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Genetic transformation of marine cyanobacterium *Synechococcus* sp. CC9311 (Cyanophyceae) by electroporation*

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Abstract *Synechococcus* sp. CC9311 is a marine cyanobacterium characterized by type IV chromatic acclimation (CA). A genetic transformation system was developed as a first step to elucidate the molecular mechanism of CA. The results show that *Synechococcus* sp. CC9311 cells were sensitive to four commonly used antibiotics: ampicillin, kanamycin, spectinomycin, and chloramphenicol. An integrative plasmid to disrupt the putative phycoerythrin lyase gene *mpeV*, using a kanamycin resistance gene as selectable marker, was constructed by recombinant polymerase chain reaction. The plasmid was then transformed into *Synechococcus* sp. CC9311 via electroporation. High transformation efficiency was achieved at a field strength of 2 kV/cm. DNA analysis showed that *mpeV* was fully disrupted following challenge of the transformants with a high concentration of kanamycin. In addition, the transformants that displayed poor growth on agar SN medium could be successfully plated on agarose SN medium.

Keyword: chromatic acclimation; electroporation; genetic transformation; *Synechococcus* sp. CC9311

1 INTRODUCTION

Synechococcus species are photosynthetic prokaryotes that contain phycobiliproteins, which serve as light harvesting antennae. Since its discovery three decades ago, the genus *Synechococcus* has been found in the illuminated upper layer of most marine ecosystems, from coastal to offshore waters, and from low to high latitudes (Zubkov et al., 1998). They are major participants in the global carbon cycle, and their contribution to primary production has been estimated to be 5%–30% (Li, 1994; Jardillier et al., 2010).

Synechococcus sp. CC9311, a unicellular marine cyanobacterium, was isolated from coastal waters of the California Current (Toledo and Palenik, 1997). This organism has a greater capacity to sense and respond to changes in its environment than *Synechococcus* strains isolated from the open ocean (Toledo and Palenik, 1997; Palenik et al., 2006; Dufresne et al., 2008). In particular, it possesses an ability to adapt to light quality. Phycoerythrin covalently links phycourobilin (PUB) and phycoerythrobilin (PEB), which serves as the chromophore. The maximum absorbance for PUB and PEB is 495 and 545 nm, respectively. Under white (green) light, the ratio of PUB:PEB is 0.66–0.71. When light is blue-shifted, this ratio increases to 0.96–1.8 (Palenik, 2001). Thus, phycobiliproteins

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Table 1 Primers for construction of integrative plasmid pMD18T-mpeV

Primer	Sequence (5′–3′)
RV1	ACCGCTGTTACCAATGTTAC
RV2	ATGGTTGCTGACTTGCGTTGTTCTTTTAAGTGT
RV3	AAAGAACAACGCAAGTCAGCAACCATAGTCCCG
RV4	GGTATCCCACTATCCGCTCAGAAGAACTCGTCA
RV5	TTCTTCTGAGCGGATAGTGGGATACCTCTTGAT
mpeVR	TACGATATCTTAATCTATTTGCTTGAGTTTCTG

efficiently harvest light energy for photosynthesis. This phenomenon is called type IV chromatic acclimation (CA4) (Palenik, 2001). The spectral quality of light in coastal sea is typically different due to the presence of terrestrial material and algal biomass. Light color distribution also changes with depth, with blue light dominating in deep water. Therefore, CA in *Synechococcus* sp. CC9311 plays an important role in environmental acclimation.

Several studies have been conducted to elucidate the mechanism of type IV CA. Based on biochemical analysis, Everroad et al. (2006) proposed a process, which involves the enzymatic activity of one or several phycobilin lyases and/or lyase-isomerases that are differentially regulated by ambient light quality. Recently, the genomes of a number of *Synechococcus* species have been sequenced. By comparative genomic study, Six et al. found that some putative phycoerythrin lyase genes, present in type IV CA strains, were absent in either high or low PUB:PEB strains. Thus, these lyases were suggested to participate in the type IV CA process (Six et al., 2007).

A method for genetic transformation of *Synechococcus* sp. CC9311 was developed in the present study to determine the molecular mechanism of CA4 in this organism. The results indicated that DNA can be genetically transformed into *Synechococcus* sp. CC9311 cells through electroporation and that a specific gene can be disrupted by the integration of a marker gene. In addition, we found that the transformants that grew poorly on agar SN medium could be successfully plated on agarose SN medium.

2 MATERIAL AND METHOD

2.1 Strain and culturing conditions

Synechococcus sp. CC9311, originally obtained from the National Center for Marine Algae and Microbiota (formerly the CCMP, East Boothbay, ME, USA), was grown in glass flasks containing SN medium (Waterbury et al., 1986) at 22–26°C without shaking. The culture was grown under 15–20 μmol/(m^2·s) white light, which was provided by daylight fluorescent tubes, with a light/dark cycle of 12 h/12 h. Liquid cultures were grown in glass flasks. For selection of transformants, the cells were spread or streaked on 0.5% agarose (MDBio) SN medium containing kanamycin.

2.2 Sensitivity determination of *Synechococcus* sp. CC9311 to antibiotics

The cultures were grown in 500 mL flasks. When the OD_{730} reached 0.1 (exponential phase), the culture was divided into 15 mL aliquots and grown in 50 mL flasks. Ampicillin (Amp), kanamycin (Kan), spectinomycin (Spe), and chloramphenicol (Cm) were individually added to the cultures, with final concentrations of 0, 20, 40, and 80 μg/mL. Two weeks post-treatment, OD_{730} was measured using a spectrophotometer.

2.3 Construction of integrative plasmid

To construct an integrative plasmid for the insertational inactivation of *mpeV*, a putative lyase gene in *Synechococcus* sp. CC9311, the upstream and downstream DNA fragments of *mpeV* were amplified using polymerase chain reaction (PCR) with primers RV1/RV2 and RV5/mpeVR, respectively, from *Synechococcus* sp. CC9311 genomic DNA. All primer sequences are provided in Table 1. A Kan-resistance gene was amplified with primers RV3/RV4 from plasmid pEGFP-N1 (Invitrogen, Carlsbad, CA, USA). *Pfu* DNA polymerase (TaKaRa, Dalian, China) was used to minimize mutation during PCR amplification. The 5′ ends of primers RV2 and RV3 shared 26 bp of overlap (Table 1, shaded characters). Similarly, the 5′ ends of primers RV4 and RV5 also shared 26 bp of overlap (Table 1, shaded characters). These PCR fragments were gel purified, mixed together, and ligated through recombinant PCR with primer pair RV1/mpeV (Horton et al., 1989). The 2.6-kb recombinant PCR product was gel purified. A-overhangs were added to the recombinant PCR product by incubation with dATP and *Taq* polymerase at 72°C for 10 min. The PCR products were then ligated into pMD18T (Takara), yielding the integrative plasmid pMD18T-mpeV. The plasmid was checked by DNA sequencing (Shanghai Sunny Biotechnology Company, Shanghai, China) and restriction enzyme analysis.

2.4 Transformation by electroporation

Genetic transformation experiments were performed when cells were at mid-exponential phase (OD_{730}=0.25, 2.9×10^8 cells/mL). The integrative plasmid was transformed into cells by electroporation, according to Mühlenhoff and Chauvat (1996). Briefly, the cells were harvested by centrifugation at 5 000 *g* for 10 min at room temperature and washed with an equal volume of buffer (2 mmol/L tricine, 2 mmol/L EDTA, pH 8.0). The cells were then washed twice with double distilled water and resuspended in water at a final density of OD_{730}=2.5. Electroporation was performed in a BTX ECM 830 device (Harvard Apparatus, Holliston, MA, USA). A total of 40 μL of cell suspension was mixed with 4 μg of plasmid DNA, injected into a 0.1 cm gap cuvette, and cooled on ice for 2 min. The mixture was then electroporated by a single pulse with a pulse width of 5 ms at 100–400 V, corresponding to a field strength of 1–4 kV/cm. The capacitance of the electroporator was 4 000 μF. The pulsed cells were rapidly mixed with 1 mL of fresh SN medium and transferred into a glass tube. After 24 h of recovery under 5–10 μmol/(m^2·s) of white light, the cells were propagated on 0.5% agarose SN plates containing 40 μg/mL Kan. Resistant colonies appeared within three to four weeks. The colonies were subjected to four to six rounds of streaking on agarose SN plates containing Kan. The resistant colonies were then picked with an inoculating needle and transferred into liquid SN medium. The culture was grown under ~15 μmol/(m^2·s) of white light.

2.5 Analysis of genomic DNA

Genomic DNA was isolated from 20 mL cultures with a Plant Genomic DNA Isolation Kit (Tiangen Biotech Company, Beijing, China) according to the manufacturer's instructions. PCR using the purified DNA was performed with primers RV1/mpeVR. The size of the PCR products for the wild-type and fully segregated transformants was 1.6 and 2.6 kb, respectively, whereas the size of the partially segregated transformants was 1.6 and 2.6 kb.

3 RESULT AND DISCUSSION

3.1 Sensitivity of *Synechococcus* sp. CC9311 to antibiotics

The sensitivity of *Synechococcus* sp. CC9311 to Amp, Kan, Spe, and Chl was investigated. As shown in Fig.1, these antibiotics exhibited an inhibitory effect on the growth of *Synechococcus* sp. CC9311 because OD_{730} decreased significantly compared to the controls. Among the four antibiotics, Amp was the most effective. The OD_{730} of the cells, even those treated with only 20 μg/mL of Amp, was zero. *Synechococcus* sp. CC9311 cells are characterized by a red pigmentation; however, we did not observe the red color three days post-treatment with 20 μg/mL Amp. Kan also inhibited the growth of the cells, as OD_{730} dropped to zero after challenge with 40 or 80 μg/mL Kan for two weeks. Moreover, the cells could not recover even when inoculated into fresh SN medium. This result indicated that the cells were killed by the antibiotic. Compared with Kan, Cm and Spe were less effective. Growth of the cells was not inhibited completely, even at a concentration of 80 μg/mL.

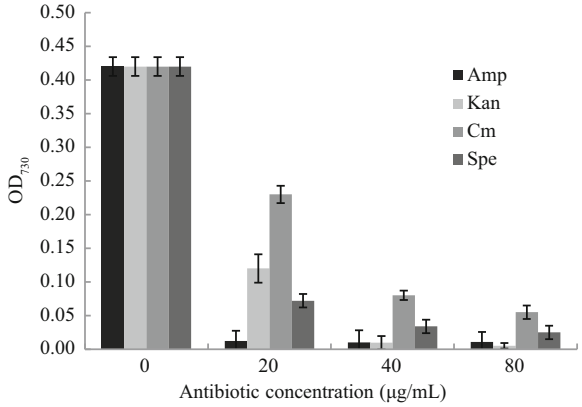

Fig.1 Inhibitory effect of antibiotics on the growth of *Synechococcus* sp. CC9311

Values are mean ± SD for three independent experiments.

Kan is often used as a selection agent in genetic engineering of *Synechococcus* strains, such as *Synechococcus* sp. PCC 7942 and *Synechococcus* sp. PCC 7002. Hence, we used Kan for the selection of *Synechococcus* sp. CC9311 transformants.

3.2 Construction of integrative plasmids and genetic transformation

Genetic transformation systems have been established for many cyanobacteria. However, these systems are available mostly for gene transfer through shuttle plasmids, and few are specifically for gene disruption (Matsunaga et al., 1990; Chiang et al., 1992; Koksharova and Wolk, 2002). In light of this, we constructed integrative plasmids to inactivate the *mpeV* gene in *Synechococcus* sp. CC9311 in the present study. A Kan-resistance gene was inserted into *mpeV* by recombinant PCR. The PCR products were then ligated into pMD18T (Fig.2). In the

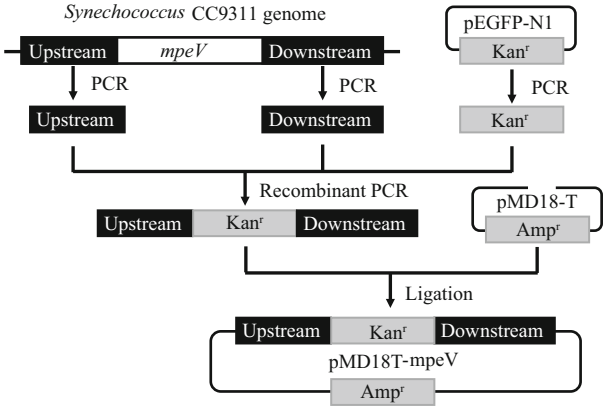

Fig.2 Construction of the integrative plasmid pMD18T-mpeV for insertional inactivation of *mpeV* in *Synechococcus* sp. CC9311

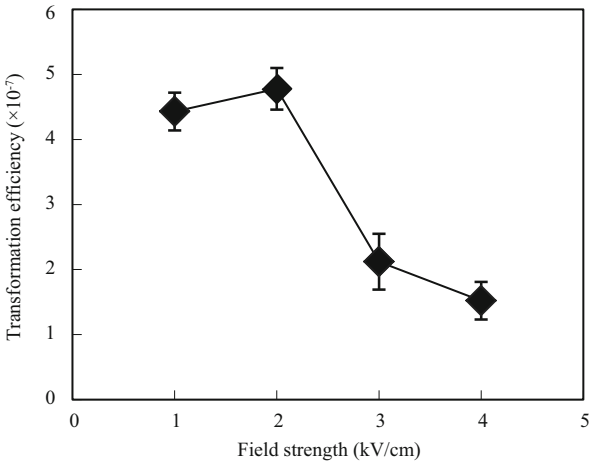

Fig.3 Effect of field strength on the transformation efficiency of *Synechococcus* sp. CC9311

Values are mean ± SD for three independent experiments.

procedure presented here, the reaction and transformation into *Escherichia coli* were performed only once, whereas these steps must be performed three times in the traditional procedure (Koksharova and Wolk, 2002). Therefore, the construction procedure was more efficient than the traditional method. Furthermore, the ligation through recombinant PCR was independent of restriction sites. This approach will be particularly useful if no appropriate restriction enzyme sites can be found in the target gene in further gene disruption studies.

We attempted to transfer integrative plasmids into *Synechococcus* sp. CC9311 cells by natural transformation following the procedure for *Synechococcus* sp. PCC 7002 (Shen and Bryant, 1995); however, no resistant clones were obtained (data not shown). It has been suggested that pilus structures on the cell surface are critical for natural transformation (Yoshihara et al., 2001; Okamoto and Ohmori, 2002). No gene homologues were found in the genome of *Synechococcus* sp. CC9311, indicating the absence of pili, which may explain the unsuccessful transformation.

Therefore, we attempted to transform the plasmids by electroporation. Electroporation uses a high-voltage current through a cell suspension to induce pores in the cell membrane, through which plasmid DNA enters the cell (Matsunaga and Takeyama, 1995). High voltage can cause excessive cell death, and thus must be optimized. We transformed the plasmid into cells under field strengths ranging from 1–4 kV/cm. Figure 3 shows the effects of field strength on the transformation efficiency in *Synechococcus* sp. CC9311 cells. The optimal field strength appeared to be 2 kV/cm, as field strengths lower or higher than 2 kV/cm resulted in lower transformation efficiency.

Marine cyanobacteria normally grow poorly on agar medium (Brahamsha, 1996), possibly because they are sensitive to contaminants in agar. Consistent with this, *Synechococcus* sp. CC9311 did not grow on SN agar plates, even when the agar was washed as suggested by Waterbury and Willey (1988). Considering that agarose is the main component of agar, and is purer, we tried plating the cells on SN agarose plates. A total of 10^8 transformed cells were spread on agarose plates. Colonies, ranging in number from 18 to 53, appeared on the plates after three weeks. Although the plating efficiency was less than 1%, this plating method may be useful in colony isolation, and thus in genetic engineering of some other marine cyanobacteria.

3.3 Selection and analysis of Kan-resistant colonies

Genomic DNA from wild-type and Kan-resistant colonies was isolated to analyze whether the *mpeV* gene had been fully segregated. PCRs were performed using the RV1/mpeVR primers. The sizes of the Kan-resistance gene and *mpeV* cassette were 1.0 kb and 1.6 kb, respectively. Therefore, the size of PCR product was predicted to be 1.6 kb in the wild-type and 2.6 kb in a fully segregated colony, while a partially segregated colony would show both 1.6-kb and 2.6-kb bands. To segregate the wild-type and mutant alleles of *mpeV*, the Kan-resistant colonies were inoculated into liquid SN medium. The concentration of Kan in SN medium was periodically increased by 20 μg/mL. At a concentration of 100 μg/mL, the cells were streaked on agarose SN plates. The isolated colonies were then used for PCR analysis.

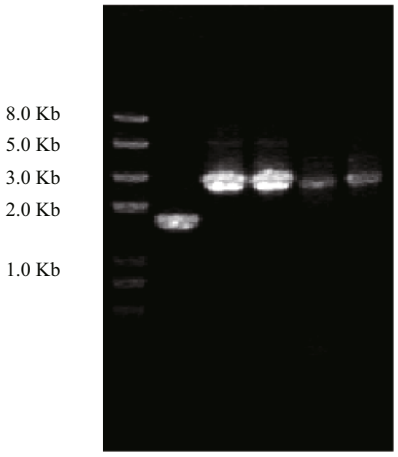

Fig.4 PCR analysis of isolated DNA for WT and Kan-resistant colonies

Lane 1: DNA marker; Lane 2: WT; Lane 3–6: Kan-resistant colonies.

Figure 4 shows that the 1.6-kb band was not present in Kan-resistant colonies. This result suggested that increasing the antibiotic concentration may promote genome homogenesis in *Synechococcus* sp. CC9311.

In conclusion, to the best of our knowledge, this is the first study to successfully transform *Synechococcus* sp. CC9311. While further studies should be conducted to improve transformation efficiency, the current study presents a convenient procedure for disrupting lyase genes or other functional genes. These findings may contribute to understanding the mechanism of type IV CA and the acclimation of marine *Synechococcus* to environmental changes.

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