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EXPERIMENTAL ARTICLES

Comparison of the Adaptive Potential of the *Arthrobacter oxydans* and *Acinetobacter lwoffii* Isolates from Permafrost Sedimentary Rock and the Analogous Collection Strains

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Abstract—A comparative study was conducted on the adaptive mechanisms of the strains *Arthrobacter oxydans* K14 and *Acinetobacter lwoffii* EK30A isolated from permafrost subsoil sediments and of those of the analogous collection strains (Ac-1114 Type and BSW-27, respectively). In each pair of the strains compared, the strains differed in terms of (i) growth-related, physiological, and biochemical properties; (ii) resistance to stress factors; (iii) capacity for generation of dormant forms (DFs) under growth arrest conditions, and (iv) intrapopulation production of phase variants. The strains isolated from permafrost displayed a lower growth rate but were more resistant to repeated freezing–thawing treatment than the collection strains. Under the same growth conditions, the permafrost strains formed larger numbers of cystlike anabiotic DFs, extraordinarily small cells, and forms that became nonculturable during long-term storage. Resuscitation of the nonculturable forms resulted in a 2- to-7-fold increase in the percentage of FISH-detectable metabolically active cells. The permafrost strains were also distinguished by increased genome lability. This facilitated their dissociation into intrapopulation variants with phenotypically distinct colonial and morphological properties and different antibiotic resistance. The phenotypic variability was more prominent in *Arthrobacter* (for which it was not reported previously) than in *Acinetobacter*. In the populations produced by plating the dormant bacterial forms, the qualitative and quantitative characteristics of the phase variant spectra varied depending on the formation conditions and the composition of the solid media used for the plating. Thus, the permafrost isolates of *A. oxydans* and *Ac. lwoffii* were distinguished from their collection analogs by a more manifest adaptive potential including stress resistance, the intensity of DF generation under growth arrest conditions, and increased intrapopulation variability.

Keywords: permafrost subsoil sediments, permafrost isolates, *Arthrobacter oxydans, Acinetobacter lwoffii*, adaptive potential, stress resistance, intrapopulation variability, phase variants, dormant forms

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Microorganisms readily adapt to environmental changes due to a complex of biochemical, physiological, morphological, and behavioral processes that constitute the adaptive potential of an organism [1]. Detailed information is available concerning the intracellular events underlying a cell's response to sublethal and lethal stress, such as the expression of stress regulon genes (*rpoS, oxyR*, the SOS-response gene, etc.), the biosynthesis of enzymes and metabolites involved in the antioxidant protection, DNA repair and stabilization mechanisms, and others [1, 2]. However, the forms and mechanisms of the long-term survival of bacteria have not been sufficiently understood up to now. Obviously, these mechanisms should be related to (i) stress resistance due to the sufficiently well-known biomolecular processes mentioned above; (ii) the formation of dormant forms (DF) responsible for species survival; and (iii) the capacity for flexibly changing the population's phase variation spectrum that forms a basis for its adaptive potential [3, 4].

The microbial community of permafrost subsoil sediments is of particular interest in terms of the survival mechanisms of microorganisms under growth arrest conditions. Permafrost was formed as a result of alternating seasonal freezing–thawing cycles: the surface soil strata gradually moved down, due to accumulation of the geologically younger sediments on their top. The more ancient layers sunk below the level above which seasonal thawing occurred, so that migration of water and dissolved substances ceased in these horizons. In all likelihood, selection of the intrapopulation microbial variants that survived under these conditions occurred during the gradual movement of

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the soil down to the permafrost level, apart from the operation of the mechanisms enabling cell survival under growth arrest conditions by forming anabiotic dormant and nonculturable forms.

The goal of this work was to compare the adaptive potential (that comprises the physiological and biochemical properties, resistance to deleterious factors, capacity for generation of dormant forms, and phase variant-forming activity) of the isolates *Arthrobacter oxydans* K14 and *Acinetobacter lwoffii* EK30A from permafrost subsoil sediments with that of their collection analogues Ac-1114 Type and BSW-27, respectively.

MATERIALS AND METHODS

The subjects of these studies were the isolates obtained from permafrost subsoil sediments (sampled in the headwaters of the Bol'shoi Khomus-Yuryakh River in the Kolyma lowlands of Siberia, Russia as described in detail in [5]) and the collection analogs of the bacteria identified by us.

The following strains were used in this work.

(i) The K14 strain of the gram-positive bacterium *Arthrobacter oxydans* was isolated from well 3/05 at a depth of 8.0 m (the storage period was ~200000 years); the analogous collection strain As-1114 was isolated from the air.

(ii) The EK30A strain of the gram-negative bacterium *Acinetobacter lwoffii* was isolated from well 3/92 at a depth of 47.9 m (the storage period was ~1.6–1.8 Ma); the analogous collection strain BSW-27 was obtained from the collection of the Saratov Medical Institute.

The bacteria were grown at 28°C in 250-mL flasks (containing 50 mL of the medium) on a rotary shaker (140–160 prm) on the following media: (i) LB (12.5 g/L) and LB/5 (2.5 g/L) (Carl Roth GmbH, Germany) and (ii) TSB (15 g/L) (Panreac, Spain). An early stationary phase culture used as the inoculum was added to the medium to the initial optical density (OD) 0.1 (Specord UV VIS, Carl Zeiss, Jena, Germany, $\lambda = 540$ nm, $l = 10$ mm).

The bacteria were isolated from permafrost subsoil sediments by directly plating the soil sample suspension (1 g of soil per 100 mL of distilled water) on petri dishes with TSA. Consecutive subculturing was used to obtain pure cultures. The strains isolated by us were stored in plastic Eppendorf tubes at –20°C as 1 mL aliquots of the stationary phase culture obtained during the first subculturing after isolation; glycerol was used as a cryoprotector (1 : 1). These strains were used as inocula in all experiments.

The isolated bacterial strains were identified using phenotypic, chemotaxonomic, and genotypic criteria as described in [6]. The phylogenetic position of the bacteria was determined by sequencing their 16S rRNA genes obtained using the universal external and internal primers [7] and analyzed with the GenBank NCBI BLASTIN database (http://www.ncbi.nlm.nih.gov/blast). The sequence spectrograms obtained were edited using the Chromas software, version 1.45 (http://www.techelysium.com.au/chromas.html).
The sequencing studies were conducted using the equipment of the Bioengineering Resource-Sharing Center Association under the Federal Topical Program "Research and Development in the Priority Fields of Russian Science and Technology for 2007–2013".

The species affiliation of the strain *Acinetobacter* EK30A was identified using a Vitek biochemical analyzer (bioMerieux, France) with the Gram Negative Identification Card test system with 30 substrates and an oxidase test according to the manufacturer's manual (bioMerieux, France).

The growth of submerged bacterial cultures was monitored by measuring their optical density ($\lambda = 540$ nm, $l = 10$ mm) on a Specord UV VIS spectrophotometer (Carl Zeiss, Jena, Germany).

The viability of vegetative and dormant cells was determined from the colony-forming unit (CFU) number obtained by plating diluted cell suspensions on agar-containing media.

For microscopy, a Zetapan (Reichert, Austria) light microscope with a phase contrast device and a JEM-100B (JEOL, Japan) electron microscope with an accelerating voltage of 60 kV (see [8]) were used.

Cell thermal stability was determined from the data on viability retention obtained by heating the cell suspensions (80 μL) in a UV-10 ultrathermostat at 50–60°C for 5–10 min and subsequently determining the CFU numbers.

Cell antibiotic resistance was determined by comparing the CFU number obtained by inoculating the stationary-phase culture aliquots on agar-containing LB medium with ampicillin (1, 2, and 5 μg/mL) or kanamycin (10, 25, and 50 μg/mL) and the CFU number on an antibiotic-free medium.

Cell resistance to freezing–thawing was determined from the CFU number in cell suspensions after 1–5 freezing (–20°C)–thawing (20°C) cycles.

The phase variant spectrum of bacterial populations was determined by plating the culture aliquots on agar-containing media mentioned above, TGY (g/L: yeastrel, 3.0; tryptone, 5.0; glucose, 1.0 and agar, 15.0) and TGYg (TGY with glucose, 10.0 g/L). The phase variation index of a population was determined as the percentage of colonies of a specific morphotype or of antibiotic-resistant colonies in the total colony number. The stability of isolated clones was determined from the data on the retention of their phenotypic traits after consecutively subculturing them at least 3 times on an agar-containing medium.

The dormant forms and non-culturable cells (NCs) of the strains of *A. oxydans* and *Ac. lwoffii* were obtained during the development cycles of their cul-

tures by exposing them to stress that simulated natural starvation and dehydration:

(i) DF1 cells were obtained by transferring a two-fold concentrated bacterial culture during the growth deceleration stage (in the LB/5 medium) to normal saline and subsequently incubating them for up to 18 months;

(ii) DF2 cells were obtained by transferring a three-fold concentrated bacterial culture during the growth deceleration stage (in the LB/5 medium) to normal saline with 0.5% KH_2PO_4 and subsequently incubating them for up to 18 months;

(iii) DF3 cells were obtained by growing a culture on a nitrogen-limited medium containing (g/L): glucose, 10.0; L-asparagine, 1.0; and K_2HPO_4, 0.5 (pH 7.5) and subsequently incubating them for up to 18 months;

(iv) DF4 cells were obtained by plating the stationary-phase culture cells (in the LB medium) on soil agar [6] and subsequently incubating them for up to 18 months. The DF suspensions were obtained by washing the DFs from the soil agar with distilled H_2O.

The DFs were resuscitated using the methods developed earlier [5].

The FISH (fluorescent in situ hybridization)—based estimation of the percentage of metabolically active cells in the DF cell suspensions of the tested strains was carried out as described in [9]. The hybridization procedure was performed using a set of fluorochrome-labeled (CyS) rRNA oligonucleotide probes (Syntol, Russia) that were specific for the representatives of the class *Gammaproteobacteria* (the GAM42a probe with the nucleotide sequence 5'-GY3 GCC TTC CCA CAT CGT TT-3', where Y is C or T; the 1027–1043 target part of the 23S rRNA molecule) and *Actinobacteria* (the HGC69a probe with the nucleotide sequence 5'-CY3 TAT AGT TAC CAC CGC CGT-3', where Y is C or T; the 1901–1918 target part of the 23S rRNA molecule) [10]. The total number of intact cells was determined microscopically by staining them with acridine orange.

The ultra-small dormant cell fraction was obtained by passing DF suspensions through a filter with a pore diameter of 0.22 μm (Merck Millipore, United States).

Specific β-galactosidase activity in the cells was determined using the standard technique based on degrading the *o*-nitrophenyl-β-galactopyranoside substrate [11].

The exopolysaccharide (EPS) content was determined by the phenol-sulfuric method [12] in stationary-phase cultures (7 days) grown on meat–peptone broth. The sugar concentration of a sample (1 mL) was determined using a calibration curve for glucose. The specific EPS content was determined by dividing their total amount by the cell number (CFU).

Three independent repeats of the experiments were conducted. The data presented in this work are average values supplemented with standard deviations for P (probability) > 0.95. The Student's test was used for the statistical treatment of the data obtained.

RESULTS

Identification of the permafrost bacterial strains. Pure cultures of 16 isolates were obtained from the permafrost sediments of the Kolyma lowlands, Siberia. Based upon the results of the 16S rRNA gene sequence analysis, they were identified as representatives of the following genera: *Acinetobacter* (2), *Arthrobacter* (4), *Brevundomonas* (2), *Exiquobacterium* (1), *Paenibacillus* (2), *Promicromonospora* (1), *Pseudomonas* (2), *Rhodococcus* (1), and *Sphingomonas* (1). For five of the isolates obtained, we sequenced the amplificates of the 16S rRNA gene fragments (the nucleotide pair numbers are given in parentheses): K14 (1494), K15 (1491), K6 (1486), KYaR1 (1511), and K22 (1427). Their species were identified. According to the sequencing data, three of the tested strains were taxonomically closest to the following species: K14 to *Arthrobacter oxydans* FM955886 (99.4%), K15 to *A. sulfonivorans* FM955860 (99.8%), K6 to *A. sulfonivorans* FM955860 (99.9%). Strain KYaR1 was related to *Pseudomonas mandelii* FM955880 (99.4%) and strain K22 was close to *Brevundomonas vesicularis* FM955876 (99.2%). Based on the identification data, strain EK30A was most closely related to the species *Acinetobacter lwoffii* (98%).

Importantly, the representatives of the genus *Arthrobacter* belong to the most widespread aerobic bacteria occurring on natural substrates [13]. They are frequently detected in permafrost subsoil sediments [13–15] and Antarctic soils [16, 17]. Therefore, we chose the isolated *A. oxydans* K14 strain and its collection analog (Ac-1114 VKM) for comparative studies on the adaptive properties of permafrost bacteria. Another pair of strains included the gram-negative *Ac. lwoffii* EK30A strain isolated from permafrost and the BSW-27 collection strain.

The growth, physiological, and biochemical properties of the Ac-1114 and K14 strains of *A. oxydans* and BSW-27 and EK30A strains of *Ac. lwoffii*. The Ac-1114 and K14 strains of *A. oxydans* significantly differed in their growth-related properties if cultivated under the same conditions both in the rich (LB) and the 5fold-diluted (LB/5) medium. The culture of the collection strain grew more intensely than that of the permafrost strain. According to the data of microscopic analysis, the cells of the permafrost strain were somewhat smaller in size, compared to those of the collection strain.

The K14 and Ac-1114 strains also differed in terms of the level of cell metabolic activity, based on the level of intracellular β-galactosidase biosynthesis during the growth of the culture (Fig. 1). Specific β-galactosidase activity in the cells of the collection strain was maximum during the exponential phase (5 h). It was virtu-

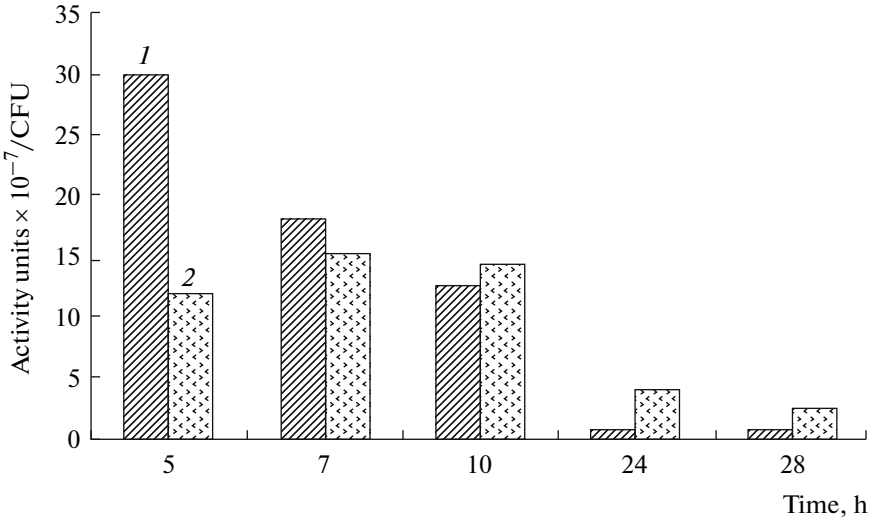


Fig. 1. Dynamics of specific β-galactosidase activity in *A. oxydans* strains Ac-1114 (*1*) and K14 (*2*).

ally absent in stationary-phase cells. In the permafrost strain, this activity was detectable throughout the whole culture development period. In its stationary-phase cells, it was 5–7 times higher than in Ac-1114 cells.

The specific exopolysaccharide content in stationary-phase cultures (7 days) was somewhat lower (by 25%) in the permafrost isolate (8.9×10^{-8} mg/cell) than in Ac-1114 (7.1×10^{-8} mg/cell).

The *Ac. lwoffii* strains BSW-27 and EK30A differed in their growth-related properties. The collection strain displayed more intense growth and biomass accumulation (data not shown). Microscopy revealed no difference between the cells of the two strains of *Ac. lwoffii*. In both strains, the specific EPS content was virtually identical $(1.0–1.2) \times 10^{-8}$ mg/cell. It was one order of magnitude below that in the *Arthrobacter* strains.

Stress resistance of the Ac-1114 and K14 strains of *A. oxydans* and the BSW-27 and EK30A strains of *Ac. lwoffii*. Comparison of the responses of the permafrost and collection strains of both bacteria to various stress factors (heat shock, freezing–thawing, and antibiotic treatment) revealed that permafrost isolates were more resistant. Thermal stability (in terms of CFU numbers) was determined from resistance to heating at 50 or 55°C for 5 min. In both exponential- and stationary-phase cells of the permafrost isolates, it was significantly higher than in the collection strain cells (Table 2). Interestingly, the thermal stability of the cells of both strains of *Arthrobacter* (not *Acinetobacter*) was higher during the exponential growth phase. However, it is known that the resistance of bacteria to various deleterious factors, including high temperature, increases towards the stationary growth phase [18]. The above peculiarities of the *Arthrobacter* strains are associated with the previously reported lesser rigidity of the cell wall of the stationary-phase coccoid forms, in contrast to that of the exponential-phase rod-shaped cells [19].

Table 1. Growth characteristics of *A. oxydans* strains Ac-1114 and K14 grown in submerged culture in LB medium at 28°C

Growth indices	Ac-1114	K14
Lag phase duration, h	2	4.5
Average specific growth rate, h^{-1}	0.18	0.14
Growth phase period, h	20	23
Minimum biomass accumulation (25 h of growth), CFU number	1.3×10^9	6.3×10^8

The cells of the permafrost strains of both bacteria were more resistant to the freezing–thawing stress. This was to be expected, taking into account their "ecological" origins (Table 2).

The molecular mechanisms involved in antibiotic resistance of the bacteria are substantially different from those described above. In the presence of antibiotics, permafrost isolates also had certain advantages over the collection strains (Table 3). The permafrost isolates typically formed microcolonies on the antibiotic-containing medium, whereas the collection strains developed normal-size colonies. This was probably due to the operation of another adaptive mechanism of the bacteria that was more prominent in the permafrost isolates. This mechanism is based upon replacing the dominant phenotype by a more resistant minor phenotype, a point to be addressed below.

Generation of dormant forms by the Ac-1114 and K14 strains of *A. oxydans* and the BSW-27 and EK30A strains of *Ac. lwoffii*. During prolonged (or lethal) stress, microorganisms are under growth arrest condi-

Table 2. Resistance of the strains *A. oxydans* Ac-1114 and K14 and *Ac. lwoffii* BSW-27 and EK30A to extreme temperatures

Variant		Viable cell number, CFU/mL (% of the CFU before the treatment)		
		before the treatment	50°C, 5 min	freezing–thawing, 5 cycles
A. oxydans Ac-1114	Exponential phase (15 h)	6.5×10^8 (100)	3.5×10^7 (5.4)	ND
	Stationary phase (26 h)	1.3×10^9 (100)	6.4×10^6 (0.49)	6.5×10^8 (50.0)
A. oxydans K14	Exponential phase (15 h)	1.0×10^8 (100)	1.3×10^7 (13.0)	ND
	Stationary phase (26 h)	6.3×10^8 (100)	4.5×10^7 (7.1)	4.6×10^8 (73.0)
Ac. lwoffii BSW-27	Stationary phase (21 h)	2.0×10^8 (100)	1.1×10^7 (5.5)	1.7×10^7 (8.5)
	Post-stationary phase (3 days)	6.0×10^7 (100)	6.0×10^3 (0.01)	1.9×10^6 (3.2)
Ac. lwoffii EK30A	Stationary phase (21 h)	1.6×10^8 (100)	8.9×10^7 (55.6)	4.4×10^7 (27.5)
	Post-stationary phase (3 days)	1.5×10^8 (100)	5.0×10^7 (33.3)	3.0×10^7 (20.0)

Note: ND stands for not determined.

Table 3. Resistance of the strains *A. oxydans* Ac-1114 and K14 and *Ac. lwoffii* BSW-27 and EK30A to antibiotics

Strain	Viable cell number, CFU/mL (% of the CFU before the treatment)					
	before the treatment (stationary phase, 26 h)	ampicillin, μg/mL		kanamycin, μg/mL		
		1	2	10	25	50
A. oxydans Ac-1114*	2.4×10^8 (100)	1.4×10^3 (0.6×10^{-3})	0	2.6×10^5 (0.11)	5.0×10^3 (2.1×10^{-3})	2.7×10^2 (0.1×10^{-3})
A. oxydans K14*	3.1×10^7 (100)	3.0×10^2 (1.0×10^{-3})	0	3.4×10^4* (0.11)	1.4×10^3* (4.5×10^{-3})	3.0×10^2* (1.0×10^{-3})
Ac. lwoffii BSW-27	2.1×10^8 (100)	7.6×10^7 (36.2)	8.0×10^6 (3.8)	0	0	0
Ac. lwoffii EK30A	3.8×10^8 (100)	1.8×10^8 (47.4)	1.1×10^8 (28.9)	0	0	0

Note: * The cells formed microcolonies on the agar-containing medium.

tions. The developmental strategy of their cultures switches from cell reproduction to cell survival as DF. The formation of cystlike dormant cell (CLC)-type DF and non-culturable cells was established in a wide variety of non-spore-forming bacteria including mycelium-lacking actinobacteria and gram-negative bacteria [8, 20–22].

CLC-type dormant forms. Comparative analysis of the intensity of DF formation and DF properties in permafrost and collection strains was performed with DFs obtained using the cultivation techniques [5, 20, 22–24] that simulated natural stress factors such as starving (DF1 cells), nutrient imbalance with P excess (DF2 cells) or N deficit (DF3 cells), and soil desiccation (DF4 cells). DF maturated and were stored at 3 or 28°C; both temperatures are within the natural temperature range. The DFs obtained with both bacteria possessed properties that were typical of the CLCs of non-spore-forming bacteria described earlier [8, 20, 22], including *Arthrobacter globiformis* [24]: (i) in morphological terms, they were small, rounded, highly refractory cells; (ii) they were resistant to stress (heating); (iii) they retained viability (colony-forming capacity) for a long time (up to 18 months); (iv) the cells had a typical CLC ultrastructure; and (v) they were formed during the developmental cycle of bacterial cultures.

DF formation intensity and DF thermal stability varied depending on the bacterial cultivation medium and maturation/storage temperature (3 or 28°C). In the permafrost strain *A. oxydans* K14, the maximum number of viable DFs (by CFU numbers) after 10 months of storage was in the case of DF2 cells (37%), in contrast to DF1 (17%) and DF3 (19%) cells. The DF-forming capacity was less manifest in the *Acinetobacter* strains than in the *Arthrobacter* strains, and the DF1 and DF2 variants prevailed (Table 4). The percentage of the DF cells that retained their colony-forming capacity for a long time was larger at 3°C. In contrast, the stress resistance (thermal stabil-

Table 4. Viable cell number (CFU/mL) and the share of dormant forms of the permafrost and collection strains of *A. oxydans* (2 months of storage) and *Ac. lwoffii* (11 months of storage). CFU number of the stationary-phase culture is accepted as 100%

Variant	DF viability, CFU/mL (% of the CFU/mL of the stationary-phase culture)			
	A. oxydans		*Ac. lwoffii*	
	Ac-1114	K14	BSW-27	EK30A
Stationary-phase culture	5.0×10^8 (100%)	1.0×10^8 (100%)	3.9×10^8 (100%)	3.8×10^8 (100%)
DF1 cells	5.3×10^7 (10.6%)	4.8×10^7 (48.0%)	2.2×10^3 (0.0006%)	1.6×10^5 (0.04%)
DF2 cells	7.7×10^7 (15.4%)	4.6×10^7 (46.0%)	0	8.0×10^4 (0.02%)

Table 5. Resuscitation of the dormant forms (DF2 cells) of *Ac. lwoffii* BSW-27 and EK30A (11 months of storage) determined by colony-forming capacity (CFU number) and the restoration of cell metabolic activity (FISH data)

Resuscitation techniques, 1 h	EK30A		BSW-27	
	CFU/mL (share of the control)	% FISH-detectable cells of total DF number (share of the control value)	CFU/mL (share of the control)	% FISH-detectable cells of total DF number (share of the control value)
Control	1.2×10^5 (1.0)	9.5 (1.0)	0	12.8 (1.0)
Washing (pH 7)	2.7×10^5 (2.3)	15.5 (1.6)	0	26.0 (2.0)
Indoleacetic acid (2.5×10^{-4} M)	1.7×10^5 (1.4)	53.6 (5.6)	0	30.4 (2.4)
C_7-Alkylhydroxybenzene (2.5×10^{-4} M)	2.4×10^5 (2.0)	48.8 (5.1)	0	33.3 (2.6)
Wheat germ agglutinin (3 μg/mL)	1.9×10^5 (1.6)	67.9 (7.2)	0	37.1 (2.9)
Yeast autolysate (1%)	3.2×10^4 (2.8)	47.6 (5.0)	0	29.5 (2.3)

ity) of DFs was higher if they maturated and were stored at 28°C. For example, the CFU number was 37% in the DF2 suspensions of the permafrost strain *A. oxydans* K14 stored for 10 months at 3°C. Storage at 28°C resulted in decreasing the CFU number to zero and to transition of the dormant cells into the nonculturable state. The DF1 cells of the K14 strain stored at 28°C exhibited the maximum thermal stability. After heating at 50 and 55°C, 28% and 0.3% of their cells remained viable, respectively. These values were two orders of magnitude higher than those for the stationary-phase cells. The percentage of heat-resistant DFs in DF2 and DF3 suspensions was 3.3 and 13.4 times lower, respectively, than that for the DF1 variant.

Nonculturable cells (NCs). A decrease in the colony-forming capacity of DFs was detected during long-term storage of the permafrost and the collection strains of both bacteria (Table 4), although microscopic analysis revealed the presence of intact cells in the DF suspensions. The detection of the NC-specific physiological state was performed using the permafrost and the collection strain of *Ac. lwoffii* as a model. The percentage of metabolically active cells in the total pool of intact cells was determined by FISH: the cells were stained with acridine orange before and after the resuscitation. The procedures were developed for resuscitation of DFs (CLCs) in laboratory cultures [20] and in the samples taken from the microbial communities of permafrost subsoil sediments and submerged soils [5]. It was revealed that, despite the absence of CFUs, the DF2 suspension of the strain *Ac. lwoffii* BSW-27 contained 12.8% of metabolically active cells (as determined by FISH) in the whole cell pool (as determined by staining with acridine orange). The DF suspension of the strain EK30A contained 9.5% of metabolically active cells (Table 5). Using resuscitating procedures increased the percentage of FISH-detectable cells of strains BSW-27 and EK30A 2.0–2.9 times and 1.6–7.2 times, respectively, in comparison to the control values (before resuscitation). The cells of the collection strain BSW-27 remained nonculturable (CFU number = 0). The CFU/mL number increased 1.4–2.8 times (compared to the pre-resuscitation values) in the DF suspension of the strain EK30A, which was accompanied by an increase in the number of metabolically active cells.

Ultra-small dormant cells. Phase contrast microscopy of the dormant form suspension of the *A. oxydans* strains demonstrated the presence of small-size cells (below 0.2 μm), which were particularly numerous in the permafrost strain K14 (Fig. 2). Electron micro-

Table 6. Viability (CFU/mL) and percentage (% of the total DF number) of ultra-small ($d < 0.22$ μm) dormant cells of *A. oxydans* K14 in surface culture on LB agar (without resuscitation)

Method of obtaining cells	Storage period, months	Total DF number	<0.22 μm fraction
DF2 cells	10	3.7×10^7 (100)	0*
DF4 cells	10	8.2×10^7 (100)	5.7×10^5 (0.7)
	18	8.1×10^5 (100)	2.0×10^2 (0.02)

Note: * Growth was also lacking on semiliquid LB agar (0.6% agar) and in LB and LB/5 media (determined by the terminal dilution method).

scopic images revealed lemon-shaped nanocells (less than 160 nm in size) with thick cell walls and an electron-dense content (Fig. 2), together with larger polymorphic DFs.

The conditions under which suspensions of the dormant cell were obtained and their age influenced the number and viability (CFU number) of ultra-small cells (Table 6). Ultra-small DFs were not detected in 10-month DF2 suspensions upon inoculation on LB agar or in semiliquid LB agar. In contrast, their number (i.e., CFU number) was 0.7% of the total CFU number in DF4 suspensions obtained on soil agar. Storing for up to 18 months resulted in decreasing the percentage of viable ultra-small DFs to 0.02% of the total DF number. Ultra-small DFs formed small (S_m type) colonies on LB agar; their reinoculation resulted in the reversion to the original colony morphology type.

Hence, both the permafrost and the collection strains of *A. oxydans* and *Ac. lwoffii* made use of their capacity for the formation of several types of dormant cells—CLCs, nonculturable cells, and ultra-small cells—under growth-arrest conditions. The permafrost strains had advantages with respect to DF formation intensity and long-term retention of their colony-forming capacity.

Population variability. The next stage of this work was aimed at comparing the capacity of the permafrost and collection strains of the bacteria *A. oxydans* and *Ac. lwoffii* to display population variability. For this purpose, we investigated their colony morphology and antibiotic resistance.

Colony morphology variants. The permafrost strain *A. oxydans* K14 differed from the collection strain Ac-1114 in terms of phenotypic instability. Importantly, plating on agar media of the growing (15 h to 5 days old) cultures of the permafrost strain (in LB medium) obtained during the first subculturing after the isolation resulted in the development of the following four morphotypes: S (98–21%), M (2–15%), R (1–70%), and S_m (1–8%) (Table 7).

The phase variant spectrum of developing K14 cultures in LB medium varied depending on their physiological age (growth phase) and the selectivity of the plating (Table 8). The share of the M phenotype cells increased in the stationary-phase culture to 15%, with an additional increase during storage. The R type variant preferentially developed on a solid medium with a high glucose content (TGYg). The phase variant spectra obtained with the DFs of the strain K14 varied depending on the DF formation conditions; they were distinguished by higher variability, which is concordant with the earlier data [22, 23]. Plating of DF1 and DF2 cells resulted in the percentage of the dominant S type not exceeding 20%; the M variant predominated in the culture on LB agar, and the R variant, on TGYg medium. The S_m type constituted a significant share in all the variants. The DF3 cells obtained under nitrogen limitation and the DF4 cells obtained under anhydrobiotic conditions were more stable phenotypically; the percentage of the minor variants (the M and the S_m type) upon DF inoculation did not exceed 35%. Hence, the permafrost K14 isolate of *A. oxydans* was characterized by high intrapopulation variability. This is an adaptive advantage for bacteria existing under drastically changing conditions, including the freezing and thawing associated with submerging of the surface soil layers.

The collection strain A. oxydans Ac-1114 exhibited a comparatively high phenotypic stability. Plating the vegetative cells of the cultures grown under diverse conditions (in terms of medium composition and temperature) on agar media yielded growing populations exclusively consisting of the colonies of the dominant S type (Table 8). However, plating of the DFs forming in 2-month cultures developing on LB medium yielded R type colonies whose percentage on LB agar and TGYg medium was 16.4% and 88.7%, respectively. Plating of the DF1 and DF2 cells of the collection strain demonstrated an enlargement of the population spectrum due to the development of the M, R, and S_m variants (Table 8). The colony morphology of the phase variants of the permafrost and collection strain was similar (Table 7). Genetic analysis of the phase variants of both strains (using the 16S rRNA gene) revealed that they belonged to the corresponding *A. oxydans* strains. It should be emphasized that the variability of arthrobacters with respect to colony morphology has not been described previously.

Strains BSW-27 and EK30A of Ac. lwoffii. No variability in terms of colony morphology was detected if aliquots of the growing cultures of the strains BSW-27 and EK30A of *Ac. lwoffii* were plated on various agar media. Only the plating of 2-month DFs of the perma-

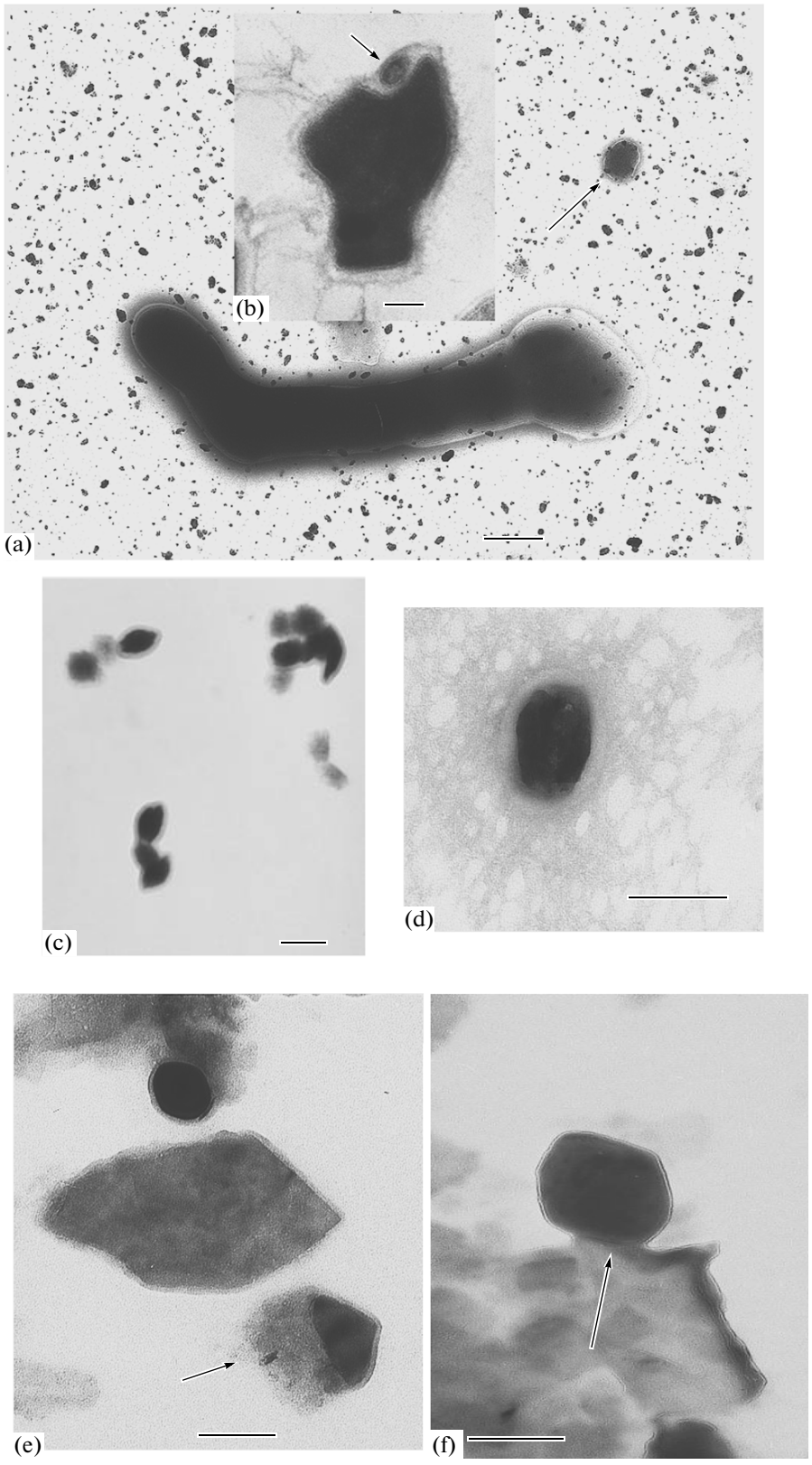
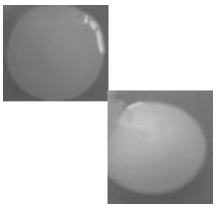
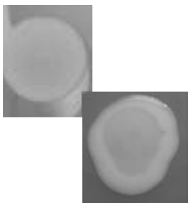
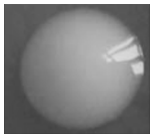
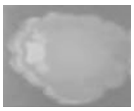
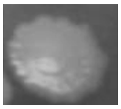


Fig. 2. Whole cells (a, c–f) and the ultrastructure (b) of the dormant forms (DF3 cells) of the strain *A. oxydans* K14. Apart from normal-size CLCs, ultra-small forms (arrows in a and b) with diameters below 0.22 μm occur. Lemon-shaped, with thickened cell walls, a capsular layer, and an electron-dense interior (c, d) resulting from the fission of larger cells (arrows in e and f), such ultra-small lemon-shaped cells also occurred in the DF4 filtrates (<0.22 μm) of the strain *A. oxydans* K14. Scale bar, 0.2 μm.

Table 7. Description of the phenotypic variants of *A. oxydans* strains Ac-1114 and K14

Type	Variant description	Colony appearance	
		Ac-1114	K14
S	Rounded cream white-colored colonies with a smooth convex glossy surface and even edges ($d = 5–7$ mm). Strain K14 on TSA medium formed darker-colored colonies; mucilage started covering the colony edges after 3–5 days (1). The addition of X-gal to the medium colored the colonies blue		 1
M	Rounded pale yellow mucilaginous colonies with a less convex glossy surface and even edges ($d = 6–8$ mm). The colonies of strain K14 on TSA medium had an indentation in the center. The addition of X-gal to the medium colored the colonies blue		
R	Rounded cream-colored rough colonies with a dull surface and an uneven edge ($d = 6–8$ mm). The colonies of strain K14 displayed intermediate features compared to those of the S and R forms (2). The addition of X-gal to the medium weakly colored the colonies		 2
S_m	Small rounded cream- or creamy-white colonies ($d = 1–2$ mm). The addition of X-gal to the medium failed to color the colonies		

frost strain EK30A made it possible to detect the following three colony morphotypes in the growing population: (i) the dominant S_1 type (rounded, homogeneously cream white-colored colonies, 3–5 mm in diameter), 83.5%; (ii) the S_2 type (distinguished from the S_1 type by an inhomogeneous colony color including dark radial strands), 8.7%; and (iii) the S_m type (small, light cream colored colonies, 1–2 mm in diameter), 7.8%. The phase variants of the S_2 and S_m types were unstable: they reverted to the dominant S_1 type after two to three transfers. Further storage of the DF of *Ac. lwoffii* strains failed to cause changes in the phase variant spectrum upon plating.

Antibiotic-resistant variants. Inoculating the DFs of the permafrost strains on media with ampicillin and kanamycin provided evidence that antibiotic-resistant clones accounted for a large percentage of the culture (Table 9). The number of kanamycin-resistant clones in 10-month DF2 cells of the permafrost isolate of *A. oxydans* was 4600 times higher than that in 2-month DF2 cells of the collection strain of *A. oxydans*.

Hence, the phenotypic variability that underlies a species' adaptive potential and manifests itself in the differences in colony morphology or physiological properties among the intrapopulation phase variants of arthrobacters and acinetobacters was more pronounced in permafrost isolates than in their collection analogs.

Properties of the phase variants. *A. oxydans* strains. Sequential transfers (three times or more) of the phase variants of strains K14 and Ac-1114 on solid media demonstrated that the S type invariably retained its traits whereas the M and S_m types completely reverted to the S type after the second transfer. If S_m type bacteria were cultivated in a liquid medium, 24% of the cells transformed into the dominant variant cells, while the M phase variant completely reverted to the S type.

The phase variants of both *Arthrobacter* strains differed, apart from their colony type (Table 7), in the biosynthetic activity of β-galactosidase. It was higher in the S type colonies as was established by growing the bacteria on the medium supplemented with X-gal, a β-galactosidase-specific chromogenic substrate. The phase variants differed also in their exopolysaccharide-producing capacity. The M cells of both the collection and the permafrost strains produced large amounts of EPSs. Their colonies grown on LB agar with ruthenium red (80 mg/mL) to conduct a qualitative test for EPS synthesis by these bacteria [25] were colored bright-red; the S, R, and S_m strains were less productive.

The M and S phase variants (which were stable and dominant) of both *A. oxydans* strains differed in terms of stress resistance that was the highest in the M variant. After a heat shock (50°C, 5 min), 44.4% of the M type cells and 5.5% of the S type cells remained via-

Table 8. Phase variation index of *A. oxydans* Ac-1114 and K14 populations obtained under various conditions

DF variant*	DF storage time, months	Inoculation medium	CFU/mL	Phase variation index, %			
				S	M	R	S_m
Collection strain Ac-1114							
DFs in LB medium	15 h	LB agar	6.5×10^8	100	0	0	0
		TGYg	6.7×10^8	100	0	0	0
	5 days	LB agar	5.2×10^8	100	0	0	0
		TGYg	5.5×10^8	100	0	0	0
	2 months	LB agar	3.1×10^8	78.1	0	16.4	5.4
		TGY	4.4×10^8	94.9	0	0	5.1
		TGYg	3.3×10^8	4.9	0	88.7	6.4
DF1 cells	2 months	LB agar	5.3×10^7	27.5	72.5	0	0
		TGYg	4.6×10^7	7.9	46.4	10.0	12.3
DF2 cells		LB agar	7.7×10^7	1.3	98.7	0	0
		TGYg	6.9×10^7	16.7	78.3	9.2	12.5
Permafrost strain K14							
DFs in LB medium	15 h	LB agar	1.0×10^8	97.8	2.3	0	0.9
		TGYg	7.0×10^7	98.3	0	1.2	0.5
	5 days	LB agar	9.4×10^7	79.3	15.1	0	5.6
		TGYg	5.6×10^8	21.1	0	70.9	8.0
	2 months	LB agar	2.1×10^8	25.2	64.2	0	10.6
		TSA	2.3×10^8	12.4	73.8	0	13.8
		TGY	2.4×10^8	18.5	79.8	0	1.7
		TGY/10	5.0×10^8	0	0	0	100
		TGYg	1.9×10^8	20.8	0	69.2	10.0
DF1 cells	2 months	LB agar	8.8×10^7	23.3	66.7	0	10.0
		TGYg	6.5×10^7	20.1	0	72.4	7.5
	9 months	LB agar	1.7×10^7	0	26.2	0	73.8
		TGYg	2.0×10^7	15.3	0	63.8	20.9
DF2 cells	2 months	LB agar	5.6×10^7	9.4	79.6	0	11.0
		TGYg	5.8×10^7	16.7	0	75.1	8.2
	9 months	LB agar	3.7×10^7	7.2	79.4	0	13.4
		TGYg	4.9×10^6	14.3	0	71.9	13.8
DF3 cells	7 months	LB agar	2.8×10^8	69.0	28.0	0	3.0
	10 months		1.9×10^7	72.9	16.5	0	10.6
DF4 cells	5.5 months	LB agar	8.2×10^7	74.1	15.0	0	10.9
		TGY	8.3×10^7	64.1	30.2	0	5.7
		TSA	7.8×10^7	87.5	6.4	0	6.1

Note: * The method of obtaining DFs is described in the Materials and Methods section.

ble in the case of the collection strain Ac-1114; the respective values were 58.6% and 33.3% for strain K14—these results are based on our CFU data (Table 10). The M phenotype was more resistant to freezing–thawing in both strains. After 5 freezing–thawing cycles, the numbers of viable cells in the suspensions of the M phase variants were 4.9 and 3.5 times higher for strains K14 and Ac-1114, respectively, than in the case of the S variants. The M phase variants also were ~2 times more antibiotic-resistant, compared to the S variants, and the difference between the variants was approximately equal for both strains (table 10).

Our research on the thermal stability of the phase variants of the permafrost isolate of *Ac. lwoffii* obtained by plating of its dormant forms (stored for 2 months) revealed that the S_2 type cells were 4.5 times more resistant to heating (55°C, 10 min) than the S_1 type

Table 9. Segregation of antibiotic-resistant strains upon reinoculating the DFs of the strains *A. oxydans* Ac-1114 and K14 on the media with ampicillin and kanamycin

Variant		Viable cell number, CFU/mL (% of the CFU before the treatment)				
		growth without antibiotics	ampicillin, μg/mL	kanamycin, μg/mL		
			1	10	25	50
Ac-1114	Stationary phase (26 h)	2.4×10^8 (100)	1.4×10^3 (0.6×10^{-3})	2.6×10^5 (0.11)	5.0×10^4 (0.02)	2.7×10^2 (1.1×10^{-4})
	DF2 (2 months)	1.5×10^8 (100)	0	8.6×10^7 (57.3)	2.9×10^4 (0.02)	4.7×10^2 (3.1×10^{-4})
K14	Stationary phase (26 h)	3.1×10^7 (100)	3.0×10^2 (1.0×10^{-3})	3.4×10^4 (0.11)	5.0×10^2 (0.6×10^{-3})	3.0×10^2 (1.0×10^{-3})
	DF2 (10 months)	5.6×10^8 (100)	5.1×10^3 (0.9×10^{-3})	2.0×10^8 (35.7)	8.6×10^6 (1.5)	7.9×10^6 (1.4)

Table 10. Viability (CFU/mL) and percentage of heat- and antibiotic-resistant cells of the phase variants of *A. oxydans* strains Ac-1114 and K14 and the *Ac. lwoffii* strain-EK30A

Treatment conditions	Viable cell number, CFU/mL (% of the CFU before the treatment)			
	A. oxydans, strain			
	Ac-1114		K14	
	S	M	S	M
Before the treatment	3.3×10^8 (100)	4.5×10^8 (100)	1.8×10^8 (100)	2.9×10^8 (100)
Heating at 50°C, 5 min	1.8×10^7 (5.5)	2.0×10^8 (44.4)	6.0×10^7 (33.3)	1.7×10^8 (58.6)
5 freezing–thawing cycles	2.3×10^7 (7.0)	1.1×10^8 (24.4)	1.9×10^7 (10.6)	1.5×10^8 (51.7)
Kanamycin, 10 μg/mL	5.5×10^7 (16.7)	1.6×10^8 (36.2)	1.8×10^6 (1.0)	6.7×10^6 (2.3)
	Ac. lwoffii strain EK30A, phase variant			
	S_1		S_2	
Before the treatment	4.8×10^7 (100)		1.1×10^8 (100)	
Heating at 55°C, 5 min	1.6×10^5(0.33)		1.6×10^6 (1.5)	
Heating at 60°C, 5 min	3.4×10^5 (0.71)		5.3×10^5 (0.48)	

cells: after heating, they retained 1.5% and 0.33%, respectively, of the CFU determined before heating.

Taking into account all these adaptive traits, the permafrost strains of the bacteria *A. oxydans* and *Ac. lwoffii* were characterized by a higher adaptive potential than their collection analogs.

DISCUSSION

Recent microbiological research has convincingly demonstrated that permafrost, dubbed the "cryobiosphere", contains a large number of viable microorganisms (10^3–10^8 cells/g) [5, 13–15]. Since the estimated permafrost area in Russia is 60 to 70% of its territory, we should acknowledge the fact that an enormous number of microorganisms on the planet exist in the cryoconserved form. Research on the microbial communities of subsoil permafrost sediments is of special importance because, apart from elucidating the cell adaptation mechanisms operating under unfavorable growth conditions, it enables us to address the question whether there is a time limit to the retention of the viability of living organisms. This issue cannot be resolved using experimental or modeling approaches. From this perspective, sediments that have been in the frozen state for a long time represent a unique system that makes it possible to observe the results of the cryoconservation of living systems on the geological time scale.

In our earlier works, we presented evidence of the presence of viable organisms (based on our CFU data) in the samples of permafrost sediments with various

geological ages. According to our electron microscopic data, the surviving microbial morphotype is represented by the anabiotic dormant forms, predominantly generating cystlike cells [15]. It was also established that cells from "sterile" samples (CFU number = 0) that lack the cells retaining their colony-forming capacity under standard conditions, could, nevertheless, be resuscitated by special techniques [5].

This work concentrated on the following features of microorganisms: (i) stress resistance (to account for cell survival upon permafrost formation); (ii) the capacity to assume the ametabolism and dormancy state (to identify the morphotypes of the dormant forms); and (iii) the lability of phenotypic variation (to account for the ability of surviving cells to retain the tested properties).

Comparison of the physiological potential of the collection strains of the bacteria *A. oxydans* and *Ac. lwoffii* and those isolated from subsoil permafrost sediment revealed that the permafrost strains were less efficient in terms of growth-related properties. However, they had advantages with respect to such a metabolic criterion as the cell β-galactosidase level. The permafrost isolate was also more productive in terms of synthesizing the exopolysaccharides that perform important protective functions: they form the biofilm/colony matrix, protect the cells from deleterious agents and desiccation, and are involved in controlling the cell aeration, translocating various substances including ions, etc. [25].

Comparative studies on the stress resistance of the permafrost and collection strains yielded results that were expected by us. The cells of the permafrost isolates were more resistant to freezing–thawing (up to 10 cycles) and to high temperatures (Table 2). Of considerable interest was also the result of comparing the antibiotic resistance of the cells of the permafrost and collection strains of *A. oxydans* and *Ac. lwoffii*. Antibiotic resistance was somewhat more manifest in the cells of the permafrost isolates (Table 3). Antibiotic resistance of permafrost-isolated bacteria was described earlier by several researchers. For instance, it was revealed in [26] that 20 of the 60 samples isolated from Arctic and Antarctic permafrost sediments with an age of 5 thousand to 3 million years were resistant to two or more antibiotics. In another work [27], it was emphasized that 5 to 17 of the 23 tested antibiotics failed to produce effects on bacteria isolated from the permafrost sediments of Antarctica. This resistance can be due to the fact that antibiotics, being the products of secondary metabolism, are characteristic of a large number of microorganisms, both bacteria and fungi, including their ancient representatives. It is possible that, due to permafrost thawing caused by global warming, we shall have to cope with the forms that are not affected by antibiotics.

It should be noted that the stronger the stress factor (high temperature, antibiotic concentration) applied, the more manifest the stress resistance of bacteria isolated from permafrost sediments becomes. Possibly, permafrost isolates are characterized by elevated levels of intra- and extracellular metabolites functioning as adaptogens; their synthesis is induced by stress factors. Such adaptogens were revealed in a number of bacteria [28–31]. Screening the permafrost isolates for metabolites serving as adaptogens and protectors holds much practical value.

Transition to the dormant state is an extreme form of microbial adaptation to unfavorable growth conditions. In both pairs of bacterial strains studied in this work, this was associated with the formation of several morphotypes of DFs. The intensity of DF formation was higher in the permafrost isolates (Table 4). Cystlike cells represented the main dormant morphotype of the tested bacteria. The CLCs of the strains K14 and Ac-1114 were quite similar in terms of their ultrastructural organization. They also resembled the CLCs of *A. globiformis* described earlier [24]. However, in the case of the permafrost isolate they were more stress-resistant and displayed a more significant potential of phase variant formation upon plating on agar media (Table 8).

Nonculturable cells represent another kind of dormant forms described in the literature. In our studies, this dormant morphotype was especially prominent in *Acinetobacter* strains, particularly in BSW-27. Its 11-month-old dormant suspensions contained no viable cells (CFU), while FISH demonstrated that 12.8% of the total cell pool was metabolically active. Resuscitation techniques (developed earlier [5, 20]) significantly increased this percentage in both *Acinetobacter* strains. However, they were insufficient for restoring the cell division capacity in the permafrost isolate. Two suggestions may be made in light of these findings. First, the formation of CLCs and NCs by the collection strain BSW-27 and the permafrost strain EK30A, respectively, seems to point to a higher resistance of the permafrost isolate of *Ac. lwoffii*, which represents its adaptive response to permafrost formation. Second, the restoration of the colony-forming capacity of NCs apparently requires specific early-exponential phase metabolites such as the Rpf factor [21], apart from germinants and cytokines.

Ultra-small (<0.22 μm) cells detected in the dormant suspensions of the permafrost strain *A. oxydans* K14 represented the third dormant form. They were structurally similar to the nanocells of soil filtrates [32]. Their number varied depending on the conditions under which the DFs were obtained and on their age. In 10-month cultures, the percentage of viable cells in the <0.22 μm fraction was higher for the DFs obtained on desiccated soil agar (DF4 cells) than for the DF suspensions formed under excess of phosphorus (DF2 cells). The "desiccated soil agar" technique we used simulates the development of unfavorable conditions in the soil habitat. The peculiarities of the ultrastructure of the small cells were characteristic of the dormant bacterial forms (cell wall thickening, cap-

sular layer formation, and an osmiophilic cytoplasm) (Fig. 2). The ultra-small cells occurring in all samples (DF2, DF3, and DF4 cells) were also characterized by a unitary lemon-shaped form that probably provides a criterion for detecting the dormant nanocells in natural habitats. The nanosize DF fraction revealed for the *Arthrobacter* strains is apparently characteristic of other soil bacteria in their natural environment.

Hence, the intraspecific polymorphism of bacterial dormant forms, an important species-level adaptive property, was particularly manifest in permafrost isolates. They were characterized by a (i) higher percentage of colony-forming DFs, (ii) their higher stress resistance, and (iii) a broader phase variant spectrum upon germination, compared to the collection strains.

Advantage of the permafrost isolates presupposes that, in addition, they exhibit genome instability (denoted as phenotype "metastability" by some researchers [3]). This enables the genome to give rise to nonidentical phenotypes, securing the adaptation of a population to environmental factors. These modifications are reversible. Therefore, it is not the multiplicity of traits that is inherited, but rather the potential capacity for their manifestation [4].

The phase variants of the representatives of the genera *Arthrobacter* and *Acinetobacter* have not been reported previously. Our research revealed a more manifest phase variant-forming activity of the permafrost strains of the tested bacteria, compared to their collection analogs, with respect to (i) the development of the variants with specific colony types on agar media and (ii) the segregation of antibiotic-resistant clones. As for the species *Ac. lwoffii*, its phase variants were detected only in the permafrost EK30A strain after plating its DFs. The high intrapopulation variability of the arthrobacters (as well as of other representatives of the *Actinobacteria* domain [33]) provides for their high adaptability to changing conditions and secures the widespread distribution of the microorganisms of this group in the soils of various areas including permafrost [13].

The phenotypic dissociation that underlies the adaptive potential of a population is based on the differences in the main adaptive features of the phase variants. This was demonstrated in our studies with the arthrobacters. The differences in terms of nutrient requirements entailed the preferential development of the phase variants on different media. The R variants preferred glucose-rich media and the M variants grew better on nutrient-limited media (Table 8). Different biosynthetic activities exemplified by β-galactosidase and EPS synthesis activity resulted in the different physiological properties of the tested strains. Resistance to deleterious factors, a prerequisite for cell viability, was more manifest in the M variant of the *Arthrobacter* strains and in the S_2 phenotype of the *Acinetobacter* strains (Table 10).

The results obtained are of paramount importance with respect to our understanding of the role of intrapopulation variability in terms of bacterial survival mechanisms operating during long-term cryoconservation. They are based on the manifestation of phenotypes characterized by (i) high cell resistance, (ii) manifest polymorphism of the anabiotic dormant forms, and (iii) high phase variant-forming activity. The phenotypes were selected for during repeated freezing–thawing cycles. Researching the mechanisms of adaptation to unfavorable conditions (including intrapopulation phase transitions) in model studies with bacteria of the microbial communities resuscitated after long-term conservation, as well as using the results of these studies for the purposeful selection and maintenance of the phenotypes with desirable traits, are of considerable microbiological, biotechnological, and medical interest.

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