ISSN 0026-2617, Microbiology, 2012, Vol. 81, No. 4, pp. 435–445. © Pleiades Publishing, Ltd., 2012.
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EXPERIMENTAL ARTICLES

Reactivation of Dormant and Nonculturable Bacterial Forms from Paleosoils and Subsoil Permafrost

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Received October 10, 2011; revised February 2, 2012

Abstract—Methods of reactivating the dormant forms (DFs) and nonculturable cells (NCs) of the bacterial communities of buried paleosoils and subsoil permafrost stored for long periods of time (thousands to millions of years), including completely sterile samples (CFU = 0), were developed. They were based on washing the DFs and NCs to remove anabiosis autoinducers (spore germination autoinhibitors) and introducing low molecular weight extracellular growth regulators of microbial or plant origin, such as alkylhydroxybenzenes of the alkylresorcinol subtype, indoleacetic acid, and wheat germ agglutinin. It was revealed that the dormant communities of permafrost and buried soils differed in their sensitivity to reactivating factors, probably due to different natural storage conditions of the tested soil substrates and the heterogeneity of dormant populations. The latter was confirmed by FISH (fluorescent in situ hybridization): applying the reactivation methods to the cells of the dormant permafrost community resulted in an increase in the number of metabolically active cells from 5 to 77% of their total number. In contrast, the addition of microbial anabiosis autoinducers (C_{12}-AHB) to background surface soil and permafrost samples caused the transition of bacterial cells to the dormant or the nonculturable state, depending on the C_{12}-AHB concentration and the sensitivity of the cells from the control soil or permafrost 'to it. The data obtained contribute to our knowledge concerning the role of intercellular communication factors and the survival of microorganisms under extreme environmental conditions.

Keywords: survival of bacteria, buried soils, permafrost subsoil deposits, dormant cells, nonculturable cells, intercellular regulators, FISH

DOI: 10.1134/S0026261712040108

Recently, the attention of microbiologists and specialists in related areas of research has been focused upon long-term survival of microorganisms in subsoil permafrost and buried soils [1–5]. This system of long-term conservation of microorganisms is unique because of (i) a lack of nutrients and energy sources; (ii) a lack of moisture (anhydrobiosis conditions); and (iii) the constant influence of subzero temperatures (cryobiosis conditions) in permafrost deposits.

The adaptive strategy of long-term viability retention by microorganisms under growth arrest-causing conditions is primarily based upon their capacity for transition into a dormant state. This state is characteristic of specialized dormant cells of spore-forming bacteria, dormant forms (DFs), and dormant nonculturable cells (NCs) of non-spore-forming bacteria [5–7]. This was corroborated by calculating the ratio between the active and the total microbial biomass in modern surface background and buried soils [8] and by direct electron microscopic analysis of permafrost samples, which demonstrated that an overwhelming majority of bacteria existed in the form of cystlike dormant cells (CDCs) [6, 9, 10]. Dormant cell populations are heterogeneous with respect to the retention of their proliferative potential and their ability to revert to actively growing forms. Therefore, special conditions are mandatory for reactivating the cells that are difficulty germinating [11]. In particular, the methods that prevent the development of oxidative stress in germinating cells and their substrate-accelerated death on nutrient-rich media are recommended [11, 12]. However, implementing these recommendations is not sufficient per se.

According to the hypothesis suggested in [13], the dormant state implicates the accumulation of spore germination autoinhibitors in dormant forms; their release into the medium results in removing the metabolic block and germination of the DFs. This should be taken into account while developing the methods of reactivating DFs and NCs. Low molecular weight extracellular autoregulators controlling specific DF germination stages are also of considerable importance for restoring the colony-forming capacity. Some of the autoregulators are perceived by dormant forms

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Table 1. Characterization of the natural soil samples used in the work

	Samples	Sampling depth	Sample age and site
Permafrost	Well 11/89	24.0 m; 25.2 m	2–3 million years (lacustrine–alluvial sandy clays of the Oler suite, upper River Bol'shoi Khomus-Yuryakh, Kolyma Plain, Siberia)
	Well 2/05	1.9 m	~200000 years (alluvial sands and sandy clays of the second half of the Middle Pleistocene, the upper River Bol'shoi Khomus-Yuryakh, Kolyma Plain, Siberia)
	Well 3/05	8.0 m	
	Well 4/05	18.0 m; 24.65 m	~600000 years (Middle Pleistocene alluvial gravelly sands, upper River Bol'shoi Khomus-Yuryakh, Kolyma Plain, Siberia)
	Well 6/06	4.15 m	~200000 years (alluvial sands and sandy clays of the second half of the Middle Pleistocene, the upper River Bol'shoi Khomus-Yuryakh, Kolyma Plain, Siberia)
Soils	Gley cryogenic soil	CR (10–25 cm)	Modern, Kolyma Plain, Siberia
	Chestnut solonetz brackish paleosoil D-534 buried under a barrow	A_1 (90–99 cm); A_1 (99–115 cm); A_{2Ca} (115–131 cm)	First half of the 3rd millennium B.C., Late Yam Culture, Volgograd Region
	Chestnut-like carbonate non-solonetz brackish paleosoil D-510	A_1 (74–86 cm)	Turn of the 2nd millennium B.C., Catacomb Culture, Volgograd Region
	Chestnut solonetz highly saline soil D-533	A_1 (0–10 cm)	Modern Age, Volgograd Region

as a signal for initiating their metabolism, whereas others perform the cytokine functions. For instance, it was established that the DFs of *Micrococcus luteus* are reactivated by an extracellular regulatory metabolite, protein Rpf (reactivation promoting factor) that is not species-specific; a number of bacteria synthesize it [14, 15].

Communicative interactions in soil biocenoses can be mediated by the regulatory substances that are synthesized, apart from microorganisms, by plants and animals. A plant lectin (wheat germ agglutinin) [16] and indoleacetic acid [17] were shown to be involved in bacterium–plant interactions. However, the function performed by plant regulators in DF germination has not been elucidated yet.

Of much interest in this context is research on the effect of microbial and plant extracellular regulators on the physiological state of microbial cells not only in laboratory cultures but also in natural microbial communities. This will contribute to our understanding of the role of chemical communication factors in nature.

The goal of the present work was to develop the methods for reactivating the cells of the bacterial communities of buried soils and subsoil permafrost and to investigate the effects of microbial and plant-produced low molecular weight regulators on the metabolic status of bacteria in samples of paleosoils of different origin.

MATERIALS AND METHODS

Research subject. In this work, we used (i) native samples of chestnut paleosoils buried under barrows, as well as subsoil permafrost in which the ancient microbial communities were stored under different conditions and for different periods of time and (ii) samples of background surface chestnut and tundra soils (Table 1).

In model experiments, collection strains of the symbiotrophic bacterium *Sinorhizobium meliloti* (VKPM P221) and the soil bacterium *Micrococcus luteus* (NCIMB 13267) were used.

The following **regulatory factors** were employed:

—Chemical analogues of microbial anabiosis autoinducers: the alkylhydroxybenzenes (AHBs) C_{12}-AHB and C_7-AHB (Sigma, United States) belonging to the alkylresorcinol group, which function as an anabiosis autoinducer and an adaptogen with antioxidant activity, respectively;

—A growth regulator produced by microorganisms and plants: indole-3-acetic acid (IAA) (Panreac, Spain);

—A growth regulator of the phytolectin group: wheat germ agglutinin (WGA) (Lectinotest, Ukraine).

Cultivation conditions for the bacteria. The bacterium *S. meliloti* was grown in the basic selective medium containing the following (g/L): yeast extract, 1.0; mannitol, 2.0; K_2HPO_4, 0.2; KH_2PO_4, 0.2; $MgSO_4$, 0.2; and $CaCl_2$, 0.02; pH 6.8. The bacterium *M. luteus* was grown in the LB medium (Carl Roth GmbH, Germany). The bacteria were cultivated at

28°C in 250-mL flasks with 50 mL of the medium on a rotary shaker (140–160 rpm). Early stationary phase cultures were used as inocula. The initial optical density (OD) of 0.2 for inoculated cultures was determined on a Specord spectrophotometer (Germany) at $\lambda = 540$ nm and $l = 10$ mm.

The **dormant forms and non-culturable cells** of the bacteria *S. meliloti* and *M. luteus* were obtained by two methods: (i) by causing a nutrient imbalance during the natural developmental cycle of the culture, as described earlier [10, 11, 19] and (ii) by employing C_{12}-AHB, a chemical analogue of microbial anabiosis autoinducer that was added to a final concentration of 10^{-4}–10^{-3} M to the culture in the growth deceleration phase [11, 19].

To activate the growth processes in the DFs and NCs of model bacterial strains, we (i) washed their cells with saline (1 : 100, vol/vol) for 1–2 h to remove spore germination autoinhibitors; (ii) preincubated their suspensions for 1 and 2 h in saline (1 : 100, vol/vol) with IAA at concentrations of 10^{-5} and 10^{-4} M; (iii) pretreated them with WGA at final concentrations of 1.0 and 2.5 μg/mL; and (iv) pretreated them with C_7-AHB at concentrations of 10^{-5} and 10^{-4} M.

Soil samples were prepared using the standard techniques [20]: (i) by grating the samples in 0.5% solution of Na pyrophosphate (Panreac, Spain) (1 : 10, wt/vol); (ii) by sonicating (22 kHz, 0.44 A, 2 min) the samples in distilled water (1 : 100, wt/vol); (iii) by grating the samples and thereupon preincubating them (1 : 100, wt/vol) for 1 h in a solution containing soil extract (the suspension of background surface cumulose tundra soil (1 : 10, wt/vol) was filtered and sterilized at 0.5 atm) or potato extract (disintegrated potatoes were squeezed, and the extract was diluted with distilled water (1 : 5, vol/vol) and sterilized at 0.5 atm).

The microbial fraction of the soil samples was obtained from sonicated soil suspensions (1 : 100, wt/vol) upon removal of the soil particles by centrifugation (3000 *g*; 10 min).

Reactivation of the cells (DFs and NCs) in soil samples was carried out as follows: (i) after preparing the samples, they were (a) washed (1.5 h; distilled H_2O) and (b) incubated for 1 h with IAA (10^{-5} M for permafrost samples after washing; 10^{-4} M for paleosoil and background surface soil samples without washing), (ii) the microbial fraction obtained was preincubated for 1.5 h with (a) yeast extract (Difco, United States) (0.01–0.1%); (b) WGA (1.0; 2.5 μg/mL); (c) yeast extract (0.1%) and glucose (1%); (d) C_7-AHB (50 μg/mL); and (e) Na pyruvate (0.1%).

The influence of anabiosis autoinducers on the physiological state of bacteria in the soil samples was investigated by adding C_{12}-AHB (5×10^{-5} and 10^{-4} M) and incubating with it for 1 h. Thereupon, the CFUs were counted and the "live" cell numbers were determined with the Live/Dead dye (Backlight, Molecular Probes, United States).

The total cell number in the soil samples (their microbial fractions) was determined by direct counting of the cells under an Axiopan (Carl Zeiss, Germany) fluorescence microscope, using acridine orange and the fluorescent dyes Live/Dead and DAPI (Biotium, United States), which make it possible to differentiate between "live" and "dead" (with disrupted cytoplasmic membrane barrier) cells.

The colony-forming capacity of the tested cells (CFU/mL, CFU/g) was determined by inoculating 10^N times diluted bacterial and soil suspensions on plates with solid (1.5% agar) and semiliquid (0.4 or 0.6% agar) media including TSA (tryptone–soybean agar) (Panreac, Spain) and the tenfold diluted TSA medium (TSA/10).

Cell number in liquid media was determined by the terminal dilution method (TDM) in the wells of a plate (450 μL of TSB/10 medium + 50 μL of suspension). The plates were incubated at 23°C for 3–21 days. The most probable number of viable cells (MPNVC) was calculated using the standard tables [21].

Assessment of the diversity and cell numbers of metabolically active bacteria in permafrost by FISH. We analyzed sample 6/06 (4.15 m), in which no colony-forming cells were detected on TSA/10 medium without the sample preparation and reactivation procedures.

The hybridization procedure was based on a set of rRNA oligonucleotide probes (Syntol, Russia) that were labeled with the fluorescent dye Cy3 and specific for representatives of the domains *Bacteria* (the EUB338 and EUB338 I probes with the 5'–3' nucleotide sequences CY3 GCT GCC TCC CGT AGG AGT GC(W) and CY3 GCC (W)CC CGT AGG (W)GT, respectively, where Y = C or T; W = A or T; the target sequence of the 16S rRNA is 338–355) and *Archaea* (the ARCH915 probe with the sequence CY3 GTG CTC CCC CGC CAA TTC CT where Y = C or T: the target sequence is 915–934) [22].

Hybridization was carried out according to [23], the preparations were additionally stained with acridine orange solution. They were analyzed with an Axioskop 2 PLUS fluorescence microscope (Carl Zeiss, Germany) with Filterset 15 filters ($\lambda = 546$ nm) for Cy3-labeled probes and Filterset 09 filters ($\lambda =$ 450–490 nm) for acridine orange-stained cells. The probe-hybridized cell number was determined in 50 fields per one well. Thereupon, the number of cells in 1 g of soil was calculated using the conventional formula [20].

The data presented in this work are the means; standard deviations for a probability $P > 0.95$ were calculated. The Student's test was used for statistically processing the data obtained.

Table 2. Numbers of viable dormant *S. meliloti* cells (4 months of storage at 4°C) during their reactivation

CDC variant	Viable dormant cell numbers, CFU × 10^8/mL (% of the CFU number in the unreactivated system)				
	Control without reactivation	Washing CDCs in saline		CDC preincubation in saline + 10^{-4} M IAA	
		1 h	2 h	1 h	2 h
CDCs on the N-limited medium	6.6 ± 0.2 (100%)	6.9 ± 0.2 (105%)	11.0 ± 0.3 (167%)	11.2 ± 0.3 (170%)	13.0 ± 0.3 (197%)
CDCs on the P-limited medium	8.2 ± 0.3 (100%)	8.3 ± 0.3 (101%)	8.9 ± 0.3 (108%)	9.6 ± 0.3 (117%)	10.4 ± 0.3 (127%)
CDCs on the N- and P-limited medium	5.1 ± 0.1 (100%)	5.9 ± 0.2 (116%)	7.9 ± 0.2 (145%)	8.3 ± 0.2 (163%)	12.0 ± 0.3 (235%)

RESULTS

A large number of issues concerning the functioning of microorganisms in natural ecosystems are resolved by modeling using laboratory cultures of collection strains. We used this approach to select an efficient method of reactivating the dormant cells in soil communities characterized by long-term persistence. The dormant cells (DFs and NCs) of collection strains of the bacteria *S. meliloti* and *M. luteus* were used as models.

Obtaining dormant forms of collection bacterial strains. The dormant forms of *S. meliloti* and *M. luteus* (CDCs and NCs) were obtained by cultivating the bacteria on N- and P-limited media, as described earlier [10, 11, 19].

The CFU number in the control cultures grown on the standard medium decreased to 0.9 and 3% of the CFU number in the standard culture, respectively, after four-month storage under programmed cell death-inducing conditions. The CFU number was higher by an order of magnitude and amounted to 7–20% (storage at 4°C) or 27–29% (storage at 23°C) in experimental cultures grown on the N- and P-limited media. The cells of 4-month post-stationary experimental cultures did not exhibit experimentally detectable levels of endogenous respiration; they were reduced in size and manifestly refractile. In conjunction with long-term survival under growth arrest-inducing conditions, this enabled classifying the cells as cystlike dormant cells (CDC) of these bacteria.

Another technique of obtaining the dormant cells of *S. meliloti* and *M. luteus* was based on inducing their formation by increasing the level of anabiosis autoregulators. This was achieved by adding the chemical analogue C_{12}-AHB to the decelerated growth phase culture as described above [11, 19]. The effects of C_{12}-AHB varied depending on its concentrations. At a concentration of 1×10^{-4} M, it induced formation of the dormant forms; at a concentration of $(2–2.5) \times 10^{-4}$ M, it elicited the transition of *S. meliloti* and *M. luteus* cells to the non-culturable state (the CFU number decreased by two and seven orders, respectively). A still higher concentration (10^{-3} M) caused an irreversible loss of the colony-forming capacity of *S. meliloti* and *M. luteus* cells. The latter effect was earlier described in other bacteria in terms of a "mummified" state resulting from critically high C_{12}-AHB concentrations [24].

Reactivation of the dormant bacterial cells of collection strains. Methods of reactivating the dormant forms were developed in the model system using *S. meliloti* DFs formed in the cultures developing on unbalanced media (Table 2). Their numbers in post-stationary cultures were $(5–8) \times 10^8$ cells/mL. Their percentage was low (7–11%).

The main reactivation methods were as follows: (i) prewashing DFs with saline and buffer solutions or water (pH 7.0–8.0) to remove the spore germination autoinhibitors [13] they contain and (ii) inducing the metabolism and growth of the prewashed DFs with the regulatory substances that exhibit growth-stimulating activity, such as IAA (Table 2). The DFs obtained under different growth conditions differed in terms of their capacity to revert to active growth. Washing in saline for 2 h and preincubating for 2 h with 10^{-4} M IAA were the most efficient methods that secured an at least twofold increase in CFU number with the DFs generated under N or N + P limitation (lim) (Table 2).

Reactivation studies were also conducted with the DFs and NCs that formed after the addition of C_{12}-AHB to bacterial cultures. Adding the plant growth regulator WGA (1.0 μg/mL) to a suspension of *Sinorhizobium* DFs with a low CFU titer (1.3×10^6 μg/mL) insignificantly (by 30%) increased the CFU number (Table 3). However, preincubation of the micrococcus (CFU = 0) with the same WGA concentration resulted in increasing the CFU number by five orders of magnitude, i.e., to 1.5×10^5 cells/mL (Table 3). These data lend support to the idea that *M. luteus* cells obtained under the influence of C_{12}-AHB (2.5×10^{-4} M) are nonculturable forms requiring special conditions for reactivation.

Table 3. C_{12}-AHB effect on the physiological state of *S. meliloti* and *M. luteus* cells and the subsequent reactivation of the DFs and NCs of the bacteria

C_{12}-AHB concentration, M	Initial CFU titer, U/mL	CFU number after adding C_{12}-AHB, U/mL	N_1*	WGA concentration, μg/mL	CFU number after adding WGA	N_2**
Obtaining CDCs of *S. meliloti*				Reactivating CDCs of *S. meliloti*		
1×10^{-4}	4.1×10^8	$(1.3 \pm 0.1) \times 10^6$	315.4	1.0	$(1.7 \pm 0.2) \times 10^6$	1.31
Obtaining NCs of *M. luteus*				Reactivating NCs of *M. luteus*		
2.5×10^{-4}	3.8×10^7	0	$>10^7$	1.0	$(1.5 \pm 0.2) \times 10^5$	$>10^5$
				2.5	$(1.8 \pm 0.3) \times 10^5$	
Obtaining mummified forms				Reactivation		
10^{-3}	*S. meliloti* 4.1×10^8	0	$<10^8$	Unviable		
	M.luteus 3.8×10^7	0	$<10^7$	Unviable		

Notes: * N_1—the ratio between the CFU number before adding C_{12}-AHB and the CFU (after adding C_{12}-AHB).
** N_2—the ratio between the CFU number after adding WGA and the initial CFU (before adding WGA).

Importantly, the preincubation of bacterial NCs with IAA (10^{-5}–10^{-4} M) did not influence the restoration of their colony-forming capacity. Accordingly, CDCs and NCs have different receptor sites that perceive the signals responsible for growth reversion (germination and subsequent division): the presence of IAA that exhibits cytokine activity is insufficient for reactivating NCs. The results obtained in the test with the Live/Dead dye were in conformity with the CFU values (data not shown).

The data that were obtained earlier [11, 19] and in the present work (Tables 2 and 3) indicate that the cells are characterized by different dormancy levels, depending on the conditions under which they were formed and stored, including the anabiosis autoinducer concentrations applied. Their capacity for reverting to the active growth state, as well as the requirements with respect to special reactivation procedures, is conditional thereupon.

Assessing the viability of bacterial communities in samples of permafrost and background surface and buried soils. A series of studies in which the standard sample preparation techniques were applied to the samples of permafrost and background surface and buried soils revealed (i) a discrepancy between the number of potentially viable cells directly counted using the Live/Dead dye and that of bacteria which formed colonies on solid media and (ii) a difference with respect to these criteria between the samples of permafrost and those of buried and background surface soils (Fig. 1). Buried and background surface soils were characterized by a high colony-forming unit number, whereas no cell growth on a solid medium occurred with a number of permafrost samples (CFU = 0). The total cell number values, determined by direct cell count, were high in all tested samples: they were ~ 10^9 cells/g and 10^{10}–10^{11} cells/g in the samples of permafrost and buried/background surface soils, respectively.

The differences with respect to growth reversion capacity between bacteria from the microbial communities of paleosoils of different origins may be due to the heterogeneity (i) of the soil system in terms of microbial numbers and (ii) of microbial populations with respect to the dormancy level of surviving cells, which necessitates specific conditions for their reactivation.

Reactivation of the cells of the bacterial communities of subsoil permafrost and buried soils. To reactivate the cells of the dormant microbial communities, we used the methods that proved to be efficient in the model studies with laboratory cultures. Removing autoinducers from the samples by washing them with saline or water (pH 7.0–7.5, 1.5 h) was a very effective technique for reactivating the permafrost cells: the CFU number in treated samples increased by 3–7 orders of magnitude, including, importantly, the samples in which no growth occurred (CFU = 0) without the preparation procedure (Fig. 2). An efficient method was based on suspending the soil samples in water extracts from potatoes or background surface cumulose tundra soil: the CFU number upon inoculating these fractions was 3–4 orders of magnitude higher, and this also applied to the fractions obtained from the samples which were completely "sterile" in the variants in which the standard preparation techniques were used (Table 4).

The reactivation level of the cells from conserved bacterial communities attained by removing the inhibitory compounds could be additionally increased with growth-stimulating compounds. The DFs obtained from different soils were characterized by different sensitivities to these metabolites. The IAA (10^{-5}–10^{-4} M) effect was especially strong with buried

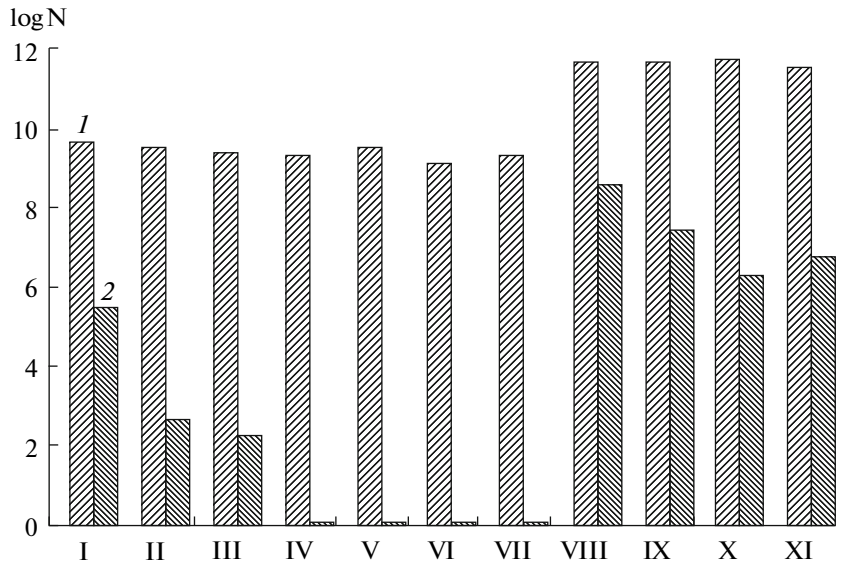

Fig. 1. Ratios between the total number (cells/g) of intact bacterial cells (*1*) determined by staining them with Live/Dead (samples I–VII) and DAPI (samples VIII–IX) and the viable cell number (CFU/g) (*2*) (TSA medium, 7 days, 25°C) in natural samples. Designations: surface background soils: I, gley cryogenic soil; VIII, chestnut solonetz highly saline soil D-533; subsoil permafrost: II, 11/89 24.0 m; III, 11/89 25.2 m; IV, 2/05 1.9 m; V, 3/05 8.0 m; VI, 4/05 18.0 m; VII, 4/05 24.65 m; paleosoil D-534, buried under the barrow, horizons: IX, A_1; X, B_1; XI, B_{2ca}. See Table 1 for detailed description of the soil samples.

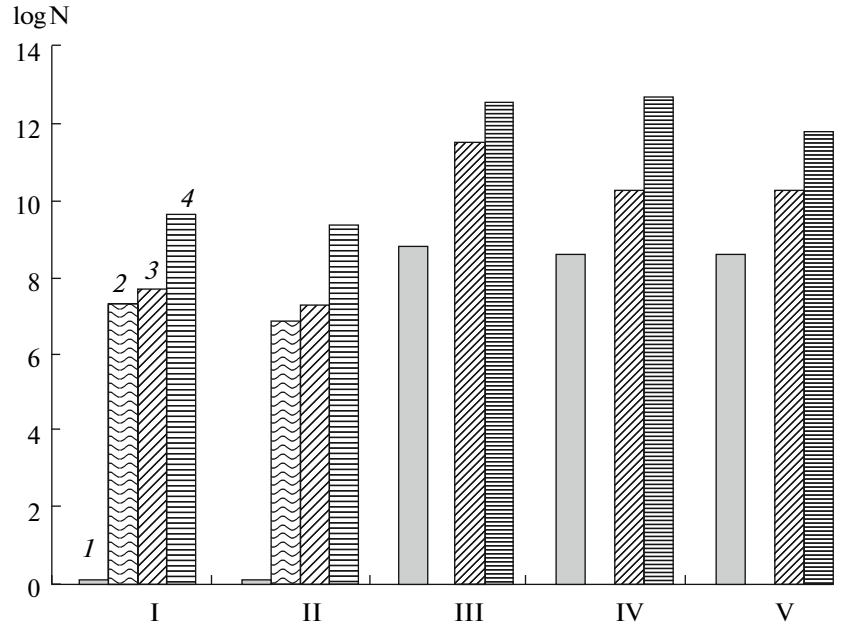

Fig. 2. Ratio between the total cell number (*4*) (cells/g) and the number of viable bacterial cells (*1–3*) (CFU/g) (TSA medium, 7 days, 25°C) in natural subsoil permafrost samples: I, 3/05 8.0 m; II, 4/05 24.65 m; buried soils: III, D-534, horizon A_1; IV, D-510; background surface soil: V, D-533. Designations: *1*, control (without reactivation); *2*, washing in distilled H_2O for 1.5 h; *3*, preincubation (1 h) with IAA (10^{-5} M for prewashed permafrost samples and 10^{-4} M for paleosoil and background surface soil, without prewashing); *4*, total cell number determined by staining with Live/Dead or DAPI.

and background surface soil samples, in which the CFU number was 2–3 orders of magnitude higher than in those without the preparation procedure (Fig. 2). IAA exerted a weak effect on the cells of the permafrost community only when it had been subjected to the preparation procedure (washed with distilled water for 1.5 h). The optimum method of reactivating permafrost DFs was preincubation with WGA (5 μg/mL). In this system, the CFU number increased to 3.5×10^5, in contrast to the completely "sterile"

Table 4. Numbers of bacteria (CFU/g) isolated from permafrost samples using different preparation methods (on the TSA medium at 25°C on day 10 of cultivation)

Well, sample depth	Numbers of microorganisms, CFU/g		
	Water suspension (control)	Potato extract	Soil extract
11/89 KHYU, 24.0 m	$(3.8 \pm 0.2) \times 10^2$	$(1.5 \pm 0.2) \times 10^5$	$(2.9 \pm 0.3) \times 10^5$
11/89 KHYU, 25.2 m	$(1.4 \pm 0.3) \times 10^2$	$(3.2 \pm 0.1) \times 10^5$	$(8.9 \pm 0.2) \times 10^5$
2/05 KHYU, 1.9 m	No growth	$(1.8 \pm 0.3) \times 10^4$	$(8.1 \pm 0.3) \times 10^4$
4/05 KHYU, 18.0 m	No growth	$(4.3 \pm 0.2) \times 10^3$	$(6.6 \pm 0.2) \times 10^4$
4/05 KHYU, 24.6 m	No growth	$(1.2 \pm 0.1) \times 10^4$	$(4.8 \pm 0.3) \times 10^4$

samples (CFU = 0) inoculated using the standard techniques (Fig. 3a). These results indicated that different cell dormancy mechanisms operated under different natural conservation conditions.

Importantly, the influence of the regulators on the bacterial communities of both buried soils and permafrost resulted in an increase in the colonial and morphological diversity of the resulting populations. This is of considerable practical importance in terms of the efficiency of environmental assessment techniques and of the quest for producers of biologically active substances, e.g., antibiotics, within microbial groups that are difficult to isolate from natural substrates.

Another method used for more precise estimation of the number of viable cells (including non-culturable ones) in permafrost was based on varying the medium composition used for their inoculation: (i) using diluted complete media to prevent the "substrate-accelerated" death of reactivated cells; (ii) adding growth-stimulating compounds and antioxidants; and (iii) using semiliquid agar to prevent the oxidative stress (Figs. 3a and 3b). Good results (increasing the CFU number to 4.2×10^4 cells/g) were obtained by supplementing the growth medium with C_7-AHB (Fig. 3b), which exhibits high antioxidant activity and possesses adaptogenic properties [18].

Hence, our experiments demonstrated the dose-dependent effects of low molecular weight growth regulators of plant and microbial origin (IAA, WGA, and AHB) that are involved in reactivating the dormant and nonculturable cells of the bacterial communities of permafrost and buried soils.

Impact of microbial autoregulators on the physiological status of the cells of soil bacterial communities. The experiments were conducted with C_{12}-AHB, a chemical analogue of microbial anabiosis autoinducers, added to subsoil permafrost and background surface tundra soil samples. It was revealed that C_{12}-AHB had a regulatory effect on the physiological state of the cells in bacterial communities. In permafrost samples, an increase in C_{12}-AHB concentration (to 5×10^{-4} M) resulted in a slight increase in the percentage of cells with disrupted membranes. This was concomitant with a decrease in the percentage of intact cells with unimpaired membranes, based on the Live/Dead test, and in the CFU number on the TSA/10 medium. The CFU number was ~60% of the control value obtained before adding C_{12}-AHB (Fig. 4a). Our studies with

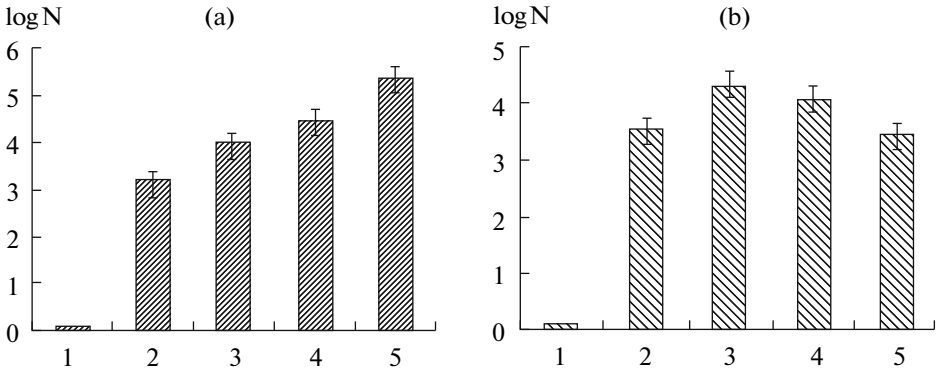

Fig. 3. Viable bacterial cell number (CFU/g) in permafrost sample 6/06 4.15 m with (a) various sample preparation methods, and (b) various media used for inoculation. Designations: (a) inoculation on TSA/10 medium: 1, without preincubation (control); 2–5, with preincubation (1.5 h): 2, in distilled H_2O; 3, with yeast extract (0.1%); 4, with C_7-AHB (50 μg/mL); 5, with WGA (5 μg/mL); (b) inoculation on the following medium (after the washing in distilled H_2O): 1, TSA (1.5% agar), control; 2, TSA/10 (1.5% agar); 3, TSA/10 (1.5% agar) + C_7-AHB (50 μg/mL); 4, TSA/10 (0.4% agar) on a supporting layer of TSA/10 (1.5% agar); 5, TSB/10 broth, using the terminal dilution method (TDM).

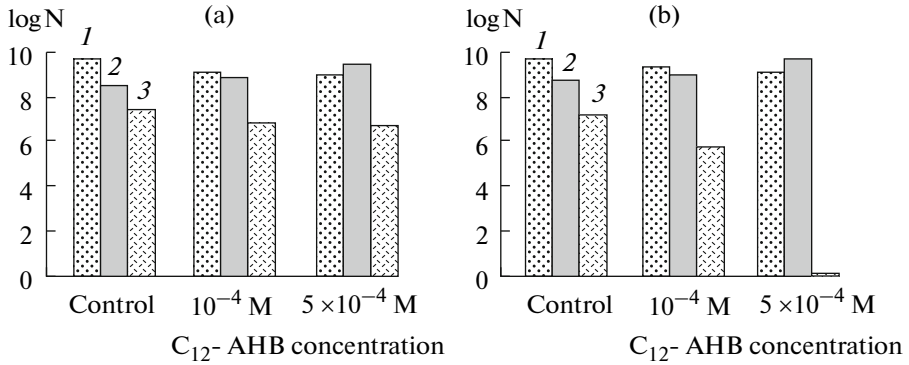

Fig. 4. Ratio between the cells (cells/g) with intact (*1*) and disrupted (*2*) membrane barrier, according to the data obtained with the Live/Dead dye and the viable cell number (CFU/g) after inoculation on the TSA/10 medium (*3*) with the addition of C_{12}-AHB to (a) permafrost sample 3/05 8.0 m and to (b) background surface tundra soil (gley cryogenic soil).

background surface tundra soil samples revealed the interesting fact that the addition of C_{12}-AHB at the high concentration of 5×10^{-4} M (Fig. 4b) resulted in a complete lack of bacterial growth upon plating on the TSA/10 medium (CFU = 0), even though the percentage of the cells with intact membranes was sufficiently high (22%). This discrepancy was probably due to the different resistance degrees of the cells persisting in permafrost deposits and seasonally freezing–thawing tundra soils, including their resistance to the effects of high C_{12}-AHB concentrations. To reiterate, a similar result—a lack of growth—was obtained with the collection strains of *M. luteus* and *S. meliloti* whose cells lost the colony-forming capacity upon increasing the C_{12}-AHB concentration in the cultures to 10^{-3} M (Table 3).

Similar processes may result when growth arrest-causing conditions develop in subsoil permafrost and background surface and buried soils. In particular, this may be due to soil freezing or desiccation and, as a consequence, to a local increase in the level of anabiosis autoinducers (alkylhydroxybenzenes) that are involved in transition of the cells to the dormant state [19].

Hence, the regulatory role of anabiosis autoinducers and growth activators of plant and microbial origin in terms of the physiological state of bacterial cells was revealed, apart from model studies with collection strains of soil bacteria, by in situ research on soil and subsoil permafrost samples.

The above results demonstrated that microbial communities characterized by long-term persistence were highly heterogeneous with respect to the capacity of the cells for the restoration of their proliferative activity, as well as to the special procedures enabling them to revert to active metabolism. The latter point was confirmed by independent studies using FISH (fluorescent in situ hybridization).

Assessment of bacterial diversity and cell numbers in permafrost deposits with FISH. We analyzed the permafrost sample 6/06 (4.15 m), in which no cells capable of forming colonies on the TSA/10 medium were detected (CFU = 0). In this sample, we compared the total cell number (determined microscopically by staining with acridine orange) with the number of metabolically active cells determined by the FISH method using the probes specific for representatives of the *Bacteria* and *Archaea* domains [22, 23].

It was established that, without the special preparation and reactivation procedure, the number of metabolically active prokaryotes in a thawed and moistened permafrost sample was 10^6 cells/g, which accounts for 5% of the total microbial cell number (Fig. 5). If washing with distilled water for 1.5 h was used as the sample preparation method, the percentage of metabolically active cells increased by two orders of magnitude, i.e. to 2×10^8. Sample preincubation with WGA (5 μg/mL) was the optimum procedure for reactivating DFs: the active cell percentage reached 77% of the total cell number. Eubacteria and archaea accounted for 71.2 and 6.2%, respectively (Fig. 5).

Hence, most bacterial cells were potentially viable during long-term conservation in soil biotopes. Although existing in a dormant state, they retained the capacity to revert to active metabolism and cell division upon development of growth-promoting conditions.

DISCUSSION

Paleosoils are regarded as peculiar models of conserved soils that formerly represented open, actively functioning systems. Soil ecotopes, including soils buried under barrows and subsoil permafrost deposits, are heterogeneous systems whose loci differ in the number of soil microorganisms that inhabit them and persist under growth arrest-inducing conditions [25].

Importantly, the overwhelming majority of cells in microbial communities of background soils and paleosoils are not detectable by conventional methods, which results in underestimating the numbers of microorganisms and their diversity [3–5].

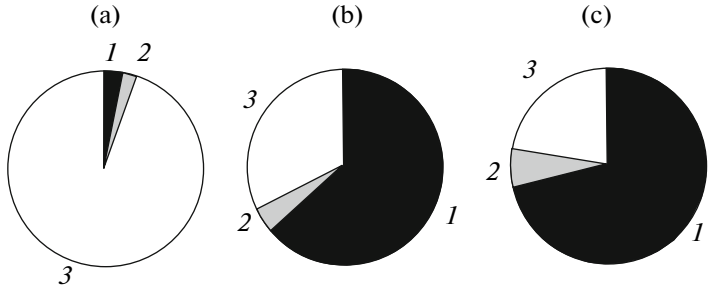

Fig. 5. Percentage of metabolically active cells of eubacteria (*1*) and archaea (*2*) and of inactive cells (*3*) determined by the FISH method in the total prokaryotic cell fraction determined with the acridine orange dye in permafrost sample 6/06 (4.15 m) in the control (a), after washing with distilled H_2O for 2 hs (b), and after the subsequent reactivation with WGA (2.5 μg/mL) (c).

Our studies on the dormant microbial communities of permafrost deposits and buried soils revealed differences between them in terms of total "live" cell number and the number of CFUs obtained on solid media (Fig. 1). These differences were apparently due to the different conservation conditions of bacterial communities in the tested natural substrates: the communities were either slowly submerged into the permafrost zone or quickly buried under a barrow. Another possible factor was the different age of the samples: permafrost deposits were much more ancient (several hundred thousand to several million years old).

The fact that paleosoil samples may exhibit low CFU values or be "sterile", i.e. yield no colonies on solid media, is sufficiently familiar to soil microbiologists. It lends support to the above assumption that soil substrates are heterogeneous. This fact may be also due to the specific dormancy state of the cells that inhabit them: they are able to revert to an active lifestyle under certain conditions [11, 14, 15, 26]. The persisting microbial communities are heterogeneous with respect to the level of cell dormancy that may be so deep that nonculturable forms appear, which require special procedures for "resuscitating" them [5, 11, 14, 15].

Our developments concerning the methods of cell reactivation in dormant microbial communities included two aspects. One aspect was concerned with the necessity for release of the spore germination autoinhibitors from DFs [13] in order to restore their metabolic activity. It was shown earlier that alkylhydroxybenzenes perform autoinhibitory functions [18]. AHB transfer into/out of the cells involves diffusion along the AHB concentration gradient [18]. Therefore, the viable cell number (CFU) increased (Table 2) upon removing the AHBs from the DFs of collection strains and soil samples by washing them with sufficiently large water (buffer) volumes (1 : 100, vol/vol). This was particularly conspicuous in the system with "sterile" samples (CFU = 0), where CFU number increased by several orders of magnitude (up to 10^3–10^7 cells/g) (Table 4, Fig. 2).

Apart from removing the growth autoinhibitors from DFs, their germination and growth efficiency within the population (community) also depended on the intercellular communicative interactions mediated by low molecular weight regulatory metabolites (phenols, alkylhydroxybenzenes, organic amines, peptides, indoles, butyrolactones, etc.). The interactions are aimed at coordinating the development and the adaptation of the whole population (community) in conformity with the changes in the environmental conditions [18, 26, 27]. Since low molecular weight regulators are not species-specific, microorganisms are capable of responding to the molecules synthesized by other biocenosis components including plants. In this fashion, they "eavesdrop" on the numerous signals spreading in the environment [17].

The other aspect of our developments dealt with research on the effects of plant and microbial extracellular regulators on reversion of the dormant cells to active growth. We revealed a differentiated influence of the regulatory metabolites on the reactivation of the DFs and NCs of collection strains and soil microbial communities (Tables 2, 3; Figs. 2, 3). While the IAA effect on the DFs formed during the bacterial development cycle resulted in an increase in CFU number within an order of magnitude, IAA did not affect the NCs formed with C_{12}-AHB. Their reactivation required preincubation with WGA that caused the CFU value to increase by 5 orders of magnitude. These data suggest sufficiently important differences in the mechanisms of dormancy state development in (i) DFs constitutively formed in starved post-stationary bacterial populations and (ii) anabiotic forms generated under the influence of high concentrations of anabiosis autoinducers. The latter mechanism of formation of the dormant and non-culturable cells may operate in permafrost cryopegs as a result of an increase in their anabiosis autoinducer concentration.

Our techniques of washing the cells of the soil microbial communities in order to remove growth autoinhibitors and reactivating them with low molecular weight regulators were the most efficient methods with respect to "sterile" paleosoil samples (CFU = 0)

(Figs. 2, 3). Dormant cell reactivation (CFU = 10^3–10^7 cells/g) occurring in this system should be regarded as resuscitation [14, 28], and the cells per se are to be classified as nonculturable.

The activating effect of IAA is characterized by different patterns in (i) buried and background surface soils where the CFU number increased by two orders of magnitude and (ii) permafrost communities where it increased only several times. These different patterns may be due to the difference in the natural pools of biologically active compounds with growth-inhibiting and growth-stimulating activity in soils of various origins. This suggestion is supported by the results of experiments in which permafrost and background surface tundra soil samples were supplemented with an anabiosis autoinducer (C_{12}-AHD). Our findings demonstrated that the effect of C_{12}-AHB on the metabolic state of soil community cells was dose-dependent (Fig. 4), similar to that revealed with bacterial collection strains (Table 3). Interestingly, high C_{12}-AHB concentrations (5×10^{-4} M) that caused "mummification" of the cells of collection strains (Table 3) and a viability loss (CFU = 0) in the cells of the microbial fraction of background soils (Fig. 4b), had virtually no effect on the cells of the dormant community of permafrost deposits: there was almost no difference in CFU numbers between the control and the experimental system. The above differences were apparently due to the fact that the permafrost microbial community was represented by forms existing in a profound dormant state. They were resistant to physical, chemical, and biological factors [11, 18, 19], including C_{12}-AHB, unlike the cells of background soil communities which were sensitive to C_{12}-AHB "overdoses" and irreversibly lost proliferative activity.

This conclusion was confirmed by the FISH method. It revealed that an overwhelming majority of the cells of the permafrost deposit samples were dormant and nonculturable cells that could revert to active growth only after carrying out the reactivation procedures.

Hence, specific regulatory metabolites secreted by microorganisms and plants may be involved in controlling the physiological state of microorganisms under natural conditions, including those promoting the long-term persistence of microbial communities. It follows from the data obtained that the effect of the regulatory metabolites on the reactivation of bacterial DFs in situ may be mediated by alterations in the rhizosphere concentration of a plant hormone (IAA), a plant lectin (WGA), or a microbial regulator performing the adaptogen function (C_7-AHB) (Tables 2, 3). These biological activities of regulatory metabolites have not been previously reported. As a rule, such substances are characterized by non-species-specific and dose-dependent effects that manifest themselves at the community level. The loss of proliferative activity of the cells at high anabiosis autoinducer concentrations in the soil is not necessarily associated with cell death; it may be caused by transition of the cells to a nonculturable state, their reversion to active growth requiring specific conditions. The effects of anabiosis autoinducers vary depending both on their concentration and the competence and sensitivity of bacterial cells to them. In general, the results of our studies contribute to our knowledge concerning the impact of intercellular communication factors in terms of biodiversity conservation and the survival of bacterial communities in situ in soil substrates of various origins.

ACKNOWLEDGMENTS

The authors wish to thank most sincerely Dr. V.A. Demkin and Dr. D.A. Gilichinsky†, Institute of Physicochemical and Biological Problems in Soil Science, Russian Academy of Sciences, for providing the samples of buried soil and subsoil permafrost samples.

This work was supported by the Russian Foundation for Basic Research (grant no. 09-04-00699).

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