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EXPERIMENTAL ARTICLES

Factors Inducing Transition from Growth to Dormancy in Rhizobacteria *Azospirillum brasilense*

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Abstract—The factors suppressing division of the cells of the rhizobacterium *Azospirillum brasilense* and inducing their transition to a dormant state were analyzed. These included the presence of hexylresorcinol or heavy metals (Cu and Co) in the medium, oxygen stress, and transfer of the cells into the physiological saline or phosphate buffer solution. The results were used to develop a protocol for obtaining of nonculturable cells of *A. brasilense* Sp245, a natural symbiont of wheat. The cells lost their ability to grow on synthetic agar medium, but could revert to growth when incubated in freshly prepared liquid medium. Needle-shaped crystals differing from struvite, which has been previously reported for this strain, were found in the dormant culture of *A. brasilense* Sp245.

Keywords: Azospirillum brasilense, stress, hexylresorcinol, copper, cobalt, dormancy in bacteria

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Abundant data have been accumulated confirming that most of bacterial cells in natural habitats are usually in a state of proliferative quiescence, i.e., in the state alternative to intense cell division [1]. In dormant bacterial forms, apart from division, the metabolic activity is also suppressed; their ultrastructure is different from that of the vegetative cells (dividing or recently divided ones). Transition into a dormant state results in numerous intracellular rearrangements, which facilitate their survival under stresses of various nature, including cold winters [2]. Investigation of the molecular signals and factors inducing a change in the physiological state (from dormancy to growth and vice versa) is of interest for basic science and biotechnological practice.

Microorganisms at the deepest stage of suppression of their activity lose completely their ability to grow on optimal media, so that special conditions are required for their reversion to growth. Such forms are called viable nonculturable cells (VNC). Progress in the understanding of bacterial transition into different physiological states is hindered by the absence of a unified experimental protocol, which could have made it possible to suppress growth of any microorganism completely and reversibly. For each species, fitting of the factors inducing VNC formation is required.

Non-spore-forming bacteria *Azospirillum brasilense* colonize the root system of higher plants [2–5]. For them, rapid transition from winter persistence to growth is an important survival factor. Microbial colonization of plant roots is known to be a highly competitive process. For azospirilla to successfully colonize the rhizosphere, they should commence active division and reach high population density by the time of root emergence.

For *A. brasilense*, however, conditions were described when growth was suppressed, although easily restored after inoculation into optimal media [2–3]. Division of azospirilla and other microorganisms is inhibited at unfavorable pH and temperature, as well as in the presence of heavy metals [6–8] and alkylresorcinols [9–12] or after prolonged maintenance without transfer to fresh media [3]. For some *A. brasilense* strains, capacity for long-time persistence (survival of unfavorable conditions), including dryout of the culture, was shown [2, 3, 13]. Although azospirilla have been under study for over 30 years, the factors causing complete and reversible loss of their capacity for division were not revealed for any of 16 *Azospirillum* species.

The goal of the present work was to determine the factors inducing deep inhibition of cell division in *A. brasilense* and causing the transition of the cells into a dormant state, which is accompanied by their loss of ability to grow on solid or liquid nutrient media.

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MATERIALS AND METHODS

The subject of the study were bacterial strains *A. brasilense* Sp245 and SR75, natural symbionts of wheat (*Triticum aestivum*), from the collection of Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (Saratov, Russia). Bacteria were grown at 31°C on solid or in liquid synthetic malate medium (SMM) containing the following (g/L): $K_2HPO_4 \cdot 3H_2O$, 3.0; KH_2PO_4, 2.0; NaCl, 0.1; $NaMoO_4 \cdot 2H_2O$, 0.002; NaOH, 2.24; malic acid, 3.76; NH_4Cl, 0.5; yeast extract, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.02 (as a chelate with nitrilo-3-acetic acid); pH 6.8. Early stationary phase cultures were used as inocula in all experiments. The initial cell density in liquid cultures was adjusted to 10^8 cells/mL. Cell density of the inocula was determined using the turbidity standard. Microbiological purity of the cultures was confirmed by light microscopy (Olympus C011, Japan).

To study the effect of stress factors on *A. brasilense* growth, the tested compounds ($CuSO_4$, $CoCl_2$, or 4-*n*-hexylresorcinol) from stock solutions were added to the autoclaved liquid SMM medium. The 4-*n*-hexylresorcinol stock solution (1 or 0.1 M) was prepared by dissolving the reagent (Sigma) in ethanol. For initial assessment of the effect of hexylresorcinol on growth of *A. brasilense* Sp245, its concentrations of 0.01, 0.1, and 0.5 mM were used. Bacteria were grown under aerobic conditions on a shaker (180 rpm) at 31°C for three days.

To test the effect of individual compounds and their mixtures on the growth of *A. brasilense* SR75, the following concentrations were used: 0.3 or 0.4 mM for $CuSO_4$ and $CoCl_2$ and 0.2 or 0.3 mM for hexylresorcinol. To create starvation conditions, bacteria were incubated in the physiological saline (0.85% NaCl) or in phosphate buffer saline containing the following (g/L): NaCl, 8.5; $K_2HPO_4 \cdot 3H_2O$, 1.34; KH_2PO_4, 0.39; pH 7.4. Both solutions contained also the following (g/L): $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02. The cultures were incubated under static conditions for 41 day at 31°C. Bacterial suspensions in saline or buffer were incubated for 11 months.

Complex stress conditions were created by transferring the cells of *A. brasilense* Sp245 into the physiological saline with 0.2 mM hexylresorcinol and 0.4 mM $CuSO_4$, as well as $MgSO_4$ and $CaCl_2$ according to the composition of the medium. Prior to inoculation (10^6 cells/mL), bacteria were washed three times using a MiniSpin centrifuge (Eppendorf, Germany; 10000 *g*, 10 min). The flasks were incubated on a shaker for 11 days (180 rpm, 31°C) and then for 6 months in the static mode at room temperature. For reactivation, the stressed culture was inoculated (5%) into the fresh SMM medium.

The state of bacterial cultures was assayed by measuring their OD_{595} on a Spekol 221 spectrophotometer (Carl Zeiss, Germany). The number of viable cells was determined by the standard procedure of colony-forming units (CFU) enumeration on solid SMM.

Crystal formation in the cultures was studied by light and electron microscopy. The shape and size of the crystals were determined using the Leica DM2500 and Leica DM6000B microscopes (Germany) equipped with ×40 and ×100 lenses. Transversal size of the crystals was determined under a Libra 120 transmission electron microscope (Germany) on Formvar-coated metal grids (100 perforations per 1 mm^2).

Statistical treatment of the data was carried out using the Microsoft Office Excel 2003 and Microsoft Office Excel XP software. Confidence intervals were determined for the 95% confidence level.

RESULTS AND DISCUSSION

Effect of hexylresorcinol on *A. brasilense* growth was determined at two oxygenation modes: with (Fig. 1) and without aeration (Fig. 2). In the first experiment, the sensitivity of azospirilla to this phenolic lipid was determined in a broad range of its concentrations (0.01–0.5 mM) with a relatively short cultivation time (36 h).

In the presence of 0.01 mM hexylresorcinol (Fig. 1, curve *2*), growth of *A. brasilense* Sp245 was not inhibited: OD of the culture did not differ from the control values. Moreover, at 15–17 h of cultivation they were even higher than in the control. Higher hexylresorcinol concentration (0.1 mM) resulted in a longer lag phase. Afterwards, the culture grew similar to the control one, reaching the same maximal density 7 to 8 h later (Fig. 1, curve *3*). When hexylresorcinol concentration was increased to 0.5 mM, bacterial growth was completely suppressed (Fig. 1, curve *4*). The cells did not form colonies when plated on agar medium (CFU = 0). Hexylresorcinol in concentrations of 0.01 and 0.1 mM had no effect on the colony-forming capacity: 3-day experimental cultures contained the same number of cells as the control ones: on average $(1.8–2.8) \times 10^9$ cells/mL.

In the subsequent experiments, hexylresorcinol was added to the medium for *A. brasilense* SR75 at the concentration of 0.2 or 0.3 mM. The state of the culture was monitored for over a month (Figs. 2a, 2b). In all cases, azospirilla were found to overcome the effect of the alkylresorcinol. Thus, during the first two days of cultivation with 0.2 mM hexylresorcinol, OD_{595} was lower than in the control (Fig. 2a). After day 5, the optical density of the experimental culture was the same as or higher than that of the control, i.e., the insignificant inhibitory effect of hexylresorcinol was completely overcome. In the presence of 0.3 mM hexylresorcinol the picture was similar (Fig. 2b). Its

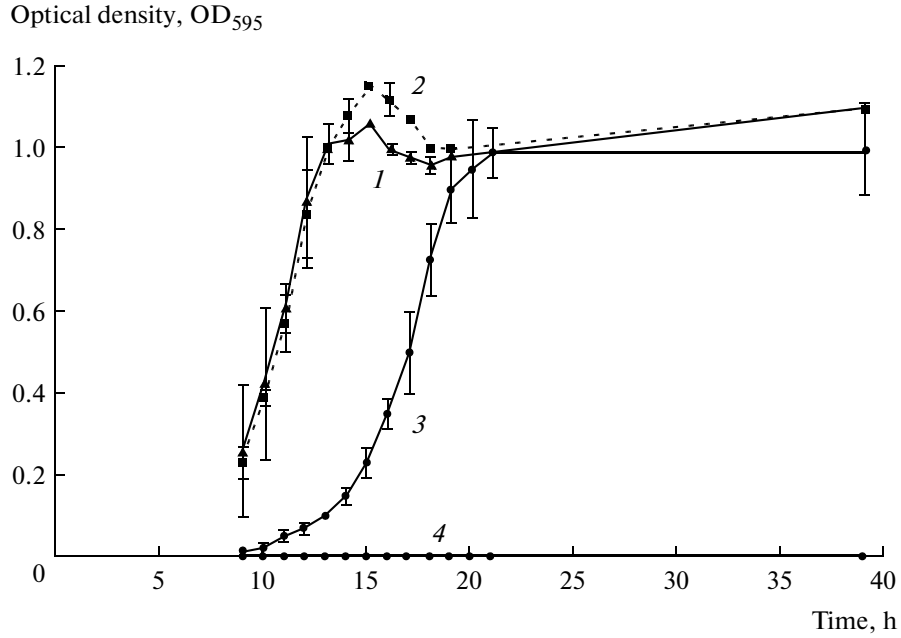

Fig. 1. Growth of *A. brasilense* Sp245 under aerobic conditions in SMM medium without (*1*) or with hexylresorcinol: 0.01 (*2*), 0.1 (*3*), and 0.5 mM (*4*).

higher concentration resulted in a longer lag phase (up to 5 days).

Since high concentrations of heavy metals are known to have a cytotoxic effect, two of them (copper and cobalt) were included in the program of analysis of the stress factors modulating the growth of azospirilla. Primary assessment of toxicity on solid media revealed that 0.1 mM $CuSO_4$ or $CoCl_2$ in the medium was sufficient to suppress growth of *A. brasilense* Sp245, while the concentrations of 0.1–0.4 mM were toxic, but not lethal [6].

Growth-inhibiting effect of the Cu^{2+} and Co^{2+} ions (0.4 mM) was also observed in azospirilla cultures in liquid medium (Figs. 2c, 2d). *A. brasilense* SR75 was more sensitive to copper ions (Fig. 2c) than to cobalt ions (Fig. 2d). A decrease in the concentration of these salts to 0.2 mM did not decrease their toxicity (data not shown).

The negative effects of 0.4 mM $CuSO_4$ were also determined in the presence of 0.2 mM hexylresorcinol. At this concentration, the latter had an insignificant effect on the growth characteristics of the bacterium (Fig. 2a). The combination of two factors resulted in a more pronounced suppression of *A. brasilense* growth (Fig. 2e) than $CuSO_4$ alone (Fig. 2c). In this variant, the most pronounced decrease in cell viability was observed: on solid media, the colonies were either absent (CFU = 0) or sporadic. The effect of the Cu^{2+} ions in the combination with hexylresorcinol induced phenotypic phase variation in *A. brasilense* SR75: the few colonies formed had diverse morphology not typical for *Azospirillum*.

The last two variants of the experiment, prolonged (6 months) starvation in the physiological saline (Fig. 2f) and in phosphate buffer saline (data not shown), yielded somewhat unexpected results. In both cases, from day 5 on, the culture exhibited very low (close to zero) OD_{595} values. Exclusion of the nutrients from the medium did not result in the death of azospirilla: after 6 months the cultures of *A. brasilense* SR75 retained high viability (up to 10^6 CFU/mL). Similar results were obtained with *A. brasilense* Sp245, another natural wheat endophyte: the number of colony-forming cells remained relatively high (up to 10^5 CFU/mL) throughout 11 months of the experiment.

Based on the data obtained, the experiment was carried out in order to investigate the response of *A. brasilense* Sp245 to a complex of stress factors: substitution of the physiological saline for the growth medium, addition of growth-hampering compounds (0.2 mM hexylresorcinol and 0.4 mM $CuSO_4$), oxygen stress (shaker), removal of the intercellular communication factors by washing the cells prior to their introduction into the saline, and a decrease of the initial density of the bacterial suspension to 10^6 cells/mL. The state of the culture was ascertained by inoculating the liquid and solid SMM medium.

In the presence of a combination of stress factors the cells of *A. brasilense* Sp245 were found to lose their capacity for growth on solid medium. Numerous platings of the stressed culture on SMM always resulted in the absence of bacterial growth. When inoculated into liquid medium, bacteria, however, resumed division. The efficiency of post-stress reactivation of

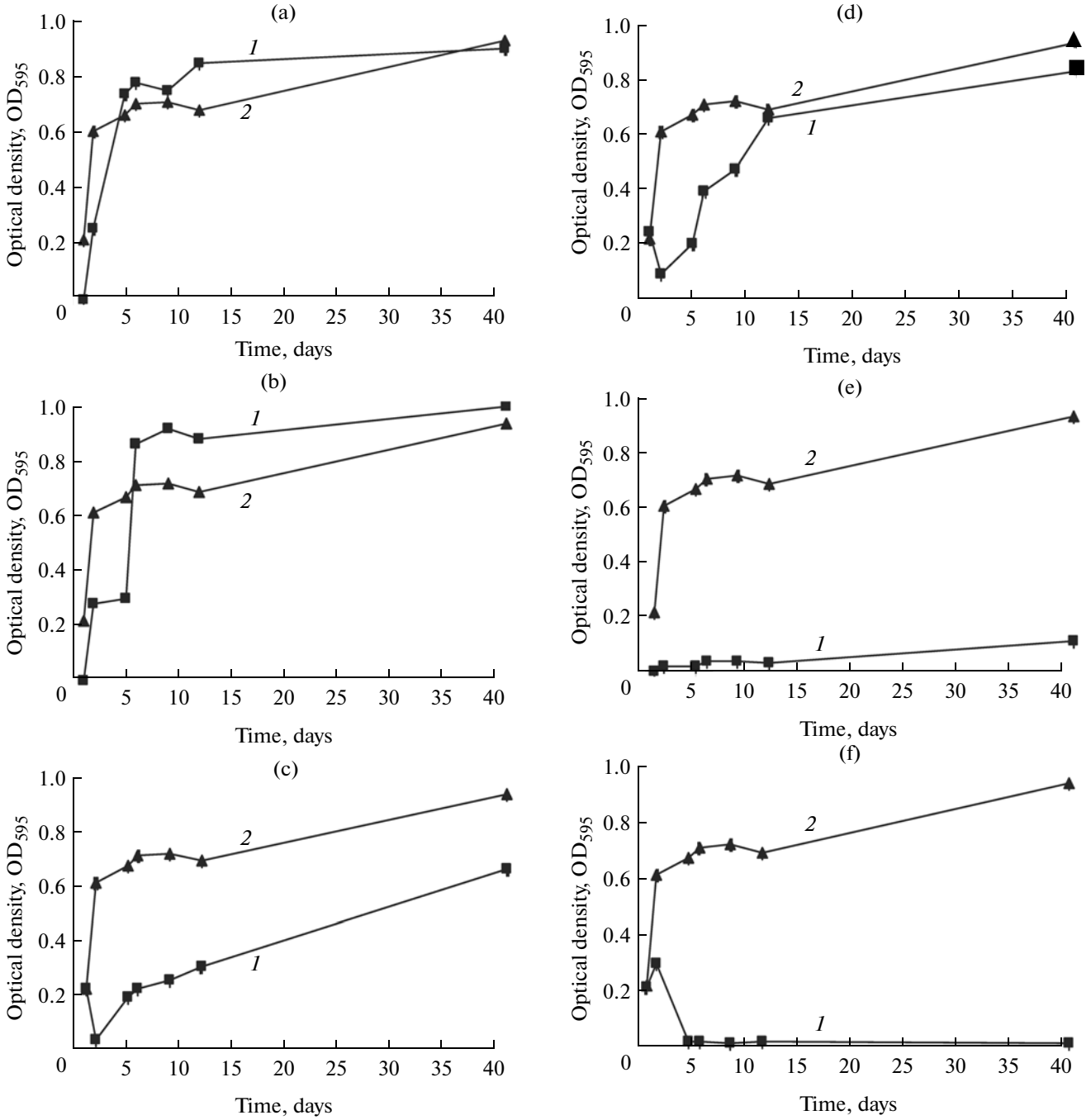

Fig. 2. Optical density of *A. brasilense* SR75 cultures growing under stress conditions (*1*) compared to the control (SMM medium, *2*): 0.2 mM hexylresorcinol (a); 0.3 mM hexylresorcinol (b); 0.4 mM $CuSO_4$ (c); 0.4 mM $CoCl_2$ (d); 0.2 mM hexylresorcinol + 0.4 mM $CuSO_4$ (e); and physiological saline (f).

A. brasilense Sp245 depended on stress duration and was different for the suspensions stored for 5 months or less and those incubated for 6.5 or 7 months (table).

In the first case, two days after inoculation into fresh medium the CFU number was typical of actively dividing azospirilla grown under optimal conditions. Similar treatment of longer stored suspensions (6.5 and 7 months) resulted in only 10–20 CFU/mL after 1–3 days (table, two lower lines).

To determine whether a subpopulation of slowly growing cells was responsible for resumed cell division after stress, liquid cultures of *A. brasilense* in fresh medium were observed for a month. After 17 days, at least 10^5 CFU/mL was revealed in fresh culture (plat-

Reactivation of the cells of a deeply stressed culture of *Azospirillum brasilense* Sp245 in liquid SMM medium (CFU numbers)

Age of stressed *A. brasilense* Sp245 cilture	Number of viable cells in 1-, 2-, and 3-day cultures, CFU/mL		
	1 day	2 day	3 day
5 months	3.3×10^4	1×10^9	8.8×10^{10}
6 months	0	0	20*
7 months	10–20*	–	–

0 stands for no growth; (–) stands for not determined; * indicates the data from individual repeats: one out of three (6-month culture) or two out of five (7-month culture), with no growth on other plates.

ing of a 6-month culture), while after 20–30 days growth could be observed visually (by turbidity).

Thus, in *A. brasilense* Sp245 stress induced by combined action of several stress factors resulted in transition of the cells to a dormant state. Inoculation of 5-month stressed culture into fresh medium resulted in relatively rapid restoration of growth. Longer incubation (6 to 7 months) caused transition of bacteria into a nonculturable state. Only a small fraction of the cells could be reactivated after transfer into fresh medium; these cells probably originated from a clone of slow-growing azospirilla.

In the deeply stressed culture of *A. brasilense* Sp245 needle-shaped crystals were observed. The crystalline nature of these formations was confirmed by polarized interference microscopy. The size of the crystals varied significantly, reaching 50 μm (light microscopic measurements). Their transverse size measured by transmission electron microscopy was 1.5 μm.

As was already mentioned, the conditions resulting in complete and reversible suppression of growth in *Azospirillum* have not been determined. This made it practically impossible to investigate transitions between the nonculturable state and physiological activity in this biotechnologically important group of bacteria. Our experiments revealed the conditions creating deep stress in *A. brasilense* associated with inhibition of cell division and transition into the dormant state and subsequently into the nonculturable state. These data were used to develop the techniques for complete and reversible inhibition of the metabolic activity (as determined by the absence of ability to grow on agar medium).

The proposed approach for obtaining nonculturable *A. brasilense* cells was based on combined action of a complex of stress factors (starvation, removal of the extracellular inducers and adaptogens by washing of the cells, and excessive aeration) together with a toxicant (Cu^{2+} ions), potentiated by the action of hexylresorcinol, which acts as an anabiosis inducer in microorganisms [9, 14, 15]. Similar to plants, microorganisms are known to synthesize and excrete alkylresorcinols as a mixture of isomers and homologues [12]. The biological activity of these compounds depends on the length and position of the alkyl radical, as well as on their concentration in the environment [9, 10, 12].

At 0.5 mM, hexylresorcinol completely inhibited cell division in *A. brasilense* Sp245 (Fig. 1), which agreed with the data on other microorganisms [9, 10, 15]. According to the literature data, lower concentrations of this phenolic lipid induced transition of microbial cells to the anabiotic state [10, 15]. Still lower concentrations either had no effect on division of *Azospirillum* cells (0.01 mM), or their effect was overcome by bacteria (0.1, 0.2, and 0.3 mM) (Figs. 1 and 2a–2c). The molecular mechanisms used by azospirilla to neutralize the growth-inhibiting effect of nonlethal concentrations of hexylresorcinol remain unknown. Detoxication is probably carried out by phenol oxidases, which oxidize it to inactive forms similar to the degradation of other phenolic compounds. While production of phenol oxidases by a number of *A. brasilense* strains has been confirmed experimentally [16], their involvement in bacterial adaptation to the presence of phenolic lipids in the medium requires additional investigation.

Copper and cobalt compounds (0.3 and 0.4 mM) were used as the toxicants affecting the growth of *A. brasilense* SR75. At low concentrations, both elements are essential for life, while at high concentrations both are toxic. Our results (Figs. 2c, 2d) and the published data [6, 7] show that growth of *A. brasilense* is inhibited by 10^{-4} M and higher copper and cobalt concentrations. The sensitivity to Cu^{2+} and Co^{2+} depended on *A. brasilense* strain and cultivation conditions, which is in agreement with accepted concepts concerning the reactions of microorganisms to unfavorable factors.

Combined action of two factors, hexylresorcinol (0.2 mM) and $CuSO_4$ (0.4 mM), i.e., in concentrations at which any of these agents alone had little or no effect on bacterial growth, resulted in significant inhibition of *A. brasilense* SR75 growth. This effect may be due to the stress-potentiating effect of hexylresorcinol, which suppresses the physiological activity of the cells and thus makes them more sensitive to toxicants. The combination of hexylresorcinol with a number of stress factors (starvation, Cu^{2+} ions, etc.) was used to create compound stress in *A. brasilense* Sp245, resulting in their loss of ability to grow on solid medium, i.e., in transition to the nonculturable state.

It should be mentioned that $CuSO_4$ concentration inducing VNC formation in *Escherichia coli* was determined [8]. Although the molecular bases of the inhibitory effect of copper cations on bacterial cells are partially known, specific details of the mechanisms

of growth inhibition in *E. coli, A. brasilense*, and other bacteria remain unknown.

Transfer of azospirilla cells into the physiological saline or phosphate buffer saline resulted in good adaptation of both wheat symbionts, *A. brasilense* SR75 and *A. brasilense* Sp245 to starvation stress, with their ability to form colonies retained for a long time (6–11 months). In winter, azospirilla are known to inhabit cereal seeds [5], which contain nutrients in a physiologically unavailable state. Bacterial survival under such conditions results from their dormant state, as was shown previously [2] and confirmed by the results of the present work. Thus, *A. brasilense* strains possess well-developed molecular mechanisms for adaptation to the absence of nutrients, in agreement with the ecological niche they occupy during the period of proliferative rest.

Importantly, starving (not growing) *A. brasilense* cultures retained high colony-forming capacity (at least 10^5 CFU/mL after 11 months of starvation) (Fig. 2e) Light microscopy of these variants (starvation in the physiological saline and in phosphate buffer saline) confirmed the presence of intact bacterial cells. Since the initial cell density was 10^8 cells/mL, it may be concluded that about 0.1% of *A. brasilense* cells were resistant to starvation stress. These may be persister cells which are thought to be always present in bacterial cultures [17]. It is considered that while most of the cells divide under optimal conditions, when persister cells do not divide (or grow at an extremely low rate), they are the ones to survive under stress conditions. It is also possible that only some of the cells retaining viability belongs to persisters, while the remaining ones are vegetative cells undergoing transition to a dormant state under the action of stress factors. Existence of persister cells in *A. brasilense* vegetative cultures remains an open issue. To our knowledge, no publications exist dealing with the subject, while the properties of dormant cystlike cells and condition for their formation have been studied [2, 3].

Interestingly, phenotypic phase variation was observed for *A. brasilense* strains SR75 and Sp245 in all experimental variants. It was especially pronounced when bacteria were grown in the media with hexylresorcinol and $CuSO_4$. This finding agrees with the previous data on the dormant forms of strains Sp245 and Sp7 [18]. Phenotypic phase variation, resulting from intragenomic rearrangements, broadens the adaptive capacities of bacteria under changed conditions and facilitates the preservation of the species.

Needle-shaped crystals were revealed in the culture of *A. brasilense* Sp245, which lost its capacity for growth on solid media. Their chemical nature was not investigated; it is probable, however, that they do not consist of struvite, which has been observed in the vegetative cultures of this strain [19]. In the case of *Azospirillum* cultures, struvite ($MgNH_4PO_4 \cdot H_2O$) contains a number of ions as isomorphic admixtures. The crystals reported in the present work differed in shape, while the medium did not contain phosphates and ammonium, which are required for struvite formation. Moreover, struvite is formed under alkaline conditions [19], while pH in the dormant culture of *A. brasilense* Sp245 decreased from 6.0 to 3.6 in the course of 2.5 months (i.e., by the time the crystals were observed).

Thus, our experiments made it possible to develop the protocol for obtaining nonculturable *Azospirillum* cells, which lost completely their ability to grow on synthetic agar medium but were able to resume growth after incubation in fresh liquid medium.

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