

Experimental Associations of Cyanobacteria and Actinomycetes*

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Abstract—Associations of cyanobacteria with actinomycetes are not being investigated. The purpose of this study is to investigate the biological aspects of coexistence of the free-living *Anabaena variabilis* with actinomycetes isolated from apogeotropic roots of *Strangeria eriopus* and *Cycas micholitzii*; with the cyanobacterium *Oscillatoria terebriformis* (Ag.) Elenk. emend., which were isolated from the natural cyanobacterial mat taken from the Kamchatkan thermal spring; and with actinomycetes isolated from the accumulating culture of cyanobacterium. Positive tropism of actinomycete hyphae to cyanobacterial trichomes and that of the cyanobacterium to streptomycetes were observed. Stimulation of growth of *O. terebriformis* in the associated culture with the streptomycete was recorded. The increase of fixation of nitrogen by *A. variabilis* and of photosynthetic activity of *O. terebriformis* in the associated culture with the streptomycete was recorded.

DOI: 10.3103/S0096392507010014

Communities involving cyanobacteria are widely distributed in nature. Examples of such communities can be found in the cyanobacterial mats of hydrothermal waters and lagoons, and in algocyanobacterial communities in the event of the “blooming” of the soil (Domracheva, 2005).

Communities of cyanobacterial mats were formed during the early stages of the development of the Earth, and have shown surprising resistance throughout the history of the world (Zavarzin et al., 1993; Zavarzin, 1995). Ancient analogs of recent cyanobacterial mats are stromatoliths, the oldest paleontological evidence of life on the Earth, which demonstrate that life originated and was supported in the form of communities (*Bacterial Paleontology*, 2002). These cyanobacterial communities flourished in inland water bodies during the Precambrian period and formed the initial biosphere through a stable turnover of matter (Zavarzin, 1997). They were the first colonizers of land and the predeterminers of life on Earth as well as the dynamics of all chemical and physical processes beginning more than 3 billion years ago.

At present the notion of the evolution of the organic world in the form of communities, i.e., systems of ecologically interconnected functional groups, is generally accepted. Moreover, isolated species cannot exist without the community for any extended period of time. This aspect demands an investigation not of pure cul-

tures of microorganisms, but of the entire community as an evolutionary unit of prokaryotes (Zavarzin, 1987).

Cyanobacterial communities are also widely distributed at the sites of primary soil formation. It is known that structures exist in a form reminiscent of mats (carpets) made of a thin, gelatinous layer covering up to 60% of the soil surface of fields during the autumnal period (Domracheva, 2005). The phototrophic nucleus of such communities consists of cyanobacteria, which is related to a wide assortment of saprophytic partners through metabolic relationships. Slimy sheaths of cyanobacteria are populated by an enormous quantity of various microorganisms that make use of slime, intravital excreta, and moribund bacterial cells. The close metabolic relationship between cyanobacteria and its oligonitrophilous companion bacteria is of a symbiotic nature (Domracheva, 2005). It is impossible to negate the existence of mycelial procaryotes within them.

On sites of primary soil formation—algal excrescences on cliffs with outcrops of carbonate—actinomycete mycelium is found in abundance where it forms associations with green algae similar to actinolichen (Zvyagintsev and Zenova, 2001). Presence of a saprotrophic complex is required condition in order for phototrophs to flourish.

Application of a traditional microbiological approach (the method of pure cultures) does not enable us to understand the autecology and synecology of these organisms. Such metabolic relationships cannot be modeled in pure cultures, but rather may be realized only in a natural consortium of microorganisms.

Actinomycetes are found in natural cyanobacterial communities and it is necessary to investigate experimental cyanomycete associations in order to reveal the

* Presented at the conference “Interrelations of Lower Plants (Fungi, Algae, and Lichens) with Other Plants in Biocenosis.” Department of Biology, Moscow State University, January 31–February 3, 2006.

Table 1. Associations of actinomycetes and cyanobacteria

Actinomycete	Cyanobacterium	Source of isolation of pairs of associations
<i>Streptomyces odorifer</i>	<i>Oscillatoria terebriformis</i>	Actinomycete from accumulation culture of <i>O. terebriformis</i> Cyanobacterium from the cyanobacterial mat from hydrothermal waters in Kamchatka
<i>S. olivaceoviridis</i>	<i>Anabaena variabilis</i> ATCC 29413	Actinomycete from apogeotropic roots of <i>Strangeria eriopus</i> (Cycadales) Cyanobacterium—museum culture
<i>S. xanthochromogenes</i>	<i>A. variabilis</i> ATCC 29413	Actinomycete from apogeotropic roots of <i>Cycas micholitzii</i> (Cycadales) Cyanobacterium—museum culture

functional role of mycelial procaryotes in these ecosystems.

The present study is aimed at the isolation of actinomycete cultures from the cyanobacterial mat and from coralloid roots of cycadales, as well as at the investigation of relationships with cyanobacteria in mixed laboratory cultures.

MATERIAL AND METHODS

The objects of our study were as follows: the culture of the cyanobacteria *Oscillatoria terebriformis* (Ag.) Elenk. Emend., which were isolated from the natural mat of hydrothermal waters in Kamchatka and supported in the laboratory over a span of several years; the culture of a free-living cyanobacteria *Anabaena variabilis* ATCC 29413 obtained from the museum of the Chair of Physiology and Microorganisms, Department of Biology, MSU; actinomycetes isolated from apogeotropic roots of hothouse cycadales *Strangeria eriopus* and *Cycas micholitzii* (Tsitsin Central Botanical Garden, RAS, Moscow) and identified as *Streptomyces olivaceoviridis* Strain 1, *S. xanthochromogenes* Strain 1; an actinomycete isolated from the accumulating culture of cyanobacteria *O. terebriformis* and identified as *S. odorifer* Strain 1. The listed cultures of cyanobacteria and streptomycetes were used as components of paired associations (Table 1).

Actinomycetes were isolated from the accumulating culture of *O. terebriformis* and from triturated apogeotropic roots of cycadales by inoculation and spreading (respectively) on agar nutrient medium. The isolation and cultivation of streptomycetes was done on Gauze-1 medium (Gauze et al., 1983).

The cultures of cyanobacteria were supported: *O. terebriformis*—on Zavarzin medium (Orleanskii and Gerasimenko, 1982); *A. variabilis*—on medium BG-11 (Stanier et al., 1971). Cyanobacteria were cultivated in liquid and solid nutrient media (the latter con-

tained 2% agar) at the temperature 24° and illumination 780 lx.

The mixed cyanobacterial cultures (Table 1) were produced on medium BG-11 of the seven-day-old mycelium of streptomycetes grown as submerged in medium G-1 and of cyanobacteria (ratio of biomass 1 : 1). Biomass of monocultures of cyanobacteria was preliminarily grown as follows: *O. terebriformis* on Zavarzin medium over three days and *A. variabilis* over the course of three weeks on medium BG-11.

The following parameters were used as criteria for associative interaction of streptomycetes and cyanobacteria in mixed cultures: (1) presence of positive tropism of the streptomycete to the cyanobacterium; (2) stimulation of growth of the cyanobacteria in association with the streptomycete; (3) discovery of the streptomycete on the surface and in deep layers of the experimental cyanobacterial mat; (4) intensification of photosynthetic and nitrogen fixing activity in association with the streptomycete; (5) modification of antimicrobial properties of the association in comparison with monocultures of streptomycetes and cyanobacteria.

(1) Presence of positive tropism of the streptomycete *Streptomyces odorifer* to the cyanobacterium *O. terebriformis* was determined by the following method: nucleopore filters (d 0.75) were placed with one bacterial loop of biomass of the culture of cyanobacteria grown in liquid medium on the freshly inoculated "lawn" of the streptomycete on lean agar. The control filters were left without cyanobacteria. The area occupied on the filter by the mycelium of streptomycete was counted under the microscope (400×), the filters having been preliminarily stained with carbolic erythrosine. The presence of positive tropism of the streptomycete to the cyanobacterium was estimated by the value of the association coefficient (K_{as}), which was calculated as the ratio of the area occupied by the mycelium of streptomycete penetrating to the filter with the cyanobacterium to the area occupied by the mycelium of strepto-

mycete penetrating to the filter without the cyanobacterium. At $K_{as} > 1$, we assumed that the streptomycete exhibits positive tropism to the cyanobacterium. At $K_{as} < 1$ there is no specific interaction.

Presence of positive action of the cyanobacterium *A. variabilis* on growth of streptomycetes *S. olivaceoviridis* and *S. xanthochromogenes* was recorded by intensive proliferation and surrounding of the colonies of cyanobacteria by streptomycetes for joint inoculation of monocultures as a "lawn" on Petri dishes with medium BG-11.

Presence of positive taxis of *A. variabilis* to streptomycetes *S. olivaceoviridis* and *S. xanthochromogenes* was determined by placing the biomass of the streptomycetes grown on medium G-1 at the center of the agar plate of medium BG-11, and placing the biomass of *A. variabilis* around the plate at a distance of approximately 2 cm from the inoculum of the streptomycete. The presence of taxis of the culture *A. variabilis* was estimated by the value of the orientation coefficient, i.e., by the ratio of the areas occupied by trichomes of cyanobacteria growing towards the streptomycete versus that growing in the opposite direction (Gorelova, 2005). The value of the orientation coefficient >1 demonstrated positive taxis of the cyanobacteria to the streptomycete.

(2) The growth of the cyanobacteria *Oscillatoria terebriformis* in the culture mixed with *S. odorifer* was assessed by placing the inoculate on the surface of clay minerals montmorillonite, caolinite, and bentonite, and comparing the radius of the spot of growth of the association to the monoculture of the cyanobacterium on the surface of the minerals.

The radial growth rate of colonies of streptomycetes *S. odorifer* on the medium with culture liquid of the monoculture of *O. terebriformis* (1% by volume of the medium) as a source of carbon was compared with the radial growth rate on media with starch, sucrose, and glucose. The control was the growth of colonies of streptomycete on the mineral medium G-1.

The diameter of colonies of streptomycetes *S. olivaceoviridis* and *S. xanthochromogenes* at proliferation through an entire "lawn" of *A. variabilis* in the case of instantaneous joint inoculation on agar medium BG-11 was compared with the size of colonies of the monoculture of streptomycetes.

(3) The streptomycete was detected on the surface and in deep layers of the cyanobacterial mat formed in the laboratory of the accumulating culture of *O. terebriformis* and *S. odorifer*. The experimental cyanobacterial mat was formed during one series of experiments of the mixed culture of *O. terebriformis*, and the streptomycete in another series of the cyanobacterium only. To do this, the inoculum of the mixed culture or of the monoculture of the cyanobacterium was placed on the agar support and a powder of CaCO_3 was poured over it. When the powder particles were overgrown by the cyanobacterium and a film had formed on the surface of

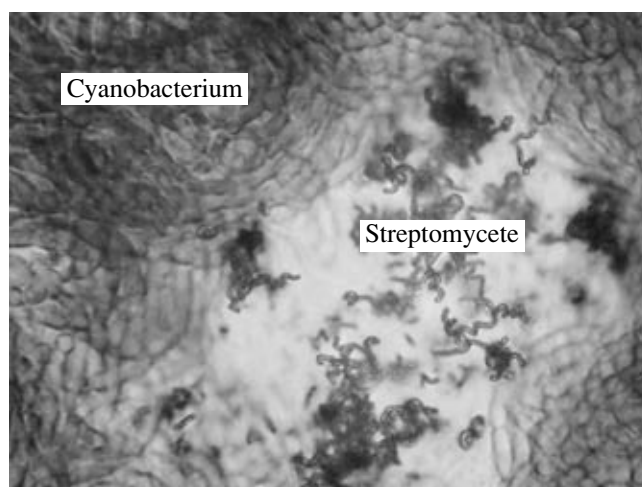


Fig. 1. Active growth of the cyanobacterium *Anabaena variabilis* and the streptomycete *Streptomyces olivaceoviridis* in association.

the powder, it was again dusted over with a layer of chalk, with the final formation of the mat consisting of eight to ten layers of the mineral alternated with layers of the cyanobacterium. Presence of the streptomycete on the surface and deep layers of the experimental cyanobacterial mat was determined by presence of colonies of *S. odorifer* on Petri dishes with medium G-1 inoculated with fragments of the material.

(4) The photosynthetic activity of the cyanobacterium *O. terebriformis* in association with the streptomycete *S. odorifer*, as well as in the monoculture, was determined by the quantity of chlorophyll *a* formed in the course of raising cultures in the light on a medium of Zavarzin over seven days (Fedorov, 1979).

(5) The antimicrobial properties of the association and its components—the streptomycete *S. odorifer* and the cyanobacterium *O. terebriformis*—towards the investigated test cultures of bacteria, streptomycetes, and yeast, were checked by the method of blocks (Egorov, 1980). The radius of the zone around the block of the tested culture in which there was an absence of growth of the test microbe was measured. In experiments to determine antibiotic activity, the mixed culture of the streptomycete and the cyanobacterium was preliminarily kept in the light for three to five days.

(6) The nitrogen fixing activity of the cyanobacterium *A. variabilis* in association with the *S. olivaceoviridis* and in monoculture was determined by acetylene reduction in a gas chromatograph (Hardy et al., 1973).

RESULTS AND DISCUSSION

The investigated cultures of streptomycetes *S. odorifer* Str.1, *S. olivaceoviridis* Str. 1, and *S. xanthochromogenes* Str. 1 did not manifest antagonism toward cyanobacteria *A. variabilis* (Fig. 1) and *O. terebriformis*.

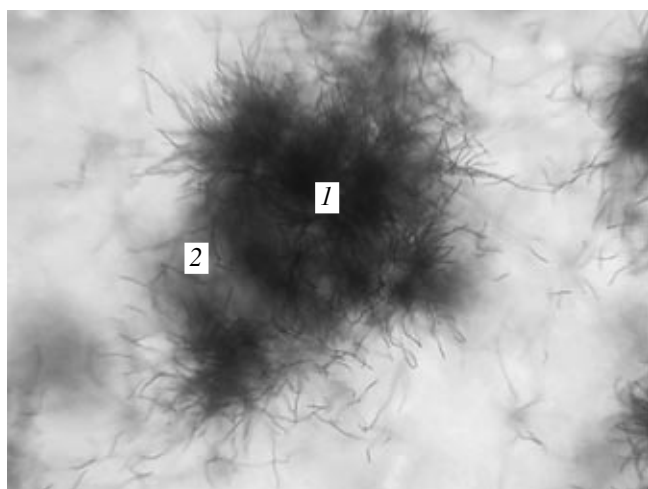


Fig. 2. Growth of colonies of the streptomycete *Streptomyces xanthochromogenes* (1) on the colony of the cyanobacterium *Anabaena variabilis* (2).

The hyphae of the streptomycete *S. odorifer* demonstrated a positive tropism to cells of the cyanobacterium *O. terebriformis*. The association coefficient (K_{as}) in the experimental variants with this streptomycete exceeded 1. The hyphae of the streptomycete penetrated the filter with the cyanobacterium and surrounded the filaments of the latter. The filaments of *O. terebriformis* and the hyphae of the streptomycete do not show any lysis, thus demonstrating the active life form of the components in the association.

Positive taxis of streptomycetes *S. olivaceoviridis* and *S. xanthochromogenes* to the culture of *A. variabilis* was recorded.

Table 2. Concentration of chlorophyll *a* in monoculture of *O. terebriformis* (Ag.) Elenk. emend. and in association with streptomycete *S. odorifer* (mg/g of wet weight)

Replications	Monoculture of cyanobacterium	Association
1	36.8	43.8
2	35.5	47.8
3	41.8	41.8
4	33.5	47.8
5	30.8	47.0
6	34.8	44.3
7	30.8	43.8
8	36.3	35.5
9	37.5	35.5
10	29.3	43.0
Average	34.7 + 3.74	43.0 + 4.46

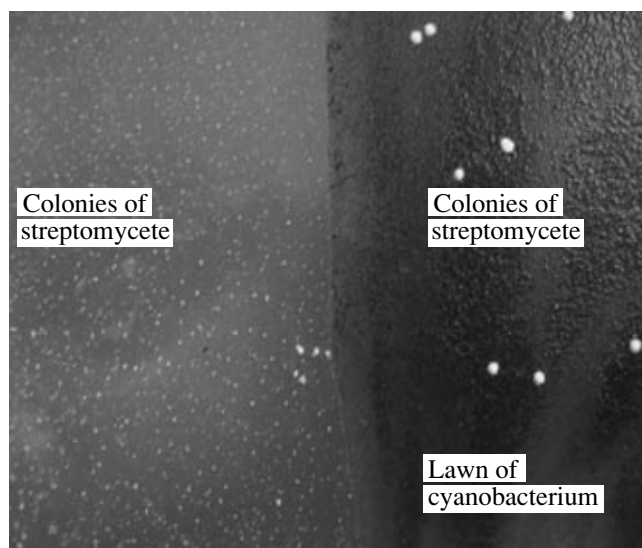


Fig. 3. The relative diameter of colonies of *Streptomyces xanthochromogenes* (1) on the solid nutrient medium and those proliferated from the lawn of the cyanobacterium *Anabaena variabilis*.

The growth of colonies of streptomycetes was concentrated on the colonies of cyanobacteria (Fig. 2).

The culture of *A. variabilis* also manifested positive taxis to streptomycetes *S. olivaceoviridis* and *S. xanthochromogenes*, as shown by the orientation coefficient over 1.

Comparison of growth of the association of *O. terebriformis* and *S. odorifer* and of the cyanobacterium on clay minerals demonstrated that both for surficial overgrowing of fragments and for pouring over of the biomass material with a triturated powder of the mineral the radius of the green spot on the surface was wider ($r = 4$ cm) in case of the association than in case of the monoculture of the cyanobacterium ($r = 1$ cm). This indicates a more vigorous growth of the cyanobacterium in the association.

It is found that the diameter of colonies of the streptomycetes *S. olivaceoviridis* and *S. xanthochromogenes* which grew through the lawn of *A. variabilis* for consecutive inoculation of the streptomycete and the cyanobacterium on a solid medium BG-11 was greater than the size of the colonies of monocultures of streptomycetes grown under the same conditions (Fig. 3). The maximum growth rate of the streptomycete *S. odorifer* ($K_r = 0.71$ mm/day) was on the medium with culture liquid of the cyanobacterium *O. terebriformis* as a source of carbon in comparison with growth parameters of the culture on media with different carbohydrates ($K_r = 0.58$ – 0.65 mm/day).

The photosynthetic activity of the cyanobacterium *O. terebriformis* increased significantly in the culture mixed with the streptomycete *S. odorifer* in comparison

Table 3. Antimicrobial properties of the streptomycete *Streptomyces odorifer*, the cyanobacterium *Oscillatoria terebriformis* (Ag.) Elenk. emend., and the association of the streptomycete and the cyanobacterium (the radius of the lysis zone of test culture, mm)

Test cultures	Monocultures		Association
	streptomycete	cyanobacterium	
<i>Bacillus cereus</i>	5.5	0	8.5
<i>Rhodococcus erythropolis</i>	4.0	0	2.0
<i>Micrococcus agilis</i>	4.2	0	15.0
<i>Arthrobacter globiformis</i>	4.5	0	17.5
<i>Pseudomonas</i> sp.	5.5	0	1.5
<i>Streptomyces prunicolor</i>	0	3.9	17.5
<i>S. xanthocidicus</i>	0	0	10
<i>Fusarium sporotrichiella</i>	0	0	17.5
<i>F. oxysporus</i>	0	0	0
<i>F. graminisarum</i>	0	10	0
<i>Saccharomyces</i> sp.	0	0	0
<i>Rhodotorula</i> sp.	1.5	0	12.5

with the photosynthetic activity of monoculture of the cyanobacterium (Table 2).

There were differences in the antimicrobial activity of the association of *O. terebriformis* and *S. odorifer* in comparison with monocultures of the cyanobacterium and of the streptomycete (Table 3). In comparison with the pure culture, the antibiotic activity of the streptomycete increased in the association against cultures of bacteria *Bacillus cereus*, *Arthrobacter globiformis*, and *Micrococcus agilis* and against the culture of yeast *Rhodotorula* sp. and of the fungus *Fusarium sporotrichiella*. The antimicrobial properties of the cyanobacterium *O. terebriformis* increased towards the test culture of *Streptomyces prunicolor*. The association of the streptomycete and the cyanobacterium manifested the antimicrobial activity towards test-cultures of *Bacillus subtilis*, *Staphylococcus aureus*, *Streptomyces xanthocidicus*, *Spirillum* sp., *Pseudomonas* sp., and *Fusarium oxysporum*, towards which the monocultures did not manifest any antagonism.

Development of the cyanoactinomycete association of *O. terebriformis* and *S. odorifer* on the clay mineral kaolin led to changes in the structure of the mineral. Roengenodiffractometrically, comminution of the dominant mineral kaolinite was indicated by a lowered intensity of reflexes of the mineral. In the rock, chlorite, the mineral most easily eroded and richest in nutrients, was transformed.

Thus our experimental results indicate the associative nature of interaction of the investigated cultures in the mixed model system.

The cultures of cyanobacteria and actinomycetes investigated in the present study are isolated from natural ecosystems, from the cyanobacterial mat and roots of cycadales, forming a unique symbiosis with nitrogen-fixing cyanobacteria (Lindblad and Costa, 2002). Our data on the interaction of the investigated organisms confirms the well-known opinion (Sirenko and Kozitskaya, 1998) that positive interrelations between organisms coexisting in microcosms for a long period of time are more probable than between randomly selected components.

We based our work on the concept of efficient microorganisms, or mixed cultures, which may be used as support for the stability of the environment and for conservation of natural resources. The final aim of our study is to identify an assortment of microorganisms that are compatible with each other both physiologically and ecologically, and which may be used as associations for practical purposes.

ACKNOWLEDGMENTS

This study was supported by the Scientific Schools program, grant NSH-8797.2006.4.

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