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EXPERIMENTAL ARTICLES

Long-Term Storage of Collection Cultures of Actinobacteria

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Abstract—A total of 553 collection cultures of actinobacteria, including 453 reference ISP strains, were studied after long-term storage in a lyophilized state, as soil cultures, and under mineral oil. It was established that their viability reached a near-critical level. A number of methodological approaches to optimization of activation and proliferation of actinobacteria made it possible to restore the viability and major cultural and morphological properties of 65% of actinobacteria stored in a lyophilized state or under mineral oil. The actinobacteria stored as soil cultures almost completely lost their viability. Resuscitated actinobacteria exhibited a high level of genetic instability, which resulted in the emergence of more than three phenotypically different types of colonies. The population spectrum shifted towards an increase in the content of minor phenotypes with low spore-forming capacity. Significant changes in the cultural and morphological properties of a number of resuscitated strains were observed. Desirability of the application of antioxidants or growth-stimulating compounds in order to restore the viability of *Actinobacteria* cultures and to stabilize them after long-term storage was demonstrated.

Keywords: actinobacteria, long-term storage, restoration of culture viability, stabilization of properties

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Preservation of cell viability and stabilization of the main properties of microorganisms during long-term storage is the primary task of all strain collections. To solve this problem, it is necessary to gain insight into the main mechanisms of the survival strategy of microorganisms. The fact that the viability of actinobacteria can be restored after long-term storage indicates the high efficiency of the reparation processes of these microorganisms. Resuscitation of collection strains of actinobacteria, especially of those on which the original descriptions of the taxa are based, as well as the restoration of those of their characteristics which are of practical importance, is the research priority for taxonomical and biotechnological investigations.

As a result of our long-term investigations, an extensive collection of actinobacteria was created and studied in the Laboratory of Classification and Storage of Unique Microorganisms (Winogradsky Institute of Microbiology, Russian Academy of Sciences). This collection includes representatives of various taxonomical groups of both mycelial and nonmycelial forms of actinomycetes. This collection was set up on the basis of the collection created by Prof. V.D. Kuznetsov. A major part of it consists of the type (reference) strains of actinomycete species described under the International Streptomyces Project (ISP) for the emendation of descriptions of the type and neotype strains of the genus *Streptomyces*. The ultimate goal of ISP was to create a universally accessible collection of taxonomically described strains of actinomycetes [1–4], considered as reference strains and used for comparative studies and identification of novel strains isolated from environmental sources. Due to the reorganization of the taxonomy of the genus *Streptomyces*, some type strains described under the international project of the type strain redescription have presently lost their status in the nomenclature. However, the importance of this collection has not diminished at all as it is always in great demand to carry out investigations into the biology of *Streptomyces* species or their close relatives.

In the present work, we present the results of our studies on long-term storage of 453 collection ISP strains and 100 collection strains of actinobacteria from the Winogradsky Institute of Microbiology, Russian Academy of Sciences, stored for 40 and 22 years, respectively, in lyophilized state, under mineral oil, and as soil cultures.

MATERIALS AND METHODS

The subjects of study were 453 collection ISP strains of the genera *Streptomyces* (449), *Saccharopolyspora* (1), *Amycolatopsis* (2), and *Nocardiopsis* (1) that had been stored since 1969 in lyophilized state, under mineral oil, and as soil cultures [5]. In addition, attempts were made to restore the viability and cultural and morphological properties of 100 strains of actinobacteria belonging to 15 different genera and stored since 1988 in lyophilized state, under mineral oil, and as soil cultures. Preservative-

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free normal horse serum was used as a protective medium; the cultures were then stored at 4°C. The cultures stored under mineral oil and soil cultures were kept at room temperature.

Reactivation of the lyophilized cultures of actinobacteria after long-term storage was carried out by plating the dry material onto nutrient medium or after preliminary rehydration of lyophilized cells by (a) incubation in a moist chamber for 24 h; (b) resuspension in sterile tap water; and (c) resuspension in the rehydrating medium. A water solution used as the rehydrating medium contained the following: peptone, 0.5%; yeast extract, 0.3%; and $MgSO_4 \cdot 7H_2O$, 0.1% (National Biological Resource Center, NBRC, Japan). For rehydration of lyophilized cells of some cultures (that had been stored since 1982), water solutions of *Callisia fragrans* sap (1 : 100) were used [6].

The natural phenolic antioxidants quercetin (3,3',4',5,7-pentahydroxyflavone dehydrate), α-tocopherol, dopamine (3-hydroxytyramine hydrochloride) (Fluka), and adrenalin ((–)-epinephrine; (R)-(–)-3,4-dihydroxy-α-(methylaminomethyl)benzyl-3,4-alcohol) (Fluka) were used during the experiments aimed at restoring the viability of the lyophilized cultures of actinobacteria (stored since 1968). The effects of these antioxidants were studied using the following technique: Water suspensions from 5 ampoules of the lyophilized material of each strain were mixed together and then divided into 5 equal parts; one of them was used as a control, whereas the other parts were supplemented with antioxidants to the final concentration of 10^{-5} M.

For comparative experiments, the ampoules containing lyophilized cells of all strains stored for the same period of time were used. Cell viability was determined by plating the cell suspensions in duplicates on the ISP-3 medium with 0.25% of yeast extract (Difco) and by subsequent enumeration of colony-forming units (CFU).

To determine the viability and cultural and morphological properties of actinobacteria after long-term storage, the standard nutrient media suggested by the International Streptomyces Project (ISP) [7] (ISP-1, ISP-2, ISP-3, ISP-6, and ISP-7), as well as the synthetic CP1 medium (liquid and agarized) with glucose and the starvation medium [8], were used. To designate the cultures, the registration numbers of the laboratory collection with the acronym RIA were used.

RESULTS

Our studies revealed that the viability level of the studied collection cultures reached a near-critical level after long-term storage. The percentage of unviable strains of the actinobacteria stored as described above was quite high (35%).

During storage of actinobacteria as soil cultures at room temperatures, the studied strains lost their viability almost completely. According to the published data, storage as soil cultures is not suitable for long-time storage of most streptomycetes and their relatives; however, this method appeared to be quite efficient for stabilization of their main cultural and morphological properties [9, 10]. Previous investigations into the viability of the reference ISP cultures determined that 51% and 88.5% of strains had lost their viability after 9-year and 12-year storage as soil cultures, respectively [10]. After long-term storage under mineral oil, viability of 63% of the studied cultures was restored. These strains produced predominantly colorless, mycelium-free colonies. To restore their ability to produce aerial mycelium and pigments, the strains were subcultured on agarized nutrient media and, once or twice, on the soil enriched with the vitamin B complex and glucose [11].

After plating lyophilized material, most of the studied strains of actinobacteria were capable of sparse growth as separate colonies, which was probably due to the death of the majority of the cells during storage.

Previously, it was demonstrated that lyophilization and subsequent storage could result in an increase in the phenotypic variation of actinomycetes [5]. The number of phenotypes with low spore-forming capacity (or an inability to form spores at all), that is, of minor variants ("oligospore-forming", "white", or "onspore-forming" [12]), exhibiting low levels of the biosynthetic activity or not capable of this activity [13], increased. According of the results of our experiments, the intrapopulation variation of 83% of the studied strains increased due to an increase in the amount of minor phenotypes. Changes in the cultural and morphological properties of the studied strains after long-term storage may result in changes in the physiological and biochemical properties of the cells as a result of unfavorable storage conditions. It was established that after long-term storage of lyophilized cultures, 25% of all the studied strains exhibited limited capacity of inability to produce aerial mycelium and spores; 36% of them were unable to produce pigments or were characterized by low intensities of pigment formation.

The effect of damaging factors at different stages of storage of lyophilized cultures (freezing, sublimation, and subsequent rehydration) can disrupt the balance of the biochemical reactions resulting in lethal damage to affected cells and their subsequent death. Reducing the damaging effect of these factors during reactivation of the studied lyophilized cultures after long-term storage was attempted through the application of various methods and techniques. The rate of dehydration is of considerable importance for the restoration of viability. The published data on the effects of the rate of rehydration on the viability of different groups of microorganisms are rather contradictory [5, 14, 15]. However, in the case of mycelial organisms (fungi and actinobacteria), incubation of lyophilized cells in a humid atmosphere before inoculation had the most

favorable effect [5, 13, 14]. In this work, rehydration of the lyophilized material in a moist chamber for 24 h before incubation allowed us to increase the number of resuscitated cultures up to 1.5 times. Using an array of 61 cultures represented mainly by the type strains of various *Streptomyces* species, we demonstrated that 20 cultures were resuscitated only after rehydration of the dry material in the moist chamber. The use of rehydrating liquid used for reactivation of lyophilized cultures in the NBRC collection did not yield any positive results as compared to sterile tap water. On the contrary, in some cases, it was preferable to use water for dehydration of lyophilized actinobacteria after long-term storage in order to restore their viability (Table 1). It is also well known that the consistency of nutrient media inoculated with preserved cultures of actinobacteria has different effects on the viability of different cultures [16]. Thus, we used both agarized and liquid nutrient media (ISP1 and CP1 with glucose) for resuscitation of actinobacteria after long-term storage. It was demonstrated that liquid nutrient media, especially ISP1, were more suitable for restoring cell viability of actinobacteria.

Resuscitation of lyophilized microorganisms occurs in two stages, rehydration and cultivation. At the stage of rehydration, lyophilized cells are severely affected by oxygen [17]. It is well known that intensification of the free-radical reactions of peroxide oxidation of the cell material occurs during storage [18]. A direct correlation between the intensity of free-radical reactions and cell death was shown [19, 20]. Antioxidants serving as traps for oxidative radicals are able to inhibit free-radical processes efficiently. A positive effect of adding antioxidants to protective media used for long-term storage was demonstrated [21].

Antioxidants were therefore used at the rehydration stage in order to restore the viability of lyophilized cultures of actinobacteria after long-term storage. Earlier, a method for application of water solutions of *Callisia fragrans* sap as a rehydrating liquid was developed and its optimal concentrations were determined [6]. It is well known that the sap of this plant contains relatively high concentrations of physiologically active compounds, such as flavonoids, phenolcarbonic acids, and salicylic acid, and is a strong antioxidant [22, 23]. Quercetin, the most potent antioxidant of this class of compounds, and α-tocopherol, the most potent antioxidant in the tocopherol family, were detected among flavonoids. It was also observed that the effect of *Callisia fragrans* sap was dose-dependent. At a dry solid concentration of 4.5 mg/mL in the reaction mixture, the antioxidant activity of this sap was comparable to the quercetin activity at a concentration of 0.01 mg/mL [22, 23]. The antioxidant activity of the water solution of *Callisia fragrans* sap used in our experiments correlated with the activity of the quercetin solution (0.0034 mg/mL or 10^{-5} M) [6]. The application of this phytogenic antioxidant agent for rehydration of lyophilized cultures allowed us to revive seven *Streptomyces* cultures, including *Streptomyces abikoensis* RIA 1179, *S. aureoverticillatus* RIA 679^T, *S. hiroshimensis* RIA 1052^T and RIA 1087, as well as *S. netropsis* RIA 1036, RIA 1114, and RIA 1184^T. Acceleration of growth and restoration of their key phenotypic properties were detected; the number of CFU was 1.5–7 times higher (Table 2).

Phenolic antioxidants widely occur in nature. The antioxidant properties of phenols are due to the presence of phenolic hydroxyl groups, from which hydrogen atoms can be easily dissociated under the influence of free radicals. In this case, phenols act as interceptors of free radicals and replace them with phenoxy radical of low activity. Tocopherols, flavonoids, and catecholamines, as well as some other bioactive compounds, are the most known phenol antioxidants. Using four *Streptomyces* strains, *S. cyaneofuscatus* RIA 1027^T, *S. flavovirens* RIA 1038^T, *S. massasporeus* RIA 1064^T, and *S. limosus* RIA 1058^T as an example, the effects of quercetin, α-tocopherol, and the catecholamines adrenalin (epinephrine) and dopamine on the viability of *Streptomyces* strains stored for long periods were studied. Our previous studies demonstrated that adrenalin (epinephrine) and, especially, dopamine exerted a growth-promoting and stabilizing effect on actinobacteria. Their optimal concentration (10^{-5} M) with the maximum growth-promoting effect was determined [24]. The above-listed compounds increased the viability of the resuscitated *Streptomyces* cultures by 1.5–5 times (Table 3). The extent to which the viability of different actinobacteria increased depended on the antioxidant used. For instance, the number of CFU of *S. limosus* RIA 1058^T was highest in the presence of dopamine and quercetin; the highest number of CFU of *S. cyaneofuscatus* RIA 1027 was obtained when using α-tocopherol; the number of CFU of *S. flavovirens* RIA 1038^T was highest in the presence of dopamine and α-tocopherol; the highest number of CFU of *S. massasporeus* RIA 1064^T was obtained when using dopamine and quercetin. These differences between the effects exerted by the studied antioxidants are probably due to the specific characteristics of their chemical composition and interactions with the cell structures of actinobacteria.

In this work, dopamine (10^{-5} M) was also used for resuscitation of actinobacteria (for instance, the lyophilized cultures of *S. almquistii* RIA 1207^T, and *S. chromogenus* RIA 1335^T) which we failed to activate by other methods. Moreover, the addition of dopamine to the liquid ISP1 medium during inoculation allowed us to revive *Parvopolyspora pallida* K-4113^T after 20-year storage under mineral oil.

In the course of our studies, we restored the viability and the main cultural and physiological properties of 361 strains of actinobacteria of the genera *Streptomyces* (342 strains), *Actinomadura* (1), *Actinoalloteichus* (1), *Amycolatopsis* (3), *Kibdelosporangium* (1),

Table 1. Effect of rehydrating liquids on the viability of the studied cultures of actinobacteria (CFU/mL)

No.	Species	Nos. of RIA strains	Rehydrating liquid	
			sterile tap water	NBRC liquid (Japan)
1	*Amycolatopsis orientalis*	1074^T	2×10^3*	1.2×10^3*
2	*Saccharopolyspora erythraea*	1387^T	1.8×10^4	2×10^4
		120^T	2.4×10^5	2×10^5
3	*Streptomyces albovinaceus*	1004^T	2×10^3	4×10^1
4	*S. anulatus*	1020	2×10^3	0.8×10^4
5	*S. aureofaciens*	1129^T	4×10^2	2×10^2
6	*S. bacillaris*	1448^T	4×10^3	2×10^1
7	*S. badius*	1010^T	1.8×10^3	2×10^3
8	*S. californicus*	1015^T	1.2×10^3	2×10^3
9	*S. candidus*	1131^T	1.8×10^3	1×10^3
10	*S. cinereoruber*	1021^T	2×10^2	1.8×10^1
11	*S. cyaneofuscatus*	1027^T	4×10^3	4×10^3
12	*S. cyanoalbus*	1150^T	4×10^2	4×10^1
13	*S. daghestanicus*	1028^T	1.8×10^2	1.2×10^2
14	*S. eurythermus*	1030^T	1.6×10^2	0.8×10^2
15	*S. felleus*	1033^T	4×10^3	4×10^3
16	*S. flavotricini*	1037^T	1.8×10^2	1.8×10^2
17	*S. flavoviridis*	1039^T	4×10^2	2×10^2
18	*S. fradiae*	1040^T	2×10^2	0
19	*S. glaucescens*	1041^T	1.8×10^2	2×10^1
20	*S. griseobrunneus*	1042^T	1.8×10^2	2.2×10^2
21	*S. griseolus*	1044^T	3×10^2	4×10^2
22	*S. halstedii*	1050^T	1.4×10^2	1.8×10^2
23	*S. kurssanovii*	1054^T	1×10^2	0
24	*S. lavendulae*	1057^T	4×10^3	1.4×10^3
25	*S. limosus*	1058^T	1×10^3	$`1.2 \times 10^3$
26	*S. macrosporeus*	1062^T	1.8×10^2	1.6×10^2
27	*S. massasporeus*	1064^T	4×10^3	1×10^3
28	*S. microflavus*	1059	2×10^3	6×10^2
29	*S. michiganensis*	1065^T	1×10^3	1.2×10^3
30	*S. mutabilis*	1068^T	1×10^3	0
31	*S. narbonensis*	1069^T	1.2×10^2	1.8×10^1
32	*S. nitrosporeus*	1071^T	2×10^2	1×10^2
33	*S. olivaceus*	1073^T	1×10^2	0
34	*S. parvulus*	1075^T	1×10^3	1.2×10^2
35	*S. pilosus*	1076^T	1×10^2	0
36	*S. pluricolorescens*	1077^T	4×10^2	4×10^2
37	*S. prasinopilosus*	1078^T	1×10^2	1×10^2
38	*S. prasinus*	1079^T	2×10^2	0
39	*S. puniceus*	1080^T	2×10^2	0
40	*S. ramulosus*	1081^T	2×10^3	2×10^3
41	*S. recifensis*	1082^T	4×10^2	2×10^1
42	*S. roseofulvus*	1084^T	2×10^2	2×10^2
43	*S. rubiginosohelvolus*	1136^T	6×10^2	0
44	*S. rutgersensis*	1089^T	2×10^3	2×10^2
45	*S. sulphureus*	1306	4×10^3	2×10^3
46	*S. violaceoruber*	1096^T	2×10^3	1×10^2
47	*S. virginiae*	1097^T	2×10^2	4×10^1
48	*S. viridochromogenes*	1308	2×10^3	2×10^3
49	*S. xanthochromogenes*	1098^T	6×10^2	4×10^2
50	*S. xantophaeus*	1099^T	2×10^2	2×10^2

Note: * To compare the numbers of CFU, we used 1 ampoule of each of the strains stored for the same period of time.

Table 2. Effect of water solutions of *Callisia fragrans* sap on the viability of lyophilized cultures of actinobacteria stored for 28 years

Strain	CFU/mL*	% of the control (tap water)
Streptomyces abikoensis RIA 1179	$(3.5 \pm 0.15) \times 10^3$	140
S. aureoverticillatus RIA 679^T	$(1.0 \pm 0.2) \times 10^4$	286
S. hiroshimensis		
RIA 1052^T	$(2.3 \pm 0.16) \times 10^3$	153
RIA 1087	$(5.2 \pm 0.4) \times 10^3$	700
S. netropsis		
RIA 1036	$(3.25 \pm 0.1) \times 10^2$	250
RIA 1114	$(9.2 \pm 0.4) \times 10^2$	400
RIA 1184^T	$(1.6 \pm 0.2) \times 10^4$	200

Note: * To compare the numbers of CFU, we used 5 ampoules of each of the strains stored for the same period of time.

Table 3. Effect of antioxidants on the viability of lyophilized cultures of actinobacteria stored for 44 years

Culture	Control* CFU/mL	Viability (% of the control)			
		Antioxidants			
		adrenalin (epinephrine)	dopamine	quercetin	α-tocopherol
Streptomyces limosus RIA 1058^T	2.4×10^4	208	328	376	163
S. cyaneofuscatus RIA 1027^T	3.1×10^4	222	144	155	366
S. flavovirens RIA 1038^T	1.4×10^3	210	305	237	300
S. massasporeus RIA 1064^T	7.4×10^2	324	500	462	400

Note: * Sterile tap water.

Kitasatospora (1), *Micromonospora* (1), *Nocardioides* (1), *Nocardiopsis* (1), *Nonomuraea* (2), *Parvopolyspora* (1), *Saccharopolyspora* (3), *Saccharothrix* (1), *Streptoalloteichus* (1), and *Trichotomospora* (1).

DISCUSSION

According to the published data, lyophilized bacterial cultures can be stored for 5–32 years [25, 26]. Activities associated with the maintenance of strain collections of actinobacteria indicate that different groups of microorganisms differ in their ability to survive lyophilization and storage. Among mycelial actinobacteria, microorganisms with a low spore-forming capacity were found to be the least viable [10, 14]. As was reported earlier, lyophilized cultures of nonmycelial forms, including rhodococci, can be stored for 5–33 years [27].

The results obtained indicate that, after long-term storage in lyophilized state or under mineral oil, the majority of the studied collection cultures of actinobacteria can be resuscitated using various methods at the activation and cultivation stages.

Damage to the cells caused by complete or incomplete anabiosis is not necessarily lethal. Most cells in the population are likely to be damaged, and their resuscitation depends on the activation conditions. Disruption of the membrane barrier functions of these cells may occur during activation, resulting in the release of vital metabolites, which leads to a decrease in the metabolic resources of the cells, impaired protein synthesis, and changes in the DNA structure [28]. These disturbances may be due to osmotic shock occurring during rehydration of lyophilized cultures, as well as due to the oxidative stress resulting from accumulation of oxidative damages during recovery of the cells from anabiosis and their subsequent activation. Many processes occurring in prokaryotic and eukaryotic cells, such as protein phosphorylation (including that of regulatory proteins) or activation of transcription factors and their binding to the regulatory DNA sites, are controlled by physiological changes in the intracellular redox balance, including the SH–SS exchange [29]. Changes in the redox balance, a typical indicator of the ratio between the reduced and oxidized SH-groups of microbial metabolites, due to a malfunction in the cellular antioxidant systems, may induce oxidative stress. It is well known that many thiol-containing proteins located on the cell surface may undergo redox transformations in response to changes in the redox status. These proteins include receptors, enzymes, and elements of the transport systems of cells. Changes in their activity may affect cell proliferation, differentiation and apoptosis. The relationships between the redox state of cells and phases of the cell cycle, and activity of the regulatory factors controlling the developmental cycles of the cultures [29] are presently determined. It is known that the developmental cycles of actinobacteria, particularly streptomycetes, are controlled by 65–66 different sigma factors of RNA polymerase [30, 31]. It is assumed that sigma(B) factor which, by activation of related sigma factors, induces adaptive response, plays a key role in the regulation of the osmotic and oxidative stresses, as well as in cell differentiation [30]. The stress response of streptomycetes to oxidative (thiol-disulphide) stress is controlled by the sigmaR-RsrA system. The formation of an intramolecular disulphide bond is a signal activating sigmaR. The SigR regulon consists of many structural and regulatory genes (controlling the redox balance) of the thiol-reducing system in the genomes of streptomycetes and some other actinobacteria [30, 31]. It is also assumed that, in these microorganisms, the sigmaR factor serves as a checkpoint control regulating the inhibition of spore formation under oxidative stress [31].

Long-term storage, as well as oxidative stress resulting from respiratory imbalance during reactivation of actinobacteria, may cause a malfunction in the redox homeostasis in cells, which may lead to disruption of the cell development and to cell death. It is notable that many strains of actinobacteria activated after long-term storage showed impaired morphogenesis and pigment formation. Redox imbalance in the activated cells may also result in the high level of the population heterogeneity due to an increase in the number of minor variants, including those characterized by impaired ability to produce mycelium at the stage of cell differentiation.

Under oxidative stress, application of antioxidants is justified due to the fact that the cellular antioxidant mechanisms may be damaged after long-term storage. Increased viability of the studied cultures indicates the possible compensational effect of these compounds used at the stage of cell activation. The data obtained demonstrated that dopamine could be highly efficient when it comes to resuscitation of the cultures with a low ability to survive long-term storage. The revealed growth-promoting and stabilizing effect of this compound [24] makes it possible to assume that dopamine may act as an inducer of various signal pathways in which the regulatory elements of cell proliferation and differentiation are involved.

It is notable that the actinobacteria stored for long periods as soil cultures lost their viability. Soil is a complex natural substrate containing essential elements necessary for actinobacteria, which can explain the efficiency of subculturing on soil for restoration of the cultural and morphological properties of actinobacteria that had been lost during storage. However, during long-term storage of soil cultures, the destructive processes occurring in cells can result in their death.

It was found that, as a result of long-term storage of actinobacteria in lyophilized state and under mineral oil, the majority of the studied cultures were damaged. The resuscitated actinobacteria were characterized by low levels of cell proliferation and impaired cell differentiation. The redox imbalance is one of the reasons

for the fact that the cells were found to be dead or damaged after long-term storage. Under oxidative stress, addition of exogenous antioxidants and growth-promoting compounds during the process of cell resuscitation may exert the regulatory and protective effect, which increases the viability of actinobacteria and intensifies the processes of their metabolism. Further studies of the activation mechanisms of actinobacteria will make it possible to use the adaptive capabilities of these microorganisms to optimize the preservation methods.

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