島根大農研報 (Bull. Fac. Agr. Shimane Univ.) 24 : 239–246, 1990

Barium Ion Encourages The Growth of Cyanobacteria*

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バリウムイオンはラン藻の増殖を促進する

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ABSTRACT

Potent effect of barium ion was observed on the growth of such cyanobacteria as filamentous *Phormidium lapideum* as well as unicellular *Anacystis nidulans*. Studies on the *P. lapideum in vitro* revealed that one of two DNA polymerases was activated with 5 mM barium chloride in the co-presence of magnesium ion. Activity of DNA dependent RNA polymerase that was purified as homogeneous preparation was also more than doubled on the addition of barium ion. The fact described here should be a unique circumstance as compared to the general experience with barium salts.

INTRODUCTTION

Cyanobacteria, better known as blue-green algae, are photoautotrophic prokaryotes, which are very widespread and versatile organisms in nature[1]. By using light as a sole external energy, cyanobacteria can generate ATP *via* their photophosphorylation device and reduce $NADP^+$ to NADPH *via* their electron flow systems PS-II and I. Up to date, therefore, cyanobacterial photobioreactors have been presented to regenerate ATP, to produce glutathione or hydrogen gas by the use of a thermophilic cyanobacterium *Phormidium lapideum*[2-6]. Immobilized marine cyanobacterium *Synechococcus* sp., upon illumination, excreted glutamate as a main amino acid using nitrate as a nitrogen source[7]. However, it is a practical problem that growth rate of the cyanobacteria is often too slow to use them as biomaterials. On the other hand, cyanobacteria may occasionally cause a striking 'water blooms' to nature[8]. This is a serious environmental problem in the present human society. Nevertheless, it is still difficult to induce 'water blooms' under the laboratory conditions. Therefore, from both viewpoints of rapid mass culture of useful cyanobacteria and of regulation of blooming cyanobacteria, it is very important to elucidate how to control the cyanobacterial reproduction mechanism. So far, a variety of culture media are rec-

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* Presented in most part at the meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry at Ryukyu University on October 4, 1989.

ommended to cultivate cyanobacteria. But there has been no description citing potent effect of barium ion on cyanobacterial growth. Here we report a novel enhancement effect of barium salts on the cyanobacterial multiplication and on the activities of the relevant enzymes, DNA polymerase as well as RNA polymerase.

MATERIALS AND METHOD

Cyanobacterial strains and Growth Conditions

A strain of filamentous and thermophilic cyanobacteria, *Phormidium lapideum*, isolated from Matsue hot springs[9] was used as a main biological material. *Anacystis nidulans*, one of representative unicellular forms, was also used for culture experiments. The culture medium used for *P. lapideum* was the enriched hot spring water as described previously[9,10]. Kratz-Myers's medium[11] was also used for *P. lapideum* and *A. nidulans* as an artificial one. Growth of the cyanobacteria was determined by chlorophyll accumulation rate in the culture. For chlorophyll determination, the intact cells were homogenized through sonication and treated with 80% acetone. The chlorophyll-a concentration in the acetone extract was determined spectrophotometrically according to Mackinney's[12].

Enzyme Preparation and Assay Procedures

The enzyme purification was carried out at 4°C. Unless otherwise specified, Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 0.05% 2-mercaptoethanol was used as the buffer throughout the purification. The enzyme activities were measured according to the conventional method using ^{3}H-dTTP to assay for the DNA polymerase and ^{3}H-UTP for the RNA polymerase, with slight modifications, respectively. The protein concentration was determined by the method of Lowry et al[13].

Determination of Barium

Barium content was determined by means of sequential plasma spectrometry. Cyanobacterial cells cultured with barium salts were completely washed with distilled water and then 40% glycerol. By centrifugation in 40% glycerol (3,500 X g, 5 min), concomitant insoluble barium salts were precipitated and separated from flouting cyanobacteria. This procedure was repeated three times. The cyanobacterial cells thus obtained were washed with 0.05 M acetate buffer (pH 5.0) to remove off barium salts adsorbed on the surface (sheath) of the cells (data not shown). The cells were again washed and allowed to the wet digestion with 70% perchloric acid. The residue was dissolved in water, and membrane-filtered. This solution was detected for barium by means of Shimadzu sequential plasma spectrometer ICRP 1000-III.

RESULTS AND DISCUSSION

Figure 1 shows growth curves of *P. lapideum* in the absence and presence of barium acetate of various concentrations. Evidently, the growth was enhanced with 3 mM barium acetate. No effect of acetate ion was observed on the growth rate (data not shown). With barium chloride, the growth was also promoted but to a lesser extent. In these cases, the culture medium often became turbid due to the resulted, water-insoluble barium sulfate. Hence, growth rate was determined by measuring of chlorophyll accumulation in the culture. Optimum concentration of barium acetate to be added was found in the range between 1 to 3 mM. The growth was depressed with 10 mM. Such potent effect of barium ion was also observed but to lesser extent on the growth of *Anacystis nidulans* (relative growth rate: about 130%, data not shown).

The enzymes, a couple of DNA deoxynucleotidyl transferases (DNA directed; DNA polymerase, EC. 2.7.7.7.) and RNA nucleotidy transferase (DNA directed; RNA polymerase, EC. 2.7.7.6.), were present almost exclusively in the membrane fraction. The DNA polymerases were partially purified through the routine procedures including ammonium sulfate fractionation, gel filtration, DEAE column chromatography and so on. As shown in a purification summary (table 1), one of the DNA polymerases, designated as *Pla* Pol (1), was obtained in 38% activity yield with an overall purification over 1000-fold. The other, designated as *Pla* Pol (2), was with about 400-fold. Interestingly, aphidicolin (100 μg/ml), which is a specific inhibitor of DNA polymerase α in mammalian cells, inhibited the ^{3}H-dTTP incorporation into the acid-insoluble fraction to 59% of the control level in the Pol (1) reaction, whereas no inhibition was observed in the Pol (2) reaction. Based upon comparative characterizations of the enzymes (their molecular weights, *in vitro* behaviors with various inhibitors, template DNAs and so on), the one enzyme, *Pla* Pol (1), was supposed to correspond to the *E. coli*'s pol III and the other, *Pla*

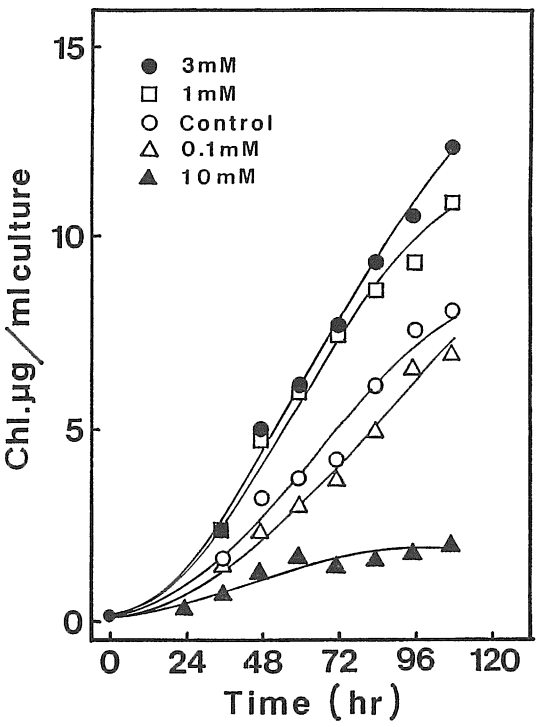

Fig. 1 Growth curves of *Phormidium lapideum*, a filamentous and thermophilic cyanobacterium, in the presence of barium acetate of various concentrations
○—○ Control, △—△ 0.1mM, □—□ 1mM, ●—● 3mM, ▲—▲ 10mM,
One μg chlorophyll/ml culture corresponds to around 100 μg of *P. lapideum* (dry weight). The culture medium used in this experiment was the enriched hot-spring water.

Table 1　Enzyme purification summary

DNA polymerases

Step	Total protein (mg)	Sp. act. (unit/mg)	Purification (fold)
Crude extract	223.6	0.187	1
$(NH_4)_2SO_4$ fractionation	118.6	20.8	11
DEAE-cellulose chromatography			
first fraction (*Pla* Pol(1))	80.7	197.1	1054
second fraction (*Pla* Pol(2))	41.9	79.4	425

unit=1 pmol ^{3}H-dTTP incorporated/30 min

Table 2　Properties of *P. lapideum* DNA polymerases

	Pla Pol(1)	*Pla* Pol(2)
Molecular weight	520K	42K
Optimum pH	8.6	8.3
pH stability	6.5-8.5	6.5-8.0
Optimum conc.		
$MgCl_2$	2.5mM	5.0mM
KCl	25mM	50mM
Heat stability		
(T_{50}, 10 min)	48℃	55-60℃
Template DNA	native double	activated double
Inhibited by		
Chelate comps.	+	±
Aphidicolin	++	−
Ethidium bromide	++	+
SH reagents	+	±
Urea	+	++

Table 3　Enzyme purification summary

RNA polymerase

Step	Total protein (mg)	Sp.act.*(unit/mg)	Purification (fold)
Crude extract	4364	0.78	1
$(NH_4)_2SO_4$ fractionation	2411	1.08	1.4
DEAE-cellulose chromatography	55.1	58.3	74.7
Phosphocellulose	3.1	812.9	1042
Heparin-Sepharose	1.3	1730.2	2218

*X 10^{-2}, 1 unit=1 μmol ^{3}H-UTP incorporated/hr

Pol (2) was the pol I, respectively, though some peculiarities have been noticed (table 2). The RNA polymerase was purified as homogeneous preparation of about 2200 fold (table 3). The enzyme had a molecular weight of 489,000 as determined by Sephadex G-150 gel filtration. α-Amanitin (800 μg/ml), an inhibitor of eukaryotic RNA polymerase II and III, did not affect the ^{3}H-incorporation by the *P. lapideum* RNA polymerase. Additional properties of the purified enzyme corresponded to those of the RNA polymerases from general prokaryotes (data not shown).

Table 4 shows the effect of metal ions on a couple of DNA polymerases partially purified from the cells. Quite interestingly, one of DNA polymerases *in vitro* was stimulated with barium ion about 2 fold, but the other not. Other metal ions except barium were all inhibitory. Replacement of magnesium ion by barium ion in the reaction mixture, however, resulted in depression of the enzyme activities; survisal activity, 25% for Pol (1) and 60% for Pol (2). Ammonium ion exerted a considerable activation to the both DNA polymerases (120% for (1), 150% for (2)).

Homogeneously purified RNA polymerase preferred magnesium ion to manganese for the RNA synthesis *in vitro*, though both metal ions were effective. As shown in table 5, however, in the presence of 10 mM magnesium ion, barium ion activated *P. lapideum* RNA polymerase up to 248%, whereas all others tested were inhibitory. Moreover, the presence of barium chloride up to 25 mM stimulated the ^{3}H-incorporation into RNA, but the RNA polymerase from *E. coli* was strongly inhibited with lower concentration of barium ion under the same condition: remaining activity in the presence of 10 mM $BaCl_2$ was less than 10% of the original, evidently as shown in Fig. 2.

In one experiment, we determined barium concentration in the cyanobacterial cells cultured with 3 mM barium acetate by means of sequential plasma spectrophotometer

Table 4 Effects of metal ions on the activities of DNA deoxynucleotidyl transferases (DNA directed) partially purified from *P. lapideum*

Compounds	Conc.(mM)	Relative Enzyme Activity *Pla* Pol(1)	*Pla*Pol(2)
(Control)	—	100%	100%
$BaCl_2$	5.0	89.8	184.2
$MnCl_2$	5.0	21.5	67.5
$CaCl_2$	5.0	6.1	30.6
$NiCl_2$	5.0	59.9	88.2
$CoCl_2$	5.0	42.9	81.6
$CdCl_2$	5.0	29.7	60.5
$ZnCl_2$	5.0	91.8	81.6
$CuCl_2$	5.0	23.7	46.1
NH_4Cl	5.0	120.1	149.1

The enzymes was preincubated with various metal ions at 37°C for 15 min in 50 mM Tris-HCl buffer (pH 8.3) in the presence of 5 mM magnesium chloride. The residual activities were then assayed under the standard conditions.

Table 5 Effects of metal ions on the activity of RNA nucleotidyl transferase (DNA directed) homogeneously prepared from *P. lapideum*

Compounds	Conc. (mM)	Relative Activity
(Control)	—	100%
$BaCl_2$	10.0	247.7
$Ba(CH_3COO)_2$	10.0	149.6
$CaCl_2$	10.0	17.7
$NiCl_2$	10.0	5.8
$CoCl_2$	10.0	2.9
$CdCl_2$	10.0	1.6
$ZnCl_2$	10.0	2.2
$CuCl_2$	10.0	2.2

The enzyme was preincubated with each metal ion at 42°C for 15 min in 50 mM Tricine-NaOH buffer (pH 8.0) in the presence of 10 mM magnesium chloride. The residual activities were assayed using ^{3}H-UTP under the standard conditions.

(Shimadzu ICRP 1000-III). Barium of about 1.62 mg/g of the dry cells was detected, whereas about ten times lower concentration (0.132 mg) was found in the control cells cultured without addition of barium salts. Barium content of the control culture medium was 0.01 ppm. In the present research, localization of the barium ion in the cell and precies mechanism of such an enhancement effect are unknown yet. But we know that the polymerases distribute almost exclusively in the membrane fractions so that we suppose that the metal probably locates in and near the membranes and activate the enzymes. Recently we reported that a couple of restriction endouncleases, *Pla* I and *Pla* II, isolated from *P. lapideum* were the membrane-bound enzymes. The

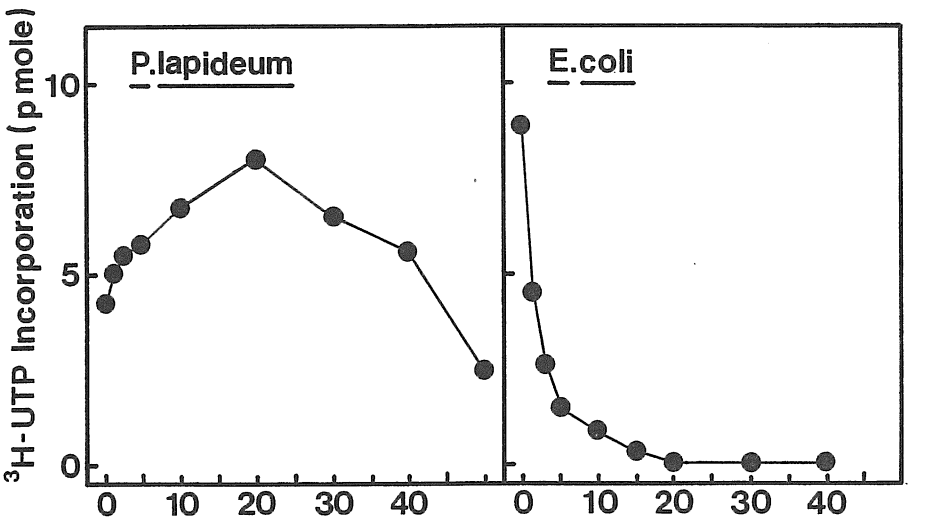

Fig. 2. Effect of barium concentration on the purified RNA polymerases
left : *P. lapideum*.　　right : *E. coli*

Pla I and *Pla* II were shown not to be inhibited by barium ion, unlike other restriction enzymes[14] originated from different sources. Moreover, various enzymes purified from cytosol of *P. lapideum* were affected with barium ion[15]. These facts support our discussions described above. The findings that barium ion encourages the growth of cynobacteria and activates the cyanobacterial DNA and RNA polymerases should be novel circumstances as compared to the general experience with barium salts upon living things. Moreover, this finding may be very important from both viewpoints of rapid mass culture of useful cyanobacteria and elucidation of blooming mechanism of nuisance ones.

ACKNOWLEDGMENT

This work was supported in part by a Grant-in-Aid (01480062, 1989) from the Ministry of Education, Science and Culture of Japan.

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摘　　要

糸状性好温性ラン藻 *Phormidium lapideum* の増殖，核酸合成酵素の活性化に対して，バリウムイオンが促進効果を示すことを見出した．即ち，1. *P. lapideum* は 3 mM 濃度の酢酸バリウムの共存下でその増殖が約2倍に上昇した．2. *P. lapideum* より約400倍に精製した DNA Polymerase はマグネシウムイオンの共存下，5 mM のバリウムイオンによって，その活性の上昇（約2倍）を示した．3. 均一標品にまで精製された RNA Polymerase も，バリウムイオンの存在下でのみ，その活性が約2.5倍に上昇した．4. 酢酸バリウムは単細胞性ラン藻 *Anacystis nidulans* の成長も促進した．5. バリウムイオンのこのような効果は新規な発見である．