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EXPERIMENTAL ARTICLES

Morphological and Physiological Modifications of Cyanobacteria in Experimental Cyanobacterium–Actinomycete Associations

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Abstract—Associations of cyanobacteria and actinomycetes were formed experimentally from the cyanobacterium *Anabaena variabilis* ATCC 29413 and the streptomycetes isolated from apogeotropic roots of sago plants. Based on their phenotypic properties and the 16S rRNA gene sequencing, the streptomycetes were identified as representatives of *Streptomyces pluricolorescens* (strains 1 and 2). Cyanobacteria developing in monoculture and in association with an actinomycete were essentially different in their morphological and physiological–biochemical characteristics. In associations, cyanobacteria showed a higher (by tens of times) nitrogen-fixing activity compared to the monoculture and the morphological modifications of which were not observed in the monoculture (increase in cell size, increase in the portion of heterocysts among vegetative cells, appearance of the forms of unbalanced growth of cyanobacteria as giant, disc-shaped, curved, and rhomboid cells). At extremely low humidity (a_w 0.50), associated cyanobacterial cells remained viable, whereas in the monoculture, chlorophyll decomposition and cells death occurred. The methods of high-resolution (H^1 600 MHz) nuclear magnetic resonance (NMR) and pulsed-gradient spin echo NMR revealed a fraction of mobile protons in lyophilized samples of the cyanobacterium–actinomycete association, which was evidence of the presence of free water. This fraction was not found in the lyophilized samples of cyanobacterial and streptomycete monocultures. The revealed differences can explain the survival of cyanobacterial cells in associations.

Key words: association of streptomycetes and cyanobacteria, phylogenetic analysis, the fraction of mobile protons.

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Microbial communities with the involvement of cyanobacteria and actinomycetes are widespread in nature: cyanobacterial communities formed in soil bloom spots [1], cyano–bacterial mats of hydrothermal springs and lagoons [2], and algo–bacterial associations with lichenlike thallome (actinolichens) in sites of primary soil formation on carbonate sedimentary rocks [3, 4]. Symbioses of nitrogen-fixing cyanobacteria with eukaryotic organisms (protozoa, invertebrates, fungi, and plants) (syncyanoses) [5–7] and symbiosis of actinomycetes with plants (actinorhizas) [8, 9] and soil animals [3] are widespread everywhere.

The scientific literature contains numerous descriptions of associative relations between organisms in multicomponent systems, which are termed "associative symbiosis" [10–12]. In the recent decade, associative microorganisms have been revealed as components of most of the studied plant symbioses; however, no common opinion exists concerning their role in the process of formation, stable existence, and productivity of symbioses (leguminous–rhizobial, actinorhizas, syncyanoses, and mycorrhizas). An example of a multicomponent symbiosis is the syncyanosis of sago plants [10]. It has been established that the bacterial associative community in coralloid roots (infected with the dominant microsymbiont, i.e., cyanobacteria) of sago plants contains mostly hydrolytic bacteria, including actinomycetes [13]. The study of interaction between cyanobacteria and mycelial actinobacteria in natural multicomponent microbial communities is complicated.

Hence, the goal of our work was to investigate the effects of interaction between nitrogen-fixing cyanobacteria and actinomycetes isolated from natural syncyanosis in model associations.

MATERIALS AND METHODS

Research objects were an axenic culture of the free-living heterocyst-forming cyanobacterium *Anabaena variabilis* ATCC 29413 and actinomycetes isolated from apogeotropic roots of greenhouse sago plants *Stangeria eriopus* (G. Runtze) Nash and *Cycas micholitzii* Dyer (Tsitsin State Botanical Garden, Russian Academy of Sciences, Moscow).

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Actinomycetes were isolated from the ground apogeotropic sago roots by the method of "scattering" over agarized nutrient medium.

Phylogenetic position of the isolated actinomycetes was determined basing on 16S rRNA gene sequencing. DNA was isolated from actinomycetes according to [14]. Two independent preparations were obtained, with DNA concentrations of 30–50 μg/ml and RNA in trace quantities (less than 1%, according to the data of electrophoretic analysis). Polymerase chain reaction (PCR) was performed with a system of universal primers [15]. The PCR reaction mixture included primers, 25 pmol of each; 10× buffer, 2.5 μl; 2 mM dNTP, 2.5 μl; Bio*Taq* polymerase (Dialat, Moscow, 5 U/μl), 0.2 μl; DNA template, 50 ng; and H_2O, 25 μl. PCR conditions were as follows: denaturation, 94°C, 0.5 min; annealing, 45°C, 1 min; synthesis, 72°C, 1 min; final polymerization, 7 min; and number of cycles, 30.

PCR products were analyzed by electrophoresis in 2% agarose gel at an electric field voltage of 6 V/cm.

The similarity of the nucleotide sequences of 16S rRNA genes in the strains under study was analyzed using the BLAST software package. Unrooted phylogenetic trees of actinomycetes were constructed by the methods implemented in the MEGA 4.0 software package.

The monoculture of cyanobacterium *A. variabilis* was maintained on a Bergey's (BG-11) medium [16] under constant illumination (780 lx, 24 ± 1°C).

Experimental cyanobacterium–actinomycete associations were formed from 7-day mycelium of streptomycete grown in submerged culture on Gauze medium 1 [17] and cyanobacterium grown on BG-11 medium [18] for 3 weeks. The inoculum components were mixed (at a 1 : 1 biomass ratio) and the association was grown in liquid or on agarized BG-11 media in the static mode in a luminostat under constant illumination (780 lx, 24 ± 1°C).

The taxis of *A. variabilis* cells to streptomycetes was assessed by the modified method [18]. The spores and mycelium of the 14-day streptomycete culture grown on mineral agar 1 were placed in the center of a petri dish ($d = 3$ cm) with agarized BG-11 medium. Drops of *A. variabilis* suspension (10^6 cells/ml) were applied at a distance of ~2 cm on the four sides relative to the streptomycete inoculum. The taxis of cyanobacteria was judged by the value of the orientation coefficient (K_{or}) calculated as a ratio of the areas occupied by *A. variabilis* trichomes growing toward the streptomycete and in the opposite direction. The value of the orientation coefficient $K_{or} > 1$ demonstrated the positive taxis of the cyanobacterium to the streptomycete.

The nitrogen-fixing activity (NFA) of *A. variabilis* in monoculture and in associations with streptomycetes was determined on BG-11 medium without sources of bound nitrogen by the method of acetylene reduction [19]. The 21-day cyanobacteria in monoculture and in association with the streptomycete were used in the experiment.

Microscopic examination of cyanobacterium in monoculture and in associations with streptomycetes was performed with optical (Zeiss Axiostar, Germany), fluorescence (Zeiss Axioscop 2 plus), and scanning electron (Hitachi 405 S, Japan) microscopes. The samples to be studied in the scanning microscope were fixed according to [20]. Instrumental magnification was 60–20000, and accelerating voltage was 15 kV.

The presence of water in the lyophilized cyanobacterium–actinomycete association was assessed by high-resolution (^{1}H 600 MHz) NMR spectra [21] on an Avance 600 NMR tomograph (Bruker, Germany) at the Educational and Scientific Interfaculty and Interdisciplinary Center of Magnetic Tomography and Spectroscopy (Moscow State University) and by the curves of spin echo decline [22] of an ampouled sample (0.5 g) in a NMR-spin echo device (Germany). The working frequency of the NMR-spin echo device was about 20 MHz; the precision of measurement was 7%. To exclude the effect of diffusion, the time of spin–spin relaxation of protons (T_2) was measured by the Carr–Parcell–Meiboom–Gill method (90°-n 180° pulses) [23]. Lyophilized samples were obtained in a freeze-drier after their freezing at –20° and water sublimation in vacuum (10^{-3} Torr, 10 h).

RESULTS AND DISCUSSION

The actinomycete strains isolated from sago roots were classified within the genus *Streptomyces* based on their phenotypic characteristics and identified as *S. pluricolorescens* by the results of genotypic analysis. BLAST analysis of the sequenced 16S rRNA gene fragment of strains 1 and 2, corresponding to *E. coli* positions from 36 to 1450, confirmed the affiliation of strains under study with the genus *Streptomyces* of the actinomycete line of gram-positive bacteria. The analogous sequences of the *S. pluricolorescens* type strain were closest to the sequence obtained (Fig. 1).

Antagonistic activity between the studied cyanobacterial and streptomycete cultures was absent.

The streptomycete–cyanobacterium associations actively grew both in liquid and on agarized nutrient media. During the growth of associations formed by *A. variabilis* and *S. pluricolorescens* strain 1 in liquid medium under static cultivation conditions, coaggregates were formed as a result of braiding of streptomycete mycelium conglomerates with the cyanobacterial filaments. Formation of stable coaggregates consisting of intertwined actinomycete hyphae and cyanobacterial trichomes, which are difficult to destroy, has been mentioned previously [24].

The orientation coefficient (K_{or}) value of 2 demonstrated the presence of positive taxis of cyanobacteria to streptomycetes.

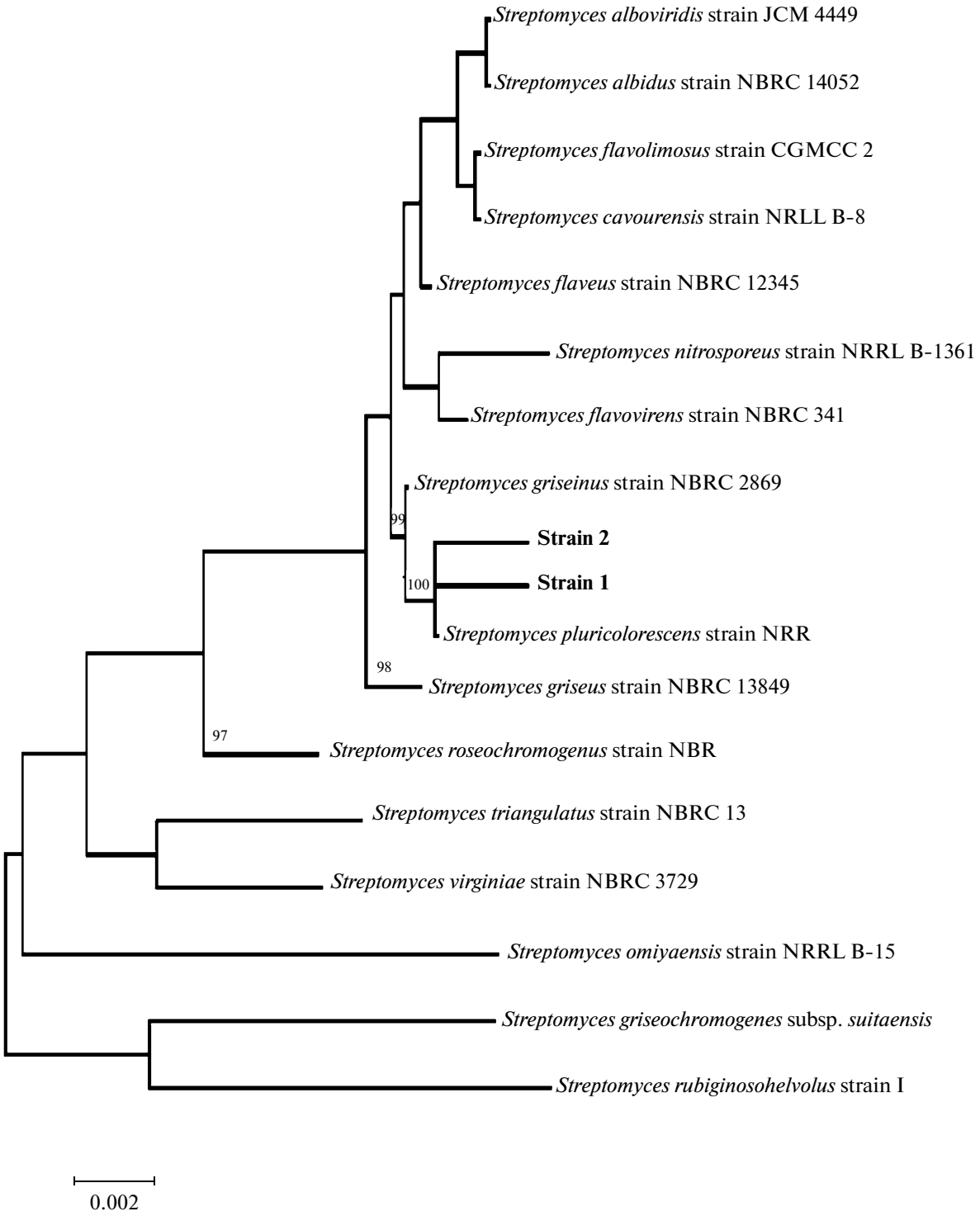

Fig. 1. Phylogenetic position of the studied actinomycete strains isolated from apogeotropic roots of sago plants. The scale shows the evolutionary distance corresponding to two nucleotide substitutions per every 100 nucleotides. The numerals indicate the reliability of branching established by bootstrap analysis of 100 alternative trees (values over 70 were considered as significant).

The development of cyanobacteria in association with streptomycetes stimulated their nitrogen-fixing ability. In experimental cyanobacterium–actinomycete associations, the nitrogen-fixing ability of the cyanobacterium was tens of times higher in monoculture (Fig. 2). It should be emphasized that the studied streptomycete strains have been isolated from apogeotropic roots of sago plants, i.e., syncyanosis, where

they are associative microsymbionts along with the dominant symbionts (nitrogen-fixing cyanobacteria).

Stimulation of the nitrogen-fixing activity of the cyanobacterium *A. variabilis* in association with streptomycetes correlated with an increased ratio of specialized nitrogen-fixing cells (heterocysts) in cyanobacterial filaments associated with the streptomycete *S. pluricolorescens* strain 1 compared to cyanobacteria in monoculture. The ratio (%) of heterocysts out of the average number of cells in cyanobacterial trichomes was 3.9 ± 2.4 in monoculture and 9.5 ± 2.9 in association with the streptomycete.

Thus, it was demonstrated that experimental associations formed of the cyanobacterium *A. variabilis* with streptomycetes isolated from the natural syncyanoses of sago plants (*S. pluricolorescens* strains 1 and 2) exhibited signs of symbiotic interaction between the streptomycete and cyanobacterial cultures, which is manifested by enhanced nitrogen-fixing activity of cyanobacteria.

Coaggregates of the model association of *A. variabilis* and *S. pluricolorescens* (strain 1) examined under the scanning electron microscope contained, together with the vegetative cells and heterocysts, the forms of unbalanced cyanobacterial growth as giant, disc-shaped, curved, and rhomboid cells (Fig. 3b–d). In the monoculture of *A. variabilis*, such morphologically modified cells were not found (Fig. 3a). In the coaggregates of associations, the portion of heteromorphic cells in cyanobacterial trichomes was up to 34% of their average number in a sample. In the cyanobacterium–actinomycete association, the mean ratio of the length of cyanobacterial cells to their width varied from 1.22 to 2.32, whereas in the monoculture this value was from 1.15 (the minimum) to 1.20 (the maximum). Overproduction of the mucous matrix as a fine-grained net with submerged cyanobacterial filaments and streptomycete hyphae was revealed on the surface of coaggregates in experimental associations, evidencing the appearance of specific associative morphostructure (Fig. 3d). In the presence of the surface mucous matrix, dissociation of such aggregates into components during repeated washing was impeded, suggesting propagation of microsymbionts in such forms for colonization of host plants under natural conditions.

Thus, the morphological features of cyanobacteria as a component of model cyanobacterium–actinomycete associations indicated modification, apart from nitrogen fixation, of other physiological properties of cyanobacteria determining the patterns of their functioning as a component of a symbiosis compared to free-living cyanobacteria.

It should be noted that the morphological modifications of cyanobacteria (shortening of filaments, increase in cell size, increase in the ratio of heterocysts among the total number of vegetative cells) have been observed both in natural symbioses with fungi (lichens) [6, 25] and higher plants [7] and in model

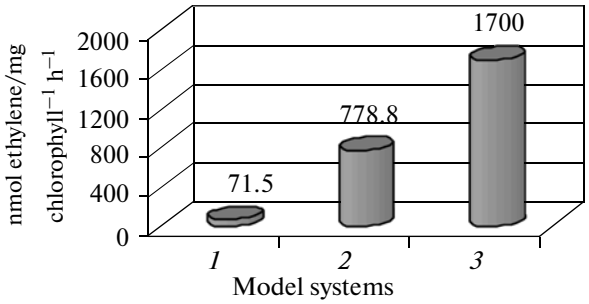

Fig. 2. Nitrogen-fixing activity of the cyanobacterium *A. variabilis* ATCC 29413 in monoculture (*1*) and in associations with *S. pluricolorescens* strain 1 (*2*) and *S. pluricolorescens* strain 2 (*3*).

associations with plant cell cultures during compartmentation within the tissues of macrosymbionts [26, 27]. In the course of associated growth, cyanobacteria also display the signs of disturbances in cell division. In the zones of localization of symbiotic cyanobacteria in plant syncyanoses, microsymbionts form cells specializing in overproduction of mucous substances. Extracellular polymers (mainly polysaccharides) are believed to play the structure-forming role in the intercellular metabolite transport [7, 28]. Such modifications of cyanobionts are considered typical of their natural symbioses with higher plants. The similarity of morphophysiological modifications of cyanobacteria in the constructed model associations and in natural systems suggests that close contact between cyanobacteria and actinomycetes and the influence of actinomycete metabolites are required for effective functioning of cyanobionts.

The adaptive changes in cyanobacteria revealed during their interaction with actinomycetes in model associations probably resulted from the long-term coevolution of organisms potentially capable of forming symbioses.

One more adaptive advantage of cyanobacteria associated with actinomycetes is preservation of their viability and functional activity on drying. Preservation of chlorophyll was shown for cyanobacterial cells in coaggregates of the experimental association stored for 3 weeks under low humidity conditions (a_w 0.50); this was evidenced by autofluorescence of cyanobacterial cells observed in the fluorescence microscope. Under the same conditions in monoculture, the fluorescence of cyanobacterial cells was not observed in most cases due to chlorophyll degradation. The number of fluorescent cyanobacterial cells in the association and in the monoculture was about 76 and 8.7% of all cells in the preparation, respectively.

Using nuclear magnetic resonance (high-resolution NMR and NMR-spin echo), we demonstrated that lyophilized samples of the cyanobacterium–actinomycete association formed by *A. variabilis* and the streptomycete *S. pluricolorescens* (strain 1) contained

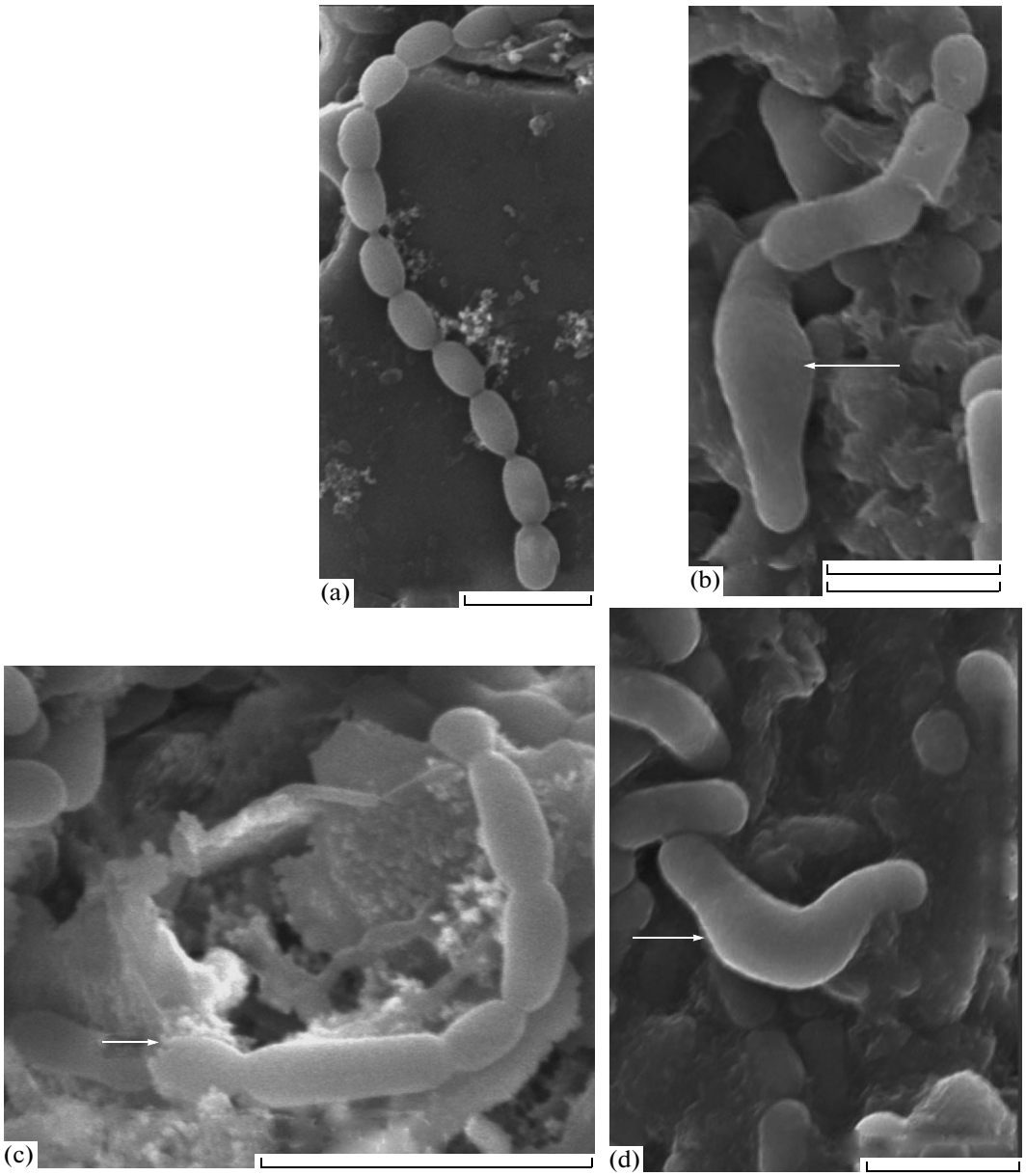

Fig. 3. Morphological forms of the cyanobacterium *A. variabilis* ATCC 29413 in monoculture (a) and unbalanced growth forms of the cyanobacterium *A. variabilis* ATCC 29413 associated with the streptomycete *S. pluricolorescens* strain 1. The arrows mark rhomboid (b), giant (c), and curved (d) cyanobacterial cells. Scale bar on the photographs: 5 μm.

a fraction of mobile protons (2.7% of sample weight, $T_2 = 16$ ms), which is preserved at low temperatures: a chemical shift of about 5 ppm from tetramethylsilane in the proton resonance spectrum of a lyophilized association sample (Fig. 4) is typical of free water protons. Free water was absent in lyophilized cells of the streptomycete and cyanobacterial monocultures used in this work to form cyanobacterium–actinomycete associations.

The presence of mobile water in dried-out microorganisms is known to facilitate their adaptation to extreme environmental conditions [29]. For example, free water was not found in lyophilized monocultures of the streptomycete and the green alga *Chlorella vulgaris* with a very low CFU titer, while the presence of mobile water protons (about 3%) was registered in the viable lyophilized lichenlike thallome formed experimentally from the above organisms [30, 31]. The enhanced stress resistance of microorganisms developing as components of binary and more complex microbial consortia (biofilms) is a general pattern [32]. In this case, significant structural changes may be assumed to occur in cellular biopolymers under conditions of development of cyanobacteria in association with actinomycetes; these changes may be responsible

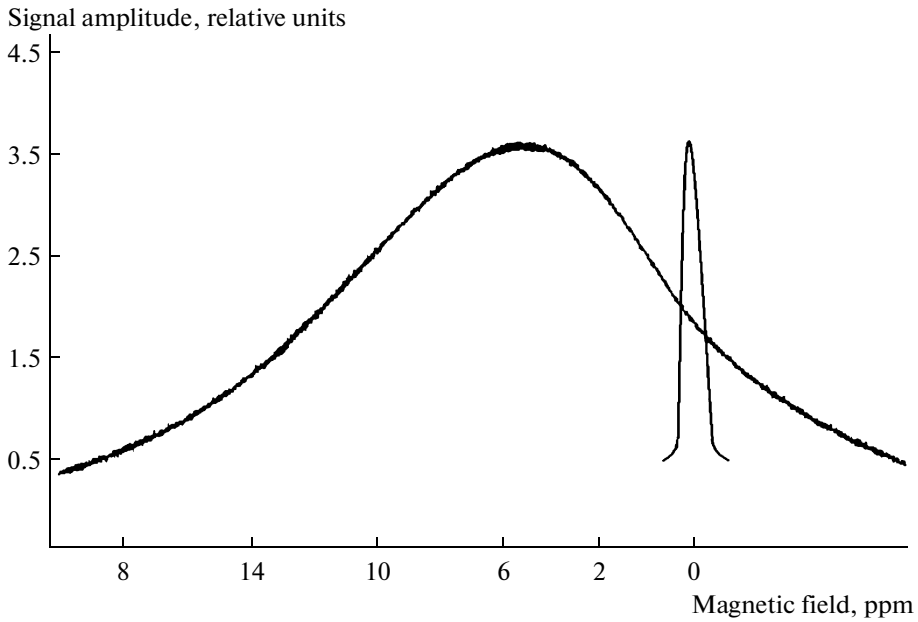

Fig. 4. High resolution-H^1 (600 MHz) NMR spectrum of the experimental cyanobacterium-actinomycete association. Lyophilized sample. Ppm, parts per million of magnetic field strength.

for the appearance of the mobile water fraction in lyophilized samples of associative thallome.

The data on the morphological and physiological modifications of cyanobacteria in a model cyanobacterium–actinomycete association demonstrate the symbiotic nature of interaction between its components: the heterocyst-forming cyanobacterium *A. variabilis* and the streptomycete *S. pluricolorescens* (strains 1 and 2) isolated from apogeotropic roots of sago plants, i.e., from a syncyanosis where streptomycetes were associative microsymbionts along with the dominant symbionts, nitrogen-fixing cyanobacteria. Actinomycetes as associative symbionts may have a positive effect on host plant development and on symbiosis as a whole owing to their stimulating effect on the nitrogen-fixing ability of cyanobacteria and to the enhanced protection of the symbiotic organism from pathogenic microorganisms due to secretion of antibiotics.

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