ELSEVIER

Bioresource Technology 70 (1999) 89–93

BIORESOURCE TECHNOLOGY

Studies on the sensitivity of *Spirulina platensis* to antibiotics and herbicide: relationship with selectable markers for genetic transformation

Jixiang Cao*, Zengfu Xu, Guohua Qiu, Baojian Li

Biotechnology Research Center, Zhongshan University, Canton 510275, People's Republic of China

Received 3 August 1998; revised 25 November 1998; accepted 16 December 1998

Abstract

The cyanobacterium *Spirulina platensis* was tested for sensitivity to 6 antibiotics: kanamycin (Km), neomycin (Neo), geneticin (G418), ampicillin (Ap), chloramphenicol (Cm), hygromycin (Hm) and to 1 herbicide: L-phosphinothricin (PPT), all of which are frequently applied as selectable markers in plant gene engineering. Growth tests, carried out at different concentrations of the agents above, showed that *S. platensis* was sensitive to Hm, Cm and PPT, while highly resistant to Km, Neo, G418 and Ap, indicating that Hm, Cm and PPT were potent inhibitors of *S. platensis* growth and able to be employed as selectable markers for further transformation studies for this alga.

Keywords: *Spirulina platensis*; Sensitivity; Antibiotics; Herbicide; Selectable markers

1. Introduction

Nowadays there have not been any approaches available related to the reproducible and stable gene transfer system for the cyanobacterium *Spirulina platensis*, though many attempts have been made during the last decade (Vachhani and Vonshak, 1997) and genetic transformations have succeeded with many other cyanobacteria (Porter, 1985; Elhai, 1993; Wu et al., 1997). This situation obviously blocks the development of new strains and new utilizations of this economic species through biotechnology which has achieved great progress in genetic improvement of various organisms.

The availability of a reliable selection protocol, which depends upon the choice of a selective agent to a great degree, is a prerequisite for the development of a practical transformation system (Nehra et al., 1994). A selectable marker gene, which confers antibiotic or herbicide resistance, should be cotransformed and coexpressed with target gene(s). This then makes it easy to discriminate the transformed cells, with resistance, from untransformed ones, without resistance (Akama et al., 1995). Therefore, this approach is beneficial to transformant screening. However, the selectable markers suitable for *S. platensis* have never been documented.

In the present study, the sensitivity of *S. platensis* to 6 antibiotics and 1 herbicide was examined in order to pick out one or more selectable markers for further gene transfer of this alga.

2. Methods

2.1. Chemicals

6 antibiotics and 1 herbicide, which are all commonly used in plant gene engineering, were investigated (Table 1). Except for G418 that was purchased from Bio-Rad Laboratories, these were from Sigma.

2.2. Cultivation conditions

Spirulina platensis strain SSP-14 was used throughout this experiment. SSP-14 was grown in Zarrouk medium (Zarrouk, 1966) in glass flasks (125 ml) on an illuminated shaker under the conditions of temperature 28°C, light intensity 90 μmol photon m^{-2} s^{-1} and light period 14:10 (light:dark). The preparation and sterilization of the medium have been described previously (Vonshak, 1997).

* Corresponding author. Present address: Microalgal Biotechnology Laboratory, Ben-Gurion University, Sede Boker Campus 84990, Israel. E-mail: jixiang@bgumail.bgu.ac.il

0960-8524/99/$ – see front matter
PII: S0960-8524(99)00002-4

Table 1
Experimental concentration of antibiotics and herbicide

Agents	Concentration (μg ml^{-1})
Antibiotics	
Kanamycin (Km)	0, 200, 400, 800, 1600
Neomycin (Neo)	0, 100, 200, 400, 800
Geneticin (G418)	0, 50, 100, 200, 400
Ampicillin (Ap)	0, 100, 200, 300, 500
Chloramphenicol (Cm)	0, 25, 50, 100, 200
Hygromycin (Hm)	0, 50, 100, 200, 400
Herbicide	
L-phosphinothricin (PPT)	0, 25, 50, 100, 200

2.3. Growth test

When they were grown to the exponential phase after inoculation from the cultures pre-cultivated as above, the strain SSP-14 cells were subjected to a series of concentrations of each antibiotic or herbicide (Table 1). To do so, the cells were concentrated and then transferred into each glass flask with 50 ml medium supplemented with different concentrations of each antibiotic or herbicide (already prepared as stock solutions in ddH_2O or ethyl alcohol (Cm)) as given in Table 1, starting with the same inoculation biomass (5.50 mg dry wt) for each agent tested, and cultivated as above. The controls were grown in Zarrouk medium without additional agent supplement. All the procedures were carried out under aseptic conditions (Vonshak, 1997).

2.4. Biomass determination

Dry weight and chlorophyll *a* were employed to represent algal biomass and growth. Dry weight was determined as described previously (Cao, 1996). To detect chlorophyll *a*, 1 ml of culture was centrifuged and, after the supernatant had been removed, 1 ml of cool 80% acetone was added and mixed; the mixture was ground in a glass homogenizer for 5 min, extracted at 4°C overnight, and centrifuged; the supernatant was adjusted to 4.0 ml with 80% acetone, and OD was determined at 663 nm; chlorophyll *a* (chl *a*) content was then calculated according to the equation reported by Myers and Krats (1955). Both determinations were performed every 48 h from the first inoculation day to day 6.

2.5. LC_{50} determination

The median lethal concentration (LC_{50}) of Cm and PPT was determined according to the growth test procedure as above. Algal cultures were subjected to concentrations of Cm and PPT of 0, 5, 10, 15, 20, 25, 50 and 100 μg ml^{-1}, respectively. The LC_{50} is here defined as the Cm or PPT concentration which produced a 50% loss for dry weight (the initial biomass) in the cultivation for 6 days. The LC_{50} was calculated by the commonly used method, the Spearman–Karber equation (Finney, 1978).

3. Results

3.1. Sensitivity

In any culture to which one of the antibiotics or the herbicide was added, the growth, compared with the control, was more or less inhibited, indicating that the experimental antibiotics and herbicide were all toxic to *S. platensis*. However, from the biomass data at day 6 this inhibition varied depending upon each agent being studied. Firstly, even the highest experimental concentrations of Km (1600 μg ml^{-1}), Neo (800 μg ml^{-1}), G418 (400 μg ml^{-1}) and AP (500 μg ml^{-1}) did not exhibit potent suppression on algal biomass (Fig. 1, D1, only Km data were illustrated), whereas Cm, Hm and PPT showed almost complete inhibition at such a low concentration as 25 μg ml^{-1} (Cm), 100 μg ml^{-1} (Hm) and 50 μg ml^{-1} (PPT) (Fig. 1, A1-C1). Secondly, the difference in biomass among various experimental concentrations was significant ($p < 0.01$) in the case of Cm, Hm and PPT, and not significant in the case of the rest of the antibiotics even between the lowest and highest concentrations. The effects on Chl *a* contents were similar to those on dry weight (Fig. 1, A2-D2), except that loss of Chl *a* was a little faster than the decline in the dry weight, implying that chlorophyll synthesis and photosynthesis were first influenced. These results showed that *S. platensis* was sensitive to Cm, Hm and PPT, and highly tolerant to Km, Neo, G418 and Ap.

3.2. LC_{50}

The amounts of the algal biomass at different concentration levels of Cm and PPT are summarized in Table 2. The $Log_{10}LC_{50}$ of Cm and PPT, determined by the Spearman–Karber method, was 1.22 and 1.29 with 95% upper and lower confidence interval 1.159–1.282 and 1.226–1.355, respectively. Correspondingly, the LC_{50} of Cm and PPT was 16.6 and 19.6 μg ml^{-1} with 95% upper and lower confidence intervals 15.77–17.43 μg ml^{-1} and 18.62–20.58 μg ml^{-1}, respectively.

4. Discussion

Kanamycin resistance is one of the most frequently used selection markers for obtaining transgenic plants (Nap et al., 1992), and the generally applied concentration is between 25–100 μg ml^{-1}. Km was also reported to be successfully used in the transformant screening of the unicellular cyanobacteria such as *Synechocystis* sp. PCC 6803 (Metz et al., 1989),

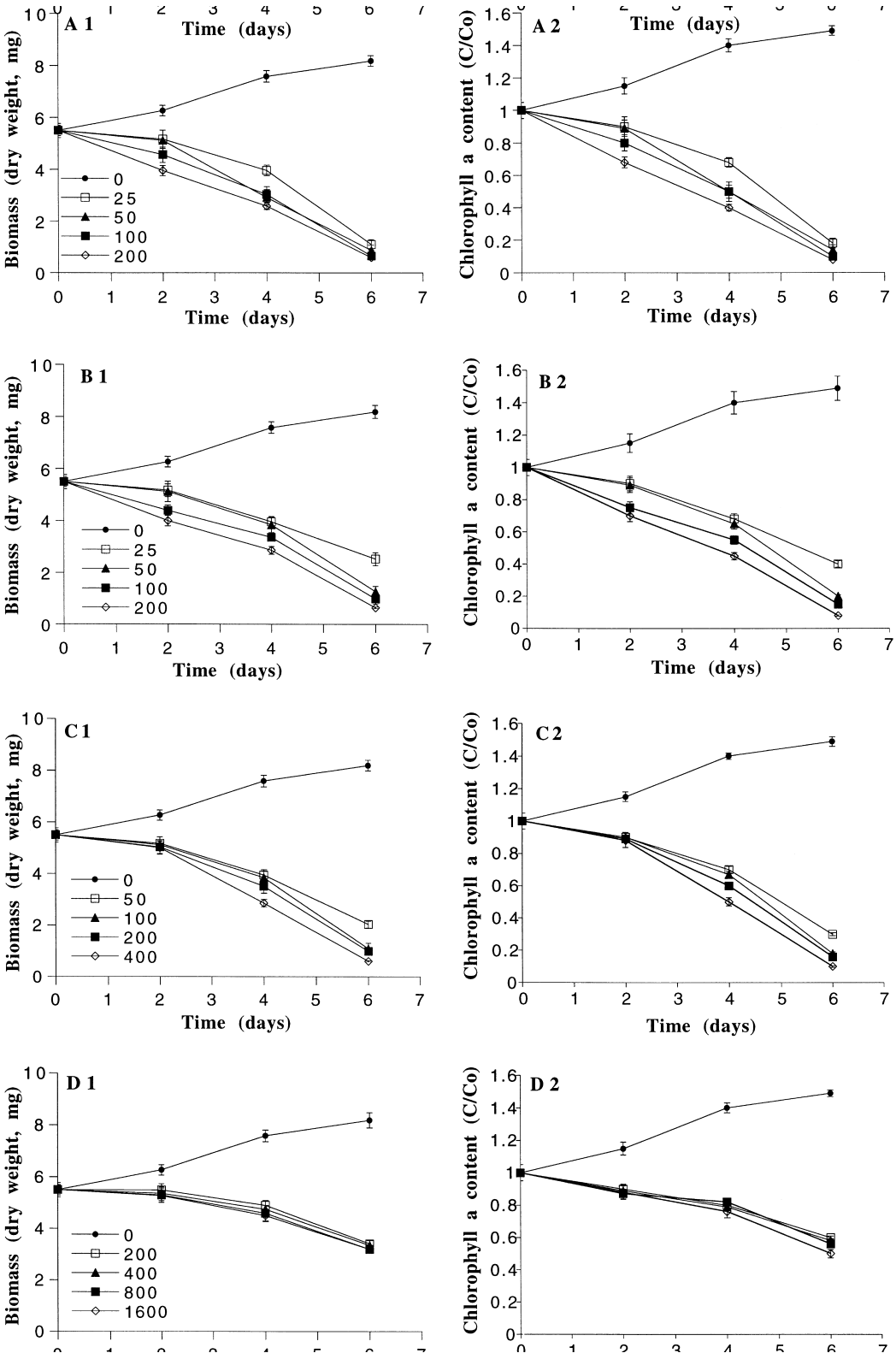

Fig. 1. Growth curves of *Spirulina platensis* strain SSP-14 when exposed to different concentrations of the experimental antibiotics and herbicide. Algal growth was expressed by the dry weight (mg) for biomass or the ratio (C/Co) for chlorophyll *a*. A1, B1, C1 and D1 represent the dry weights, and A2, B2, C2 and D2 represent the ratios of chlorophyll *a* contents to the control, when exposed to Cm, PPT, Hm and Km, respectively. Bars indicate standard error deviation (SEM) ($n = 3$). Symbols show concentrations of added agents.

Table 2
Biomass (dry weight, mg) of *Spirulina platensis* strain SSP-14 when cultivated in different concentration (μg ml^{-1}) of Cm and PPT for 6 days. Data shown are means of three trials

Concentration gradients	Cm		PPT	
	Dry wt	Ratio [a]	Dry wt	Ratio [a]
0	8.2	1.0	8.2	1.0
5	5.6	0.68	6.8	0.83
10	4.9	0.60	5.2	0.63
15	4.3	0.52	4.8	0.59
20	2.6	0.32	4.0	0.49
25	1.1	0.13	2.6	0.32
50	0.6	0.07	1.2	0.15
100	0.5	0.06	1.0	0.12

[a] Indicates the ratio (W/Wo) of the dry weight of each test (W) to that of the control (Wo) at day 6.

Synechococcus sp. PCC 7002 (Murphy et al., 1990) and *Synechococcus* sp. PCC 7492 (Van der Plas et al., 1990), and Neo with the filamentous *Anabaena* sp. PCC 7120 (Wu et al., 1997). Km, Neo and G418, all aminoglycosidic antibiotics, could be modified and then inactivated by neomycin phosphotransferase II (NPT II), a kind of aminoglycoside-modifying enzyme (Dong et al., 1995). However, *S. platensis* strain SSP-14 examined in this experiment did not exhibit sensitivity to Km, Neo and G418, on the contrary, it demonstrated powerful tolerance, even at the highest experimental concentrations. The results implied that the gene *nptII* encoding NPT II was not suited as the marker gene for *S. platensis* genetic transformation.

Ampicillin is also a frequently used selective marker, both for plant and prokaryote gene engineering (Sambrook et al., 1989), and was reported for the cyanobacterium *Synechococcus* sp. PCC 7002 (Murphy et al., 1990) and PCC 7492 (Luque et al., 1992). However, *S. platensis* did not demonstrate sensitivity to Ap from the results given above. It seems that Ap is suited as the selectable marker only for bacteria and unicellular cyanobacteria with high growth rates, and not for *Spirulina* and other filamentous cyanobacteria with low growth rates, in large part due to the fact that Ap is a thermounstable antibiotic and can maintain activity for only 24–28 h at the experimental temperature (Sambrook et al., 1989).

Strain SSP-14 studied here showed sensitivity to Cm, Hm and PPT. But the sensitivity to Cm and PPT was greater than that to Hm. Therefore, more attention was focused on Cm and PPT, and especially on Cm. In this experiment, dry weight of culture was used as an index for algal biomass and correspondingly loss of weight for sensitivity. Here, dry weight of culture actually means that of undissolved matter in the culture, simply because the determination of dry weight was carried out through filtering of the culture (Cao, 1996). In the case of Cm, PPT and Hm, the cells ceased growing, began to die and lyse, and thus the culture lost weight.

Cm inhibits translation on a 50S ribosomal subunit at the peptidyltransferase step, that is to say, Cm is an elongation inhibitor (Sambrook et al., 1989). Cm activity can be removed by acetylation, which is catalyzed by chloramphenicol acetyltransferase (CAT) encoded by the *cat* gene. Thus the *cat* could confer resistance of transformed cells to Cm in a medium and the untransformed cells would be killed. PPT is the active ingredient of the nonselective herbicide BASTA, and irreversible inhibitor of glutamine synthetase (GS) activity. Inhibition on GS causes plants to die because of the toxic effect of ammonia accumulated in the cells (Akama et al., 1995). PPT can be inactivated by phosphinothricin acetyltransferase (PAT), encoded by bialaphos resistance gene (*bar*) cloned from *Streptomyces hygroscopicus* (Akama et al., 1995; Franco et al., 1996). *cat* and *bar* are useful marker genes for *S. platensis* transformation.

It was reported that Cm was once used as the selectable marker in cyanobacteria (Metz et al., 1989; Thiel and Poo, 1989). Thiel and Poo (1989) succeeded in transforming the *cat* gene into a filamentous cyanobacterium *Anabaena* sp. M131 by electroporation for the first time, in which 25 μg ml^{-1} of Cm was applied to screen the transformants. Before the work reported here, no reports were available about PPT as the selectable marker for cyanobacteria.

References

Akama, K., Puchta, H., Hohn, B., 1995. Efficient agrobacterium-mediated transformation of *Arabidopsis thaliana* using the bar gene as selectable marker. Plant Cell Reports 14, 450–454.

Cao, J., 1996. Effect of germanium dioxide on growth of *Spirulina platensis*. Chin. J. Oceangr. Limnol. 14, 378–381.

Dong, L.Y., Ling, J., Wang, C.M., Shi, J.N., 1995. Ti plasmid-mediated transformation of tobacco with C-4PEPCase cDNA from *Zea mays*. Acta Phytophysiologia Sinica 21, 281–288.

Elhai, J., 1993. Strong and regulated promoters in the cyanobacterium *Anabaena* PCC 7120. FEMS Microbiol. Lett. 114, 179–184.

Finney, D.J., 1978. Statistical Methods in Biological Assay, 3rd ed. Macmillan, New York.

Franco, A.R., López-Siles, F.J., Cárdenas, J., 1996. Resistance to phosphinothricin (glufosinate) and its utilization as a nitrogen source by *Chlamydomonas reinhardtii*. Appl. Envir. Microbiol. 62, 3834–3839.

Luque, I., Herreto, A., Flores, E., Madueno, F., 1992. Clustering of genes involved in nitrate assimilation in the cyanobacterium *Synechococcus*. Mol. Gen. Genet. 232, 7–11.

Metz, J.G., Nixon, P.J., Rögner, M., Brudvig, G.W., Diner, B.A., 1989. Directed alteration of the D1 polypeptide of photosystem II: evidence that tyrosine-161 is the redox component, Z, connecting the oxygen-evolving complex to the primary electron donor, P680. Biochemistry 28, 6960–6969.

Murphy, R.C., Gasparich, G.E., Bryant, D.A., Porter, R.D., 1990. Nucleotide sequence and further characterization of the *Synechococcus* sp strain PCC 7002 *recA* gene: complementation of a cyanobacterial *recA* mutation by *Escherichia coli rec A* gene. J. Bact. 172, 967–976.

Myers, J., Krats, W.A., 1955. Relations between pigment content and photosynthetic characteristics in a blue-green alga. J. Gen. Physiol. 39, 11–22.

Nap, J.P., Bijvoet, J., Stiekema, W.J., 1992. Biosafety of kanamycin-resistant transgenic plants. Transgenic Res. 1, 239–249.

Nehra, N.S., Chibbar, R.N., Leung, N., Caswell, K., Mallard, C., Steinhauer, L., Baga, M., Kartha, K.K., 1994. Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J. 5, 285–297.

Porter, R.D., 1985. Transformation in cyanobacteria. Crit. Rev. Microbiol. 13, 111–132.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory press, USA.

Thiel, T., Poo, H., 1989. Transformation of a filamentous cyanobacterium by electroporation. J. Bact. 171, 5743–5746.

Vachhani, A.K., Vonshak, A., 1997. Genetics of *Spirulina*. In: Vonshak, A. (Ed.), *Spirulina platensis* (*Arthrospira*): Physiology, Cell-biology, and Biotechnology, Taylor and Francis, USA, pp. 67–77.

Van der Plas, J., Hegeman, H., de Vrieze, G., Tuyl, M., Borris, M., Weisbeek, P., 1990. Genomic intergration system based on pBR322 sequences for the cyanobacterium *Synechococcus* sp. PCC 7942: transfer of genes encoding plastocyanin and ferredoxin. Gene 95, 39–48.

Vonshak, A., 1997. Appendix III. In: Vonshak, A. (Ed.), *Spirulina platensis* (*Arthrospira*): Physiology, Cell-Biology and Biotechnology. Taylor and Francis, USA, pp. 218–219.

Wu, X., Vennison, S.J., Liu, H., Ben-Dov, E., Zaritsky, A., Boussiba, S., 1997. Mosquito larvicidal activity of transgenic *Anabaena* strain PCC 7120 expressing combinations of genes from Bacillus thuringiensis subsp.israelensis. Appl. Envir. Microbiol. 63, 4971–4975.

Zarrouk, C., 1966. Contribution à l'étude d'une cyanophycée influencé de divers facteurs physiques la croissance et la photosynthèse de *Spirulina maxima* (Setch.et Gardner) Geitler. Ph.D. Thesis, University of Paris, France.