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Production of the antibiotic cyanobacterin LU-1 by *Nostoc linckia* CALU 892 (cyanobacterium)

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Abstract

Cyanobacterin LU-1, produced by *Nostoc linckia* CALU 892, inhibits the growth of many cyanobacteria and eukaryotic algae. The minimum effective dose of a crude preparation to *Synechococcus* sp. R-2 is *ca* 1 μg ml^{-1}. The antibiotic hinders cell division and light-dependent oxygen evolution in *Synechococcus* sp. R-2 (PCC 7942) cells. It is not active against heterotrophic bacteria and fungi, and is non-toxic to mice.

Purified cyanobacterin LU-1 contains a nitrous heterocycle with sugar and phenolic substituents. Cyanobacterin LU-1 accumulates in the medium during the course of growth, although not in direct proportion to cell density. Productivity of the culture depends on temperature.

Introduction

Some filamentous cyanobacteria secrete antibiotics that kill other strains of cyanobacteria (Mason *et al.*, 1982; Flores & Wolk, 1986). Flores and Wolk (1986) observed that N_2-fixing strains produce compounds with different activity spectra and different molecular weights, but their chemical structures have not been established. The antibiotic-herbicide, cyanobacterin, produced by *Scytonema hofmanni*, is a chlorine-containing γ-lactone (Pignatello *et al.*, 1983) active against many cyanobacteria and eukaryotic algae (Mason *et al.*, 1984; Gleason & Paulson, 1984). It inhibits electron transport at a site in photosystem II (Gleason & Paulson, 1984).

Strains of cyanobacteria from the collection of algal cultures in Leningrad University (CALU) were screened for the production of biologically active compounds, and several strains of *Anabaena* and *Nostoc* were found to produce antibiotics very active against other cyanobacteria. This report describes the production of an antibiotic by a strain of *Nostoc linckia*.

Materials and methods

All algae were obtained from the collection of algal cultures in Leningrad University (CALU). The strain of *Nostoc linckia* was brought into axenic culture from a sample of park soil in Leningrad. It produced a clear region on a lawn of an indicator strain (see below) when it formed colonies on plates of nutrient agar. During laboratory cultivation in aerated liquid medium and sub-

sequent cloning on agar plates, a mutant strain was obtained which is not nitrogen-fixing, but which gives a homogeneous suspension of short filaments when grown in liquid media. This mutant was designated as *N. linckia* CALU 892.

Synechococcus sp. R-2 (PCC 7942) was used as the standard indicator strain. Other cultures examined are listed in Table 2. All but one (*Microcystis aeruginosa* (CALU MA)) were axenic.

Strains of bacteria *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus*, and of the fungi *Torula utilis* and *Saccharomyces cerevisiae* were obtained from the Department of Microbiology of Leningrad University.

An antibiotic designated cyanobacterin LU-1 was extracted from the culture medium by extraction with an equal volume of ethyl acetate at pH 10. The ethyl acetate was evaporated in a vacuum evaporator, and dry material was dissolved in minimal amount of water and lyophilized. Further purification was achieved by thin-layer chromatography (TLC) on silica gel plates (Silufol-UV 254) in neutral system chloroform/methanol (9 : 1). The active fraction was detected by bioautography on *Synechococcus* sp. R-2 and in the ultraviolet at 254 nm. The active spot was treated as recommended by Kirchner (1967).

Cultures of cyanobacteria were grown in a medium with the following composition (mg l^{-1}): KNO_3 – 1000; K_2HPO_4 – 200; $MgSO_4 \cdot 7H_2O$ – 200; $CaCl_2$ – 150; $NaHCO_3$ – 200; $ZnSO_4 \cdot 7H_2O$ – 0.22; $MnSO_4$ – 1.81; $CuSO_4 \cdot 5H_2O$ – 0.079; $NaBO_3 \cdot 4H_2O$ – 2.63; $FeSO_4 \cdot 7H_2O$ – 9.3; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ – 1.0; $Co(NO_3)_2 \cdot H_2O$ – 0.02; EDTA (Na_2-salt) – 10.

Cultures were grown at 25 °C, if not specified otherwise. Irradiation of 4000 lux was provided by cool-white tubes.

N. linckia was grown in glass vessels with 350 ml of medium, and suspensions were bubbled with 5% CO_2-enriched air. Dry weight of cellular biomass was measured after the cells were washed and dried to a constant weight at 105 °C.

The minimum effective dose (MED) of crude extract of cyanobacterin LU-1 for algae was determined by the dilution method. Consequent dilutions of the antibiotic were prepared with the dilution coefficient 2. 2 ml of each dilution probe were added to tubes containing 2 ml of test culture and incubated 10 days in the light. The MED was assumed, by a common definition, as the minimal concentration of cyanobacterin LU-1 in dilution probes to inhibit the growth of an organism being tested.

The concentration of the antibiotic in cultural medium of *N. linckia* was estimated by titration on *Synechococcus* sp. R-2 assuming that MED for the indication is *ca* 1 $\mu g\ ml^{-1}$.

The indicator *Synechococcus* sp. R-2 was grown, in the presence of cyanobacterin LU-1 in vessels as described for *N. linckia*, but at 34 °C. Cell numbers were determined by direct counting in a counting chamber, or the growth was estimated by determination of chlorophyll concentration. Chlorophyll was determined by extracting cells with 96% methanol and using the extinction coefficient $E_{666}^{0.1\%} = 72\ ml\ g^{-1}\ cm^{-1}$. Viable cell were determined by inoculation of agar plates. Rates of oxygen evolution by cells were measured with a Clark-type oxygen electrode. Microscope observations were carried out using a Zeiss phase contrast microscope. Cell length was estimated on microphotographs; 100 cells were measured in each preparation. Bacteria were grown on beef extract medium at 30 °C. Paper discs impregnated with concentrated cyanobacterin LU-1 (crude extract) were placed on the surface of the inoculated medium.

Acute toxicity of the freeze-dried crude cyanobacterin LU-1 was tested by mouse bioassay: the known concentration of a sample in physiological salt solution was given by intraperitoneal injection to female mice (20–25 g).

Results and discussion

Figure 1 shows the change in amounts of the cyanobacterin LU-1 during the course of growth of *N. linckia* culture. It is evident that cyanobacterin LU-1 is synthesized by actively

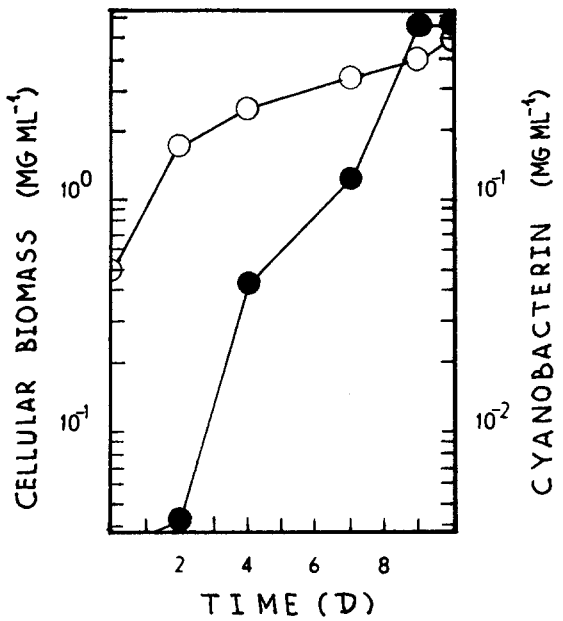

Fig. 1. Growth of *N. linckia* (○) and and cyanobacterin LU-1 accumulation (●) in aerated medium at 25 °C.

growing cells, but the accumulation of cyanobacterin LU-1 is not always directly proportional to cell density. No sign of cell lysis has been detected even in senescent cultures. The maximum accumulation of the antibiotic, achieved in 11 days culture at 25 °C, was *ca* 600 μg ml^{-1} of crude preparation.

Cyanobacterin LU-1 production is temperature-dependent. Table 1 shows that the best growth of the cyanobacterium was observed at 30 °C, and growth proceeds even at 39 °C; however, with increasing temperature, the production of cyanobacterin LU-1 was markedly decreased.

Cyanobacterin LU-1 is extracted from culture medium most effectively by ethyl acetate at pH 10. Activated charcoal as well as ion-exchange resins Dowex 1 (CL^{-1}) and Dowex 50W (Na^{+}) bind the antibiotic irreversibly.

Lyophilized crude extract of cyanobacterin LU-1 is a yellow powder with a strong characteristic flavour. MED for the indicator, *Synechococcus* sp. R-2, is *ca* 1 μg ml^{-1}.

Eight UV-absorbing fractions of cyanobacterin LU-1 crude preparation were detected on silica gel plates when developed in chloroform/methanol (9 : 1) system. The R_fs vary from 0.3 to 0.98. Bioautography assay revealed the only active fraction with the $R_f = 0.4$. The active fraction gives positive reactions with aniline-phthalate, p-anisidine-phthalate, p-antisaldehyde, 4-(dimethylamino)-bensaldehyde, formaldehyde, and iron(III)chloride.

Cyanobacterin LU-1 has a characteristic UV spectrum with maximum absorbance at 210 nm, 265 nm and 300 nm. The pH does not influence spectral characteristics.

All these data indicate that cyanobacterin LU-1 is a nitrous heterocycle with sugar and phenolic substitutes.

Cyanobacterin LU-1 is active against both prokaryotic and eukaryotic phototrophs (Table 2). The MED varies depending on organism. Only one strain of cyanobacterium, *Nostoc* sp. CALU 893, as well as several green algae, were

Table 1. Production rate of cyanobacterin LU-1 in *N. linckia* cultures at different temperatures (mean values for the late-exponential phase of growth).

Temperature	Production of biomass	Production rate of cyanobacterin LU-1	
(°C)	($g\,l^{-1}\,d^{-1}$)	($\times 10^{-3}\,g\,l^{-1}\,d^{-1}$)	($\times 10^{-3}\,g\,g_{biomass}^{-1}\,d^{-1}$)
25	0.27 ± 0.01	18.6	68.9
30	0.40 ± 0.01	10.8	27.0
35	0.35 ± 0.03	3.4	9.7
39	0.29 ± 0.07	0.6	2.1

Table 2. Relative algicidal activity of cyanobacterin LU-1 crude preparation from *N. linckia.*

Strain teste	CALU no.	MED $\mu g\ ml^{-1}$
Cyanobacteria		
Anabaena variabilis ATCC 27892	458	0.5
Anabaena sp. PCC 7120	786	0.3
Synechococcus sp. R-2 PCC 7942	895	1.0
Aphanizomenon sp.	L-118	0.6
Aphanocapsa sp. str. Floyd C-8	751	1.0
Microcystis aeruginosa	MA	0.08
Nostoc punctiforme str. Gromov 1964/1	374	0.5
Nostoc sp.	893	>85.0
Nostoc sp. str. Gromov 1967/467	467	0.7
Nostoc boryanum str. Dyer 3	465	5.6
Phormidium tenuissimum str. Gromov 1971/4	558	1.4
Synechococcus sp. str. Gromov 1978/768	768	1.0
Synechocystis aquatilis str. Gromov 1965/428	428	5.6
Eukaryotic algae		
Ankistrodesmus acicularis str. Baslerova	254	1.0
Chlamydomonas gloeogama str. Gromov 1960/8	47	20.0
Chlorella pyrenoidosa str. Pringsheim	4	>85.0
Chlorella vulgaris str. Gromov 1962/30	157	2.0
Chlorella intermedium str. Deason C1-13	474	10.0
Coelastrum proboscideum str. Jaag	339	5.0
Euglena gracilis str. Z	520	>66.0
Cosmarium sp. str. Gromov 1965/6	394	4.5
Klebsormidium flaccidum str. Pringsheim	332	>60.0
Kirchneriella obesa str. Pinevich	447	2.0
Scenedesmus obliquus str. Pringsheim	13	2.0

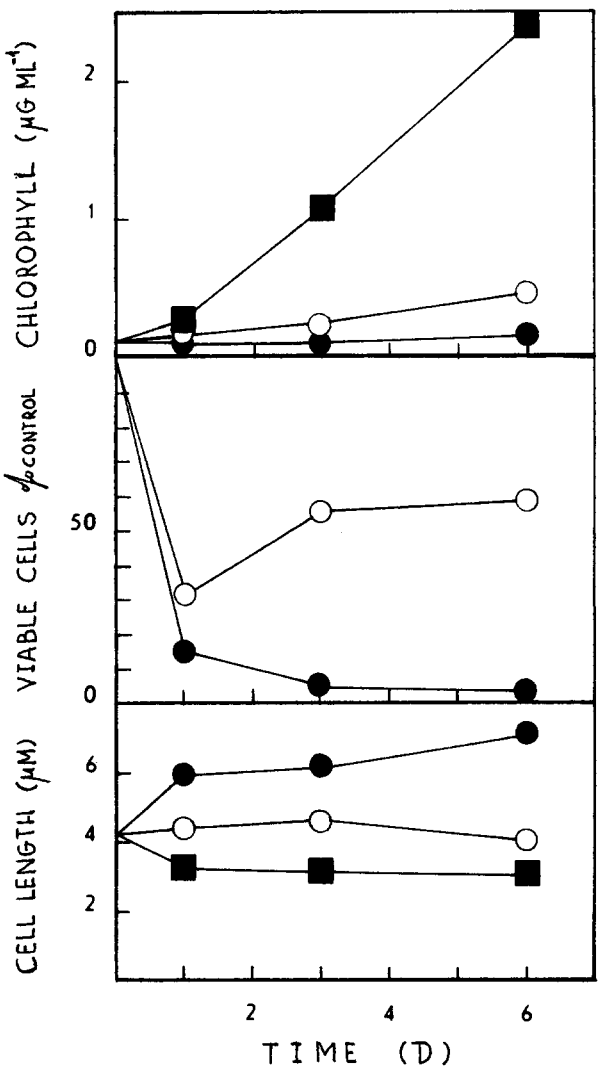

Fig. 2. Growth of *Synechococcus* sp. R-2 in aerated medium. (■) – control without cyanobacterin LU-1; (○) – 0.5 $\mu g\ ml^{-1}$ of cyanobacterin LU-1; (●) – 1.0 $\mu g\ ml^{-1}$ of cyanobacterin LU-1.

resistant. Eukaryotic algae in general are less sensitive than cyanobacteria; the bacteria and fungi examined were fully resistant. Five mg of cyanobacterin LU-1 given by intraparitioneal injection was not toxic to mice.

Figure 2 shows the effect of cyanobacterin LU-1 on the growth of *Synechococcus* sp. R-2. If cyanobacterin LU-1 is added in lethal concentration (1 $\mu g\ ml^{-1}$), the cells do not divide, but grow for some time and gradually die. Some cells are not only elongated, but become significantly deformed. At a cyanobacterin LU-1 concentration of 0.5 $\mu g\ ml^{-1}$, not all cells die, and after some period of adaptation slow growth of the culture can be observed (Fig. 2). Strains resistant to cyanobacterin LU-1 could not be obtained from these cultures.

Cyanobacterin LU-1 inhibits light-dependent oxygen evolution in *Synechococcus* sp. R-2 cells (Fig. 3).

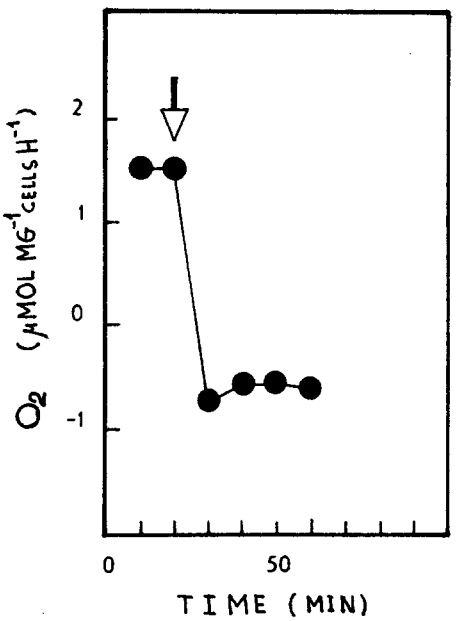

Fig. 3. Effect of cyanobacterin LU-1 (0.5 μg ml^{-1}) on oxygen evolution in *Synechococcus* sp. R-2. Cyanobacterin was supplied as indicated by the arrow.

Cyanobacterin LU-1 inhibits growth of phototrophic microorganisms, but was not active against heterotrophs. It inhibits light-dependent oxygen evolution. These data imply that cyanobacterin LU-1 is a herbicide-like compound which inhibits photosystem II electron transport. It was shown that mutants of *Synechococcus* sp. R-2 and *Anabaena variabilis* (ALU 458) resistant to cyanobacterin LU-1 acquired resistance to the classical photosystem II inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea) (Khudyakov, pers. comm.). This fact seems to suggest that cyanobacterin LU-1 binds to polypeptide Q_B (32 kD).

Cyanobacterin LU-1 acts similarly to cyanobacterin produced by *Scytonema hofmanni*, although the chemical composition of the two cyanobacterins, site of action, and the producing organisms differ (Pignatello *et al.*, 1983; Gleason & Paulson, 1984).

References

Flores E, Wolk CP (1986) Production, by filamentous, nitrogen-fixing cyanobacteria, of a bacteriocin and of other antibiotics that kill related strains. Arch. Microbiol. 145: 215–219.

Gleason FK, Paulson JL (1984) Site of action of the natural algicide, cyanobacterin, in the blue-green alga, *Synechococcus* sp. Arch. Microbiol. 138: 273–277.

Kirchner JG (1987) Thin-layer chromatography. Interscience Publ., New York, 788 pp.

Mason CP, Edwards KR, Carlson RE, Pignatello J, Gleason FK, Wood JM (1982) Isolation of chlorine-containing antibiotic from the freshwater cyanobacterium *Scytonema hofmanni*. Science 215: 400–402.

Pignatello JJ, Porwoll J, Carlson RE, Xavier A, Gleason FK, Wood JM (1983) Structure of the antibiotic cyanobacterin, a chlorine-containing γ-lactone from freshwater cyanobacterium *Scytonema hofmanni*. J. Org. Chem. 48: 4035–4038.