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# Effects of $Mg^{2+}$ on the growth and DNase activity of *Spirulina platensis*, a cyanobacterium

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**Abstract**

To investigate the methodology to inhibit DNase activity in the cyanobacterium *Spirulina*, the effects of $Mg^{2+}$ on the growth and DNase activity of *Spirulina platensis* strain SSP-14 were studied. The results indicated that strain SSP-14 could keep growing, but much slower, when inoculation and cultivation were done twice in $Mg^{2+}$/free Zarrouk medium; and the activity of both intracellular and extracellular DNases was almost completely inhibited. This manipulation could efficiently remove the high-level activity of DNases which frequently exist in *Spirulina* and block foreign genes from being introduced. It is recommended that the technique be applied for genetic transformations of this alga. © 1998 Published by Elsevier Science Ltd. All rights reserved.

*Keywords:* $Mg^{2+}$; Growth; DNase activity; Cyanobacterium; *Spirulina platensis*

## 1. Introduction

*Spirulina* is one of the economically important cyanobacterial genera due to its industrial production of proteins, $\beta$-carotene, vitamins (especially $B_{12}$), phycocyanin and $\gamma$-linolenic acid (Cohen, 1997), and up to now has become the most well-known and the most broadly cultivated species of both cyanobacteria and other algae in the world (Vonshak, 1997b).

Many species of cyanobacteria have been proved to possess a restriction-modification system which can destroy any foreign gene that enters the cells (Wolk *et al.*, 1982; He *et al.*, 1984; Takahashi *et al.*, 1996). *Spirulina platensis* has a high level of activity of DNases (deoxyribonucleases) which are believed to be the most important of the reasons why a reproducible and efficient gene transformation system, like those in unicellular cyanobacteria (Porter, 1985), has not been established (Vachhani and Vonshak, 1997). Apparently, this situation hinders the development of new strains and new utilizations of this alga by genetical engineering.

Although some attention has been paid to isolation and characterization of restriction endonucleases from different strains of *Spirulina platensis* (Kawamura *et al.*, 1986; Tragut *et al.*, 1995), none of them was focused on removing or lowering the DNase activity of this alga. It is known that the activity of each enzyme is influenced by one or more cations, e.g. $Mg^{2+}$, $Ca^{2+}$ and so on, and DNases are affected by $Mg^{2+}$, while endonucleases are absolutely affected by $Mg^{2+}$ (Sambrook *et al.*, 1989). Hence, it is easy to determine whether the absence of $Mg^{2+}$ can inhibit the activity of DNase. However, magnesium is also one of the essential macroelements for growth of algae (Harold and Sorger, 1966). Therefore, the key problem is how to determine the relationship or the balance point between the growth of the alga and the inhibition of DNase. Naturally, the effect of the removal of $Mg^{2+}$ from the cultures on DNase activity and the effect of its absence on growth should be studied together.

## 2. Methods

### 2.1. Organisms and cultivation

*Spirulina platensis* strain SSP-14, preserved in our laboratory, was used. SSP-14 was cultured in Zarrouk

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medium (Vonshak, 1997a) on an illuminated shaker (cool white fluorescent light, illumination 14 h) for 5–8 days at 28°C and then axenic cultures were prepared as follows: 1 ml of exponential culture was centrifuged and the pellet washed 3 times with Zarrouk medium and then treated with 1 ml of 5% NaClO for 2–6 min; centrifuged and NaClO removed, and the pellet washed 3 times and resuspended in 1 ml of Zarrouk medium, then 50 μl were transferred to 20 ml of Zarrouk medium and grown for 1–2 weeks.

### 2.2. Growth tests

Experiment I: SSP-14 was cultivated in 20 ml of Zarrouk medium (containing 0.8 mM $Mg^{2+}$) for 1 week, from an initial $OD_{560}$ 0.1 to a final $OD_{560}$ about 0.8, under the conditions as above.

Experiment II: Part of Experiment I cultures was centrifuged and the cells washed 3 times with Mg-free Zarrouk medium and then transferred to 20 ml of $Mg^{+2}$-free Zarrouk medium (initial $OD_{560}$ 0.1) and cultivated for 1 week.

Experiment III: Part of Experiment II cultures was centrifuged and the same procedure as in Experiment II was carried out.

Experiment IV: Part of Experiment III cultures was harvested and the procedure as in Experiment II was carried out.

$Mg^{2+}$ concentration in algal cells was determined as previously described (Fujii and Hellebust, 1992). Each day, the dry weight from each culture was determined as described by Cao (1996) to calculate specific growth rate (SGR). Contents of chlorophyll *a* and pheophytin *a* were determined by the unichromatic method (Lorenzen, 1967). Every Experiment was repeated 3 times.

### 2.3. DNase preparation

1 ml of cultures were centrifuged (12 000*g*, 15 min), and the cell-free supernatant was the extracellular DNase solution. After rinsing 5 times with cool (4°C) Zarrouk medium, the pellet was resuspended in 1 ml of modified Zarrouk medium (supplemented with 40 mM Tris-HCl, pH 8.0) and the cells were lyzed by ultrasonication (Fisher Scientific, Model 550); the cell debris was completely removed by centrifugation as above at 4°C, and the cell-free supernatant was the intracellular DNase solution.

### 2.4. DNA preparation

The DNA substrate employed was lambda phage DNA, which was purified (Sambrook *et al.*, 1989) and diluted to the concentration of 0.1 μg/μl.

### 2.5. DNase activity assay

From Experiments I and II, only intracellular DNases were tested. In eppendorf tubes, the DNase solution and DNA were mixed and digestion was conducted.

For Experiment I:

a: 15 μl Zarrouk medium +2 μl DNA,
b: 15 μl intracellular DNase solution +2 μl DNA.

For Experiment II:

a: 15 μl $Mg^{2+}$-free Zarrouk medium +2 μl DNA,
b: 15 μl intracellular DNase solution +2 μl DNA.

Both extracellular and intracellular DNases from Experiment III were tested in 5 eppendorf tubes:

a: 15 μl $Mg^{2+}$-free Zarrouk medium +2 μl DNA,
b: 15 μl intracellular DNase solution +2 μl DNA,
c: 15 μl intracellular DNase solution +0.8 mM $Mg^{2+}$ +2 μl DNA,
d: 15 μl extracellular DNase solution +2 μl DNA,
e: 15 μl extracellular DNase solution +0.8 mM $Mg^{2+}$ +2 μl DNA.

In tubes c and e, $Mg^{2+}$ was added to a final concentration of 0.8 mM, the concentration in Zarrouk medium.

The tubes were incubated at 28°C for 1 h, and then the samples were loaded on to 0.7% agarose gel.

The peak area of each DNA band on gel was measured by means of NIH Image software to show how much substrate had been digested.

## 3. Results and discussion

### 3.1. Growth

In Experiment I, $Mg^{2+}$ concentration in the cultures, including $Mg^{2+}$ in medium and inoculated algal cells, was 0.81 mM; in Experiments II, III and IV, the prepared medium was free of $Mg^{2+}$, so $Mg^{2+}$ in the cultures came only from the initial inoculum and concentrations were $8\times10^{-2}$, $7\times10^{-3}$ and $9\times10^{-4}$ mM, respectively. At the different $Mg^{2+}$ concentrations the growth of *Spirulina* was distinct.

Dry weight is a comprehensive index of algal biomass and growth (Vonshak, 1987). The biomass in Experiment I, II and III (Fig. 1) increased during the experimental period. However, SGR was very different ($P<0.01$) among tests: 0.29, 0.20 and 0.08 per day, respectively. In Experiment IV, the alga died on the 4th day. The results showed that magnesium was an essential element for the growth of *Spirulina* and when

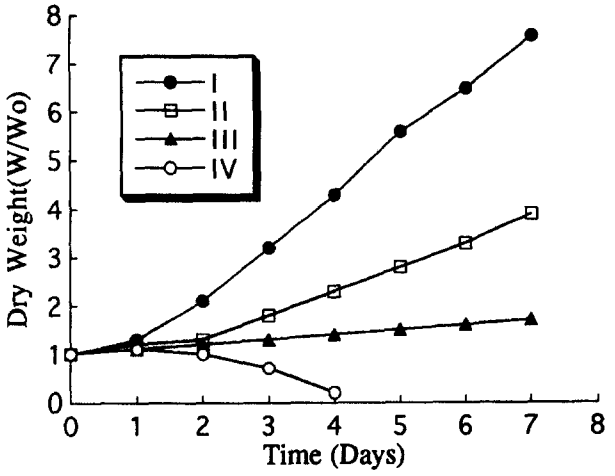


Fig. 1. Dry weight of *Spirulina platensis* strain SSP-14 in four Experiments. Data shown are means of three repeats.

it was insufficient or absent in the medium, *Spirulina* grew slower and eventually died.

Chlorophyll *a* is also used as an index of algal growth (Hansmann, 1973). The content of both chlorophyll *a* and pheophytin *a* was highest in Experiment I (the control), and lowest in III (data from IV not shown), but the ratio of pheophytin *a* to chlorophyll *a* was highest in Experiment III (Fig. 2 and Table 1). $Mg^{2+}$ is the active centre of chlorophyll *a*; pheophytin *a*, without photoactivity, is the derivative of chlorophyll *a* that has lost $Mg^{2+}$ (Lorenzen, 1967). Chlorophyll *a* can normally be degraded into pheophyin *a*, but in the absence of $Mg^{2+}$ in the medium, this conversion increased.

### 3.2. DNase activity

As shown in Fig. 3(A) and (B), the intracellular DNases from Experiment I and II digested the substrate lambda DNA thoroughly. On the contrary, both intra- and extra-cellular DNases from Experiment III degraded hardly any substrate (Fig. 3(C)). In lanes 1–5 (Fig. 3(C)), the peak areas of each lambda DNA band were 100% (the control), 98%, 6%, 99% and 68%, respectively. In lanes 2 and 4 in which $Mg^{2+}$ was removed from the medium and digestion mixture, the digestion was almost completely inhibited; in lanes 3 and 5, both intracellular and extracellular DNase activities recovered after the addition of $Mg^{2+}$. Nevertheless, the increase in the former was much greater than that of the latter.

The results presented here indicate that the DNase activity, both intracellular and extracellular, was almost completely (96–98%) inhibited when *Spirulina* was repeatedly inoculated and cultivated in $Mg^{2+}$/free Zarrouk medium. If these results were employed for transformation of foreign genes into *Spirulina*, then the genetically-engineered DNA vectors could remain undigested before integrating into the genome. Some researchers have made efforts to remove or shelter the DNAase activity by genetic manipulation. Tragut *et al.* (1995) reported that they found a gene encoding the methylase for *Spa* I, a restriction endonuclease with high-level activity in *Spirulina platensis* strain pacifica. Foreign vectors, after being modified by this methylase,

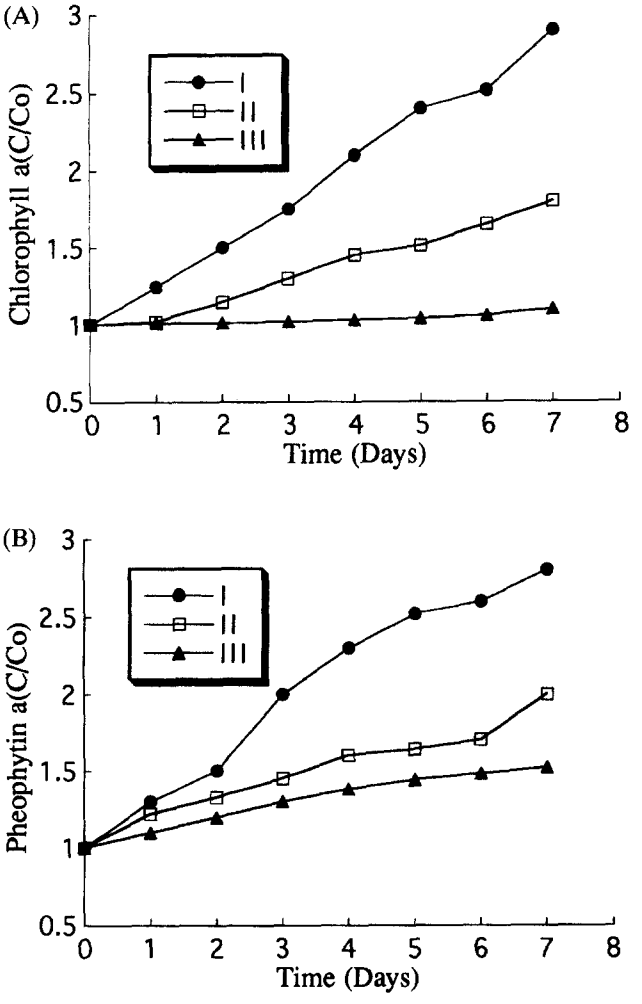


Fig. 2. The contents of chlorophyll *a* (A) and pheophytin *a* (B) of *Spirulina platensis* strain SSP-14 in three Experiments. Data shown are means of three repeats.

Table 1
The ratio of pheophytin *a* to chlorophyll *a* of *Spirulina platensis* strain SSP-14 in three Experiments

| | Day0* | Day1 | Day2 | Day3 | Day4 | Day5 | Day6 | Day7 |
|---|---|---|---|---|---|---|---|---|
| Experiment I | 1.50 | 1.60 | 1.52 | 1.60 | 1.61 | 1.61 | 1.61 | 1.61 |
| Experiment II | 1.50 | 1.51 | 1.61 | 1.62 | 1.62 | 1.61 | 1.63 | 1.62 |
| Experiment III | 1.50 | 1.62 | 1.74 | 1.81 | 1.86 | 2.0 | 2.02 | 2.02 |

*Values are averages from three repeats performed as described in the text.

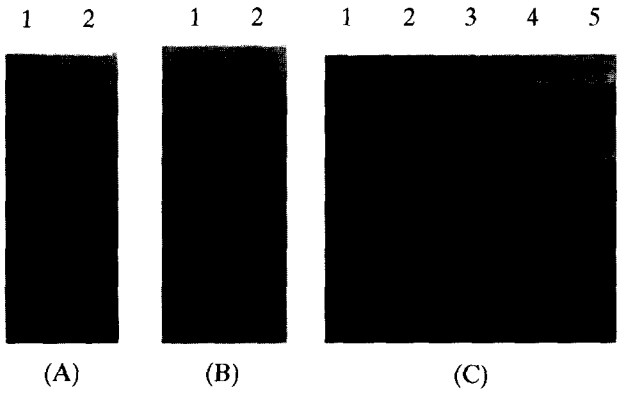


Fig. 3. Electrophoretogram showing results obtained when tests were carried out the effects of $Mg^{2+}$ on DNase activity of *Spirulina platensis* strain SSP-14 (lambda DNA as the substrate). (A) Experiment I, (B) Experiment II, (C) Experiment III. Lane numbers are identical to eppendorf tube numbers described in text.

could be prevented from being cut when transforming into *Spirulina* cells. But there are another three endonucleases in this strain and in most cases, it is difficult to find the methylase corresponding to every endonuclease. It would be much easier and more efficient to use the method reported in this paper, which needs only removal of $Mg^{2+}$ from the medium, to produce the effect.

When $Mg^{2+}$ was removed from the medium and digestion mixture, the activity of both extra- and intracellular DNases was inhibited and when it was restored, the activity also recovered (Fig. 3(C)), indicating that the DNases examined here are $Mg^{2+}$-dependent. It was also verified by EDTA test (data not shown), in which the addition of EDTA to the reaction mixture, in a concentration above 1 mM, efficiently inhibited the DNase activity. This showed that a bivalent cation is essentially required. However, when $Mg^{2+}$ had been added to tubes c (for intracellular) and e (for extracellular), the substrates were not completely digested, which could be mainly due to the fact that the added $Mg^{2+}$ concentration was much lower than that in general restriction endonuclease buffer (10 mM $Mg^{2+}$) (Sambrook *et al.*, 1989).

$Mg^{2+}$ is also one of the activators of some enzymes involved in the process of DNA synthesis, transcription and translation (Sambrook *et al.*, 1989). While inhibiting DNase activity other enzymes should maintain certain activities to guarantee the subsequent introduction, integration and expression of foreign genes. So, to keep growing is also needed. In four Experiments, only Experiment III could keep a growth level between faster growth and death; and just at this time, its DNases can be efficiently removed. It should thus be concluded that continuous inoculation and cultivation in $Mg^{2+}$/free medium for two times is just the balance point at which the alga can keep growing while the DNase activity is inhibited.

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