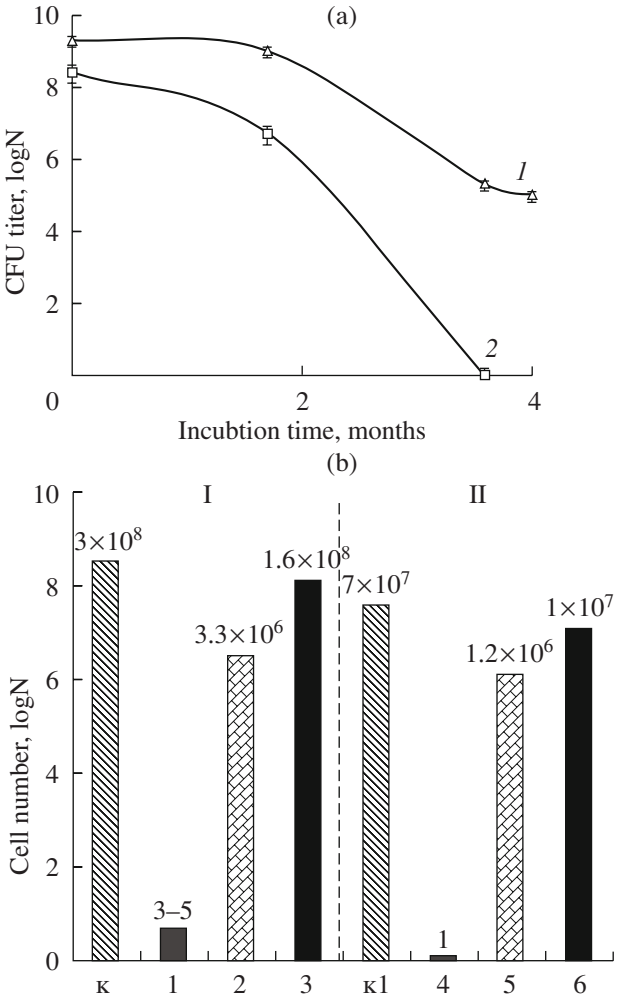

Fig. 3. (a) The number of colony-forming cells (CFU/ ml) of *A. globiformis* grown on: basal unlimited medium (*1*) and nitrogen-limited medium (*2*). (b) The total number of cells and CFU titer determined in: 4-month incubated cultures under nitrogen limitation (I) and cell suspensions incubated in 0.09% silica solution for two months (II). The designations are as follows: k and kl, initial CFU titer in the stationary-phase cultures and in cell suspensions, respectively; 1 and 4, CFU titer on LB agar; 2 and 5, CFU titer on twofold diluted LB agar; 3 and 6, total number of live cells determined by the Live/Dead test. The cell number is shown above the columns.

the inhibitory effect of ROS involved decreased oxygen access to growing cells achieved by stab inoculation of 10^N times diluted suspensions of non-platable CRC (5 μl) into semisolid LB medium containing 0.25% agar; after 3–5 days of incubation, intense formation of small colonies was observed in the place of inoculation (Fig. 5c). Enumeration of mCFU formed after a 3-day CRC incubation in semisolid agar (SA) by method 3 and simultaneous determination of the number of cells capable of growth in SA by the MPN method revealed that the number of actively proliferating cells reached 2×10^7 cells/ml, which coincided with the number of live cells (Table 4). It is important that

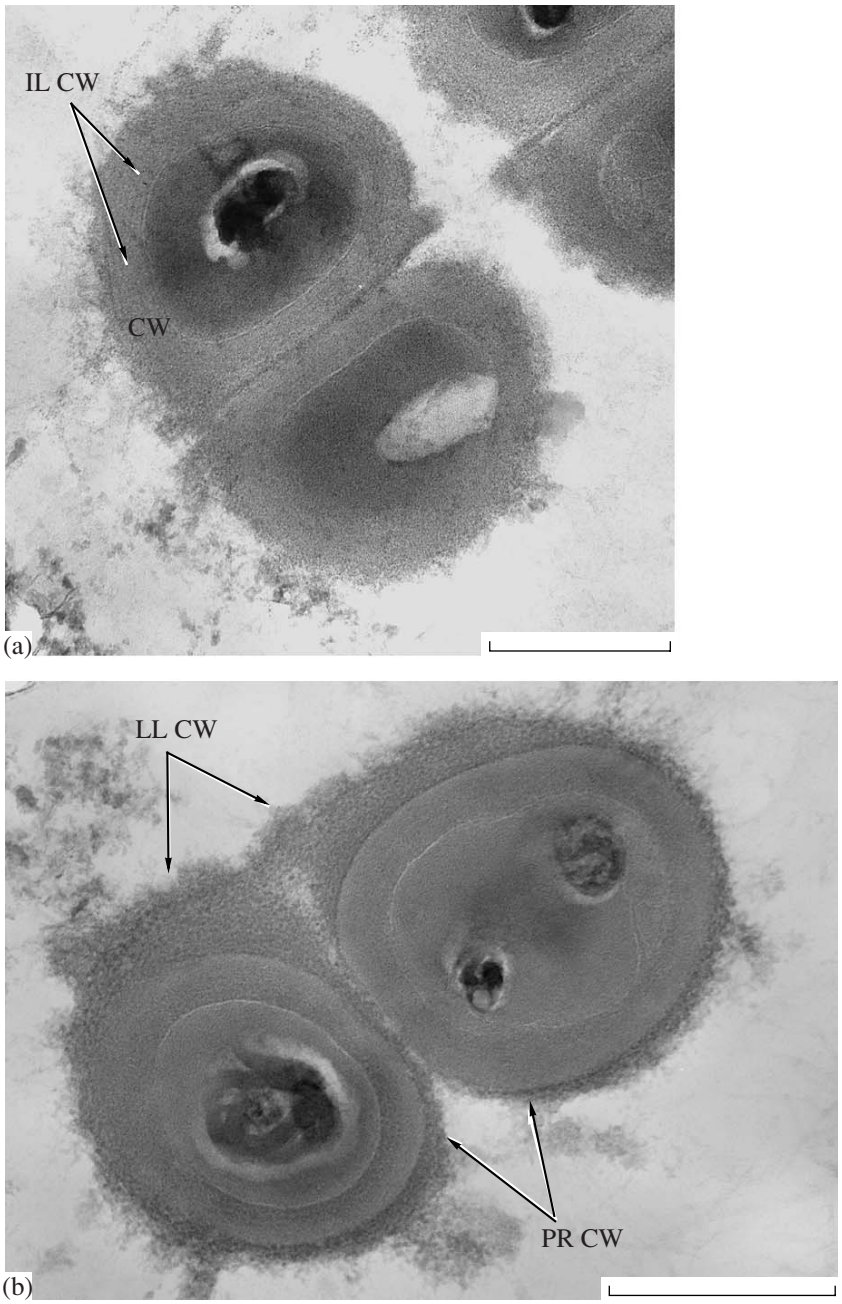

Fig. 4. Electron micrographs of the sections of CRC of *M. luteus* (obtained under carbon and nitrogen limitation) incapable of growth on LB agar before (a) and after (b) incubation with lysozyme (1 μg/ml). The designations are as follows: CW, cell wall; IL CW, individual layers of CW; LL CW loosened layers of CW; PR CW peeling-off residues of CW. Scale bar is 0.5 μm.

microcolonies plated on solid LB agar completely reversed to the formation of large colonies typical of *M. luteus*.

Thus, to revive micrococcal CRC formed under double (C + N) limitation, which were unable to grow on standard media, it is necessary to protect cells against oxygen; therefore, CRC inoculation into SA was the most effective method of their resuscitation.

DISCUSSION

The first conclusion that can be made from the results of this study is that the conditions of CRC formation determine the degree of preservation of the colony-forming ability for the resting forms of micrococci and arthrobacters incubated for a long time (up to 9 months). Thus, CRCs developed under double limita-

Table 3. Effect of the medium composition on recovery of "non-culturable" CRCs of *M. luteus*

Titer of the cell number	Variants of solid media (1.5% agar)					
	LB	LB + 0.1% pyruvate	1/5 LB	1/5 LB + 0.1% pyruvate	Lactate medium	Lactate medium + 0.1% pyruvate
Control (without pretreatment)						
CFU/ml	0.5	80 ± 20	50 ± 10	0	0	0
mCFU/ml*	$(2.1 \pm 0.2) \times 10^5$	$(2.3 \pm 0.4) \times 10^5$	$(1.9 \pm 0.2) \times 10^5$	$(1.8 \pm 0.2) \times 10^4$	$(3.2 \pm 0.6) \times 10^5$	$(3.4 \pm 0.6) \times 10^5$
Preincubation (3 h) with *L. casei* reactivating factor						
CFU/ml	0	0	0	0	0	0
mCFU/ml*	$(2.3 \pm 0.2) \times 10^5$	$(6.8 \pm 0.4) \times 10^5$	$(1.8 \pm 0.2) \times 10^5$	$(1.2 \pm 0.3) \times 10^5$	$(2.9 \pm 0.5) \times 10^5$	$(3.3 \pm 0.7) \times 10^5$

Note: *Determined by direct microscopic examination of replicas from agar surface (method 2).

Table 4. Efficiency of semisolid media for detection of the number of actively proliferating cells in a population of non-platable CRC of *M. luteus*

mCFU/ml determined on:		Viable cells determined by MPN, cells/ml:		Total number of live cells determined by the Live/Dead test, cells/ml
solid LB agar (1.5%)*	semisolid LB agar (0.25%)	LB broth	semisolid LB agar (0.25%)	
$(2.1 \pm 0.2) \times 10^5$	$(2.3 \pm 0.5) \times 10^7$	11 (3–33)	2.5×10^7 (7.5×10^6–8.25×10^7)	$(2.6 \pm 0.4) \times 10^7$
Actively proliferating cells, % of the total number of live cells				
0.8	89	4×10^{-5}	96	100

Note: *Determined by direct microscopic examination of replicas from agar surface (method 2).

tion (C + N) were unable to form macrocolonies on LB agar unlike those developed under single (phosphorus or nitrogen) limitation [7, 13]. The lack of the colony-forming ability by the CRC plated on solid media under oxygen access was apparently due to increased sensitivity of the cells to ROS because of an insufficient level and activity of the enzymes involved in antiradical protection; the other reason is an increased level of extra- and intracellular autoinducers of anabiosis, alkyl hydroxybenzenes (AHB) [13, 20], which ensure the maintenance of metabolic dormancy and the inhibition of the germination of the dormant form in high-cell-density suspensions [18]. In additional experiments, we observed an increased level of AHB in the cultures of micrococci grown under nitrogen limitation (data not shown).

The developed modified media for obtaining CRC with a different level of preservation of their colony-forming ability (double limitations by carbon and nitrogen or by carbon and phosphorus) imitate environmental conditions in various microzones of soil [1]. The CRCs of the same bacterial species encountered in natural systems in situ, including those preserved in permafrost soils and sediments [6, 7] are probably different in their capacity for revival under "standard" or "specific" conditions. Therefore, to reveal the actively proliferating cells in natural populations containing mainly dormant forms, we tested various methods of their reactivation. The washing of CRC in 0.1 M K_2HPO_4 solution (pH 7.4) restored the barrier function of the membranes in a certain part of the population, as was demonstrated by the Live/Dead test; an increase in the level of live cells with green and yellow fluorescence was accompanied by a decrease in the number of red-fluorescent cells with a disturbed barrier function of membranes (Table 2). The washing procedure promoted the removal of d_1 factors (AHB) from the cells and, as a consequence, an increase in fluidity of the cell membranes and recovery of their barrier function [18, 21]. A similar increase in the level of live cells with restored barrier function of the membranes was determined (by the test with fluorescein diacetate and ethidium bromide) in 10-month-old cultures of *Mycobacterium tuberculosis* with low CFU titers after a 2-day incubation in the presence of the supernatant from the early-stationary-phase culture of this strain [22]. The supernatant was shown to contain a specific protein RV1174c and phospholipids [23]. The addition of phospholipids (4 μg/ml) into solid medium resulted in a 66- to 400-fold increase in the number of colony-forming cells of *M. tuberculosis* in comparison with the CFU titer of 3×10^4 cells/ml in the control medium [23]. However, in our experiments, no increase in the CFU titer was observed when non-platable CRCs of

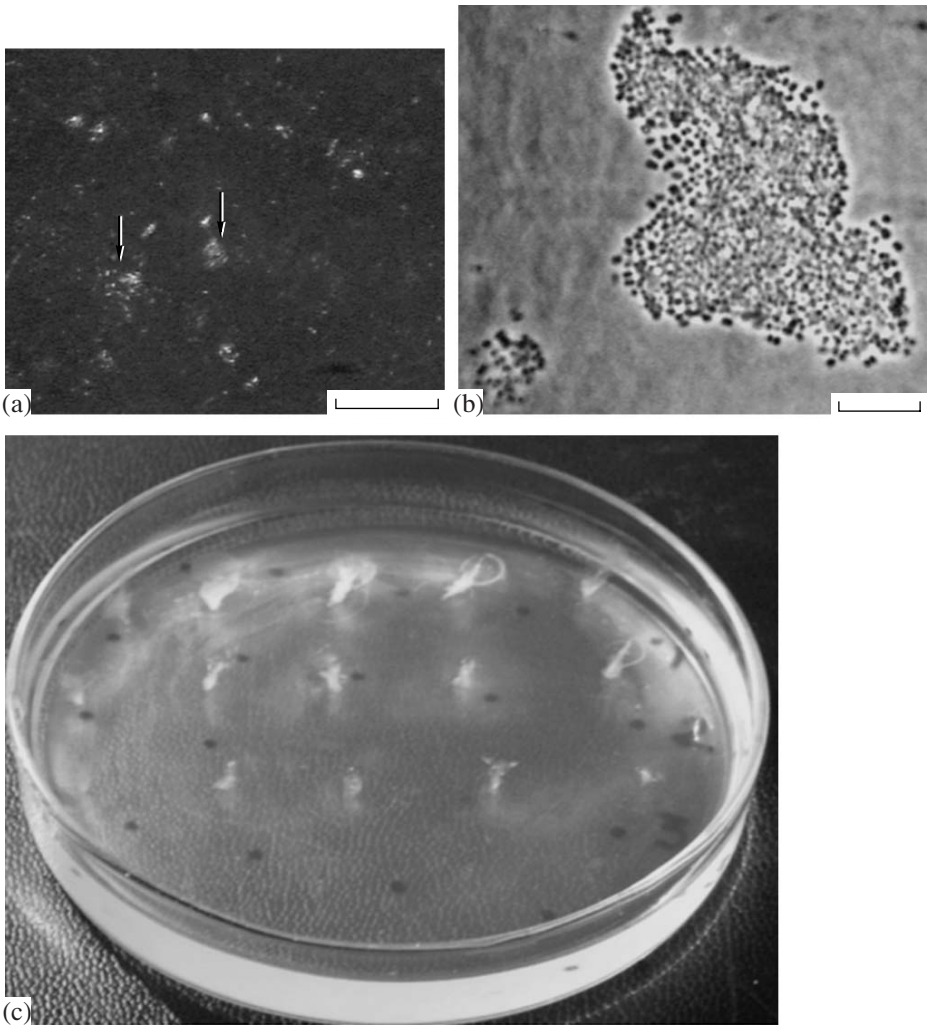

Fig. 5. Microcolonies of strain *M. luteus* on the surface of solid media observed with a binocular loupe (indicated with arrows) (a) and in the preparations of replicas of agar surface observed under a phase-contrast microscope (dividing cells are visible) (b). The bars represent 2 mm (a) and 25 μm (b). (c) Cell growth in semisolid agar.

M. luteus were incubated with phosphatidylcholine added to the reactivating solution. Phosphatidylcholine probably affected the later stages of germination, rather than activating the dormant cells.

The effect of the Rpf protein on the restoring of the colony-forming ability was demonstrated with post-stationary cultures of *M. luteus* incubated for a long time (up to 6 months); the CFU titer ranged from 10^2 to 10^3 cells/ml [9, 10]. Since under standard conditions the titer of germinating cells in these micrococcal cultures was low, but not zero, two compatible mechanisms of the action of this protein were suggested: (1) it is a factor of resuscitation of non-culturable cells with low peptidoglycanase (lysozyme-like) activity; and (2) it plays the role of a cytokine with respect to already reactivated growing cells [11, 12]. In our experiments, incubation of 'non-culturable' (non-platable) micrococcal CRCs in the presence of lysozyme (1 μg/ml) caused loosening of the outer layers of the thick cell walls of dormant forms (Fig. 3b), similar to the results observed earlier [7], but did not increase the CFU titer. A partial restoration of proliferative ability in CRCs of *M. luteus* was observed in the presence of the extracellular reactivating factor isolated from the culture liquid of *L. casei*, which is indicative of its nonspecific action [24].

In our experiments, variations in the medium composition aimed at removing the effects of substrate-accelerated death [25] and oxidative stress [17, 19] were more effective for revealing the actively proliferating cells among 'non-culturable'CRC of micrococci and arthrobacters than preincubation procedures. The CFU titers of CRCs of *M. luteus* (C + N limitation, incubation for 9 months) and of *A. globiformis* (nitrogen limitation or starvation, incubation for 2–4 months) were higher by 2 to 6 orders of magnitude on LB agar with 0.1% of sodium pyruvate and in diluted LB medium than on a standard medium. At the same time,

the titer of mCFU of micrococci revealed on different variants of solid media was higher at least by 4 orders of magnitude than the number of cells forming macrocolonies (Tables 3 and 4). The fact that the titers of mCFU exceeded those of CFU was earlier reported for laboratory cultures (microcosms) of pseudomonads and nonculturable *Escherichia coli* [26, 27], as well as for microbial communities of soil [28]. It was suggested that formation of microcolonies was due to the development of a subpopulation of viable nonculturable cells capable of only a limited number of divisions [28]. The formation of microcolonies by micrococcal CRC on the surface of solid media observed in our experiments can be evoked by not only a limited number of cell divisions and/or slow growth owing to the effect of stress factors, including oxygen supply, but also by the preferred development of a certain clone and was probably controlled by autoregulatory factors [18]. It is important that plating these clones on solid media resulted in the reversion of *M. luteus* cells to the phenotype forming colonies typical of this species. The restored capacity of micrococcal CRC for growth in semisolid agar, which was confirmed by both mCFU titer and MPN, could be explained by formation of an oxygen gradient (inward from the medium surface) and possibly by the effect of physicochemical conditions (moisture and gas exchange). Prevention of oxidative stress in reactivating cells is the main effect of semisolid media [29]. In general, the methods tested on the laboratory models (nonplatable CRCs of *M. luteus* and *A. globiformis*), which aimed at revealing the cells potentially capable of proliferation under mild conditions, can be applied for ecological studies.

The development of strategies for more complete revealing of microorganisms non- or unculturable under ordinary conditions is presently an important field of investigation. For instance, variations in the composition of solid media and protection of the cells against peroxides by introducing catalase into the growth media were used for the isolation from agricultural soils and the termite intestinal tract of representatives of the genera *Acidobacteria* and *Verrucomicrobia*, which could not be cultivated by standard methods [30]. The main conclusion drawn from this study is that dormant cells potentially capable of growth but unable to form colonies under standard conditions, as well as a large subpopulation of cells capable only of microcolony formation in the first transfer can be revealed with the use of special reactivation procedures, variations in the medium composition, and different methods of cell enumeration.

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